UNIVERSITE BIOLOGIE BRETAGNE SANTE LOIRE

UNIVERSITÉ DE NANTES

Thèse de doctorat

L'UNIVERSITE DE NANTES

Université Bretagne Loire

Ecole Doctorale n° 605
Biologie Santé
Spécialité : Immunologie
Par

Eros Alexandre Marín Millán

Novel mechanisms in tolerogenic dendritic cells

Thèse présentée et soutenue à Nantes, le 27/11/2018
Unité de recherche : INSERM UMR1064
Thèse N° :

Rapporteurs avant soutenance :

Eva María Martinez Cáceres	Professeur, Barcelone
Federica Marelli-Berg	Professeur, Londres

Membres du jury:

Président :	Gilles Blancho	PU-PH, Nantes
Examinateurs :	Olivier Herault	PU-PH, Tour
Dir. de thèse :	Maria-Cristina Cuturi	Directeur de recherche, Nantes
Co-dir. de thèse :	Aurélie Moreau	Chercheur non statutaire, Nantes

"Hay que buscar la verdad y no la razón de las cosas,

y la verdad se busca con la humildad"

Miguel de Unamuno

Acknowledgements:

Ce travail n'aurait pas été possible sans le soutien et les conseils de collègues, d'amis et des membres de la famille. Tout d'abord, je voudrais remercier mes tuteurs Maria Cristina Cuturi et Aurélie Moreau. Merci de m'accueillir dans votre groupe et aussi pour l'aide et les enseignements fournis au cours de ces trois années. Bien qu'on n'a pas toujours été d'accord, je suis heureux que la différence des opinions a toujours donné lieu à des discussions qui m'ont permis de développer un esprit plus critique en science et en même temps augmenter ma confiance lors de l'exposition de mes idées. De même, merci beaucoup à la fondation Progreffe et aux benevoles qui ont permis de me financer pendant ces trois années.

Je voudrais également remercier mes rapporteurs Federica Marelli-Berg et Eva María Martinez Cáceres d'avoir accepté de former partie du jury de thèse et de lire et juger mon travail. Merci aussi à Olivier Herault et Gilles Blancho d'avoir accepté de faire partie du jury de thèse.

Merci beaucoup aussi aux membres du comité de suivi de thèse, Jean-François Fonteneau et Ignacio Anegon pour évaluer et critiquer mon travail et de contribuer avec leur expérience à des nouvelles perspectives et idées.

Un grand merci à Yodit, Aurélie M et Laurence D pour leur aide dans les corrections du manuscrit de thèse.

Merci également à Laurence D pour ça patience pendant mon apprentissage des techniques, ainsi que pour l'aide scientifique fournie tout au long de cette période et aussi pour le soutien moral. Merci aussi à Amandine E. de m'avoir toujours et TOUJOURS aidé quand cela était nécessaire et merci beaucoup pour ton soutien moral. Aussi Aurélie, merci beaucoup d'avoir pris ton temps pour m'aider, surtout pour les questions administratives, et à avoir une vision plus critique de cette belle discipline.

Je voudrais également remercier tous les membres de l'équipe 1, Régis J, Cedric L, Frank H, Dorian M, Elise C, Céline B, Jeremy P, Flora C, Cynthia F, Ahmed A, Gaëlle B, ... et les autres qui sont encore ou sont partis, pour tous les conseils fournis lors des présentations, notamment en termes de statistiques. J'espère pas vous avoir trop ennuyé avec le métabolisme ...

Merci également aux personnes qui nous ont aidés à développer des idées, Claire P, Xavier P, Brigitte A et autres.

Je voudrais également remercier le reste des membres du laboratoire, de toutes les équipes qui ont rendu ce séjour plus agréable. Pour commencer, grâce aux gens des conversations terrasse, qui aident à déconnecter, Pierre, Malo, Alex, Amédé, Yveline, Ahmed, Gaëlle, Aurélie F, Dimitri, Raphaël, Antoine, Laetitia ... et d'autres que certains j'oubli. Merci aussi à tous ceux qui m'ont aidé dans les expériences, Flora C, Annaick P, Lola L., Séverine B, ... et tant d'autres. Aussi je vous remercie beaucoup à tous les gens souriants qui illuminent les jours gris de Nantes, Vanessa G, Charlene, Jean Marc, Manu, Claire, Jean Paul, Géraldine T, Caroline, Apo, Gwen, Virginie, Laurent et plein d'autres... Aussi un grand merci à tous ceux qui ferment les happy hours (Vous savez qui vous êtes).

Aussi un grand merci à tous les gens du vendredi pour le soutien et les rires, Severine B, Melanie L, Lola L, Antoine F, Justine, Delphine, Lea, Laetitia, Naïl et d'autres qui viennent parfois

Merci beaucoup aussi, à ceux qui m'ont soutenu et aidé quand je venais tout juste d'arriver et que je ne connaissais rien ni personne. Merci Jason, Lucas, Camille, Ahmed, Lola L, Mathieu, Maxim et tant d'autres. Merci pour les rires. De même merci Raphael pour les concerts et la décompression, ça fessait longtemps que j'avais oublié tout ça.

Je tiens particulièrement à remercier Lola et Diego de m'avoir accueilli et de m'avoir fait sentir plus proche de chez moi. Vous avez été un grand pilier pour ne pas perdre la tête. Aussi pour le reste des hispanophones, Marco, Karina, Luquita (pas trop phonique) et Nacho.

Je voudrais également remercier toutes les personnes qui ne liront jamais cette thèse, mais sans elles ça n'aurait pas été possible.

J'aimerais d'abord remercier l'équipe de Biologie du Macrophage de l'Université de Barcelone. Merci, Antonio Celada, Jorge Lloberas, Calatayud, Tania, Esther, Laura, Marti et Lorena, c'était très amusant. Surtout, Joan Tur, merci pour la patience et le soutien moral, t'as été un excellent exemple. Merci aussi à Gloria Sans et Selma Pereira de m'avoir aidé à obtenir la place doctorant.

Je voudrais également remercier tous les compagnons du Master of Immunology qui ont fait de cette année, une des plus cool de ma vie. Aussi à Jessi (Pavita) et Elena Borrego.

Un merci spécial à ceux qui m'ont aidé quand les temps étaient moins bons. Surtout, Tania et David, vous m'avez sauvé plusieurs fois et je vous en serai éternellement reconnaissant. Dans

le même contexte, Bernat et Sara ainsi que Matías et ses parents. Je me sens un peu obligé de mettre Tito, même si j'ai des doutes ... Merci quand-même.

Alexis et Eric. Vous êtes des personnes incroyables qui m'ont donné beaucoup de force pour continuer et arriver ici. Vous avais changé mon monde vers le mieux. Merci pour tous les soirs et pour tous les concerts. ALBOE. Trasher, Sara et Cris vous m'avais fait sentir trop bien, et je suis très fiers des chemins qu'on a pris. Qui l'aurrais jamais pensé ? Jovanna, je t'aurrais toujour dans mes pensées. T'est une personne admirable et c'est grace a toi que j'ai cette faim pour la connaisance. Merci

Aussi avec beaucoup d'amour, tous ces gens d'Andorre qui m'ont fait ressentir à chaque fois comme si je n'étais jamais parti, Rus, Renzito, Andy, Ivan, Pau, Mati, Niki, Robert. Olga et Aitor. Qu'aurais-je fait sans vous? Vous savez que je vous aime beaucoup.

Pour finir, je veux remercier toute ma famille pour leur soutien et leur affection à distance. À mes parents, Pedro et Marta, merci pour tout. Vous êtes toujours là.

Je suis desolé si j'oublie quelqu'un. En tout cas MERCI à tous.

Table of content

List of figures

List of tables

List of abbreviations

2-DG: 2-deoxy-D-glucose

β-Ox: β-oxidation

AA: Amino acids

Ac-Coa: Acetyl-CoA

Akt: Protein Kinase B

AMP: Adenosine monophosphate

AMPK: Adenosine monophosphate activated protein kinase

ASCT2: Neutral amino acid transporter B(0)

ATP: Adenosine triphosphate

CAMKK2: Calcium/Calmodulin-dependent protein kinase kinase 2

c-myc: Cancer-Myelocytomatosis

cAMP: cyclic AMP

CCL: C-C motif ligand

CCR: C-C motif chemokine receptor

Cit: Citrate

-CoA: -Coenzyme A

CD: Clusted of differentiation

CPT1: Carnitine palmitoyltransferase I

CTLA: Cytotoxic T-lymphocyte-associated protein

CXCR: C-X-C motif chemokine receptor

DAG: Diacylglycerol

ECAR: Extracellular acidification rate

Egr: Early growth response

ERK: Extracellular signal-regulated kinase

FADH: Flavin adenine dinucleotide

Fas: Tumour necrosis factor receptor superfamily member

FA: Fatty acids

FGFR: Fibroblast growth factor receptor

Fox: Forkhead box

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GCK: Glucokinase

Glc: Glucose

Gln: Glutamine

Glu: Glutamate

Gly: Glycerol

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GrzB: Granzyme B

GTP: Guanosine triphosphate

HIF: hipoxia inducible factor

HLA: Human leukocyte antigen

Hsp: Heat shock protein

KB: Ketonic bodies

ICOS: Inducible T-cell costimulator

IL: Interleukine

ILT: Ig-like transcript

IP3: Inositol triphosphate

Lac: Lactate

LAT1: L-type amino acid transporter 1

Lag3: Lymphocyte-activation gene

LD: Lipid droplets

LKB1: Liver kinase B1

MAPK: Mitogen-activated protein kinase

MEK: Mitogen-activated protein kinase kinase

MHC: Major histocompatibility complex

mTOR: mammalian target of rapamycin

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

NFAT: Nuclear factor of activated T-cells

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

Nu˙:Nucleotides

OCR: Oxygen consumption rate

PD: Programmed cell death protein

PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K: Phosphoinositide 3-kinase

PIP3: Phosphatidylinositol (3,4,5)-triphosphate

PKM: Pyruvate kinase M

PLC: Phospholipase C

PPAR: Peroxisome proliferator-activated receptor

PPP: Pentose phosphate pathway

Pyr: Pyruvate

RAG: Recombination-activating gene

SIRT1: NAD-dependent deacetylase sirtuin-1

Slc: Solute carrier family

TCA: Tricarboxylic acid cycle

TCR: T-cell receptor

TGF: Transforming growth factor

Th: T helper cell

TLR: Toll-like receptor

TNF: tumour necrosis factor

Introduction

1. Transplantation

1.1. Historical overview

The history of transplantation starts in the IIIrd century BC, when two Christian martyrs twins, Saint Cosmas and Saint Damian, miracoulosuly transplanted a black leg from an Ethiopian onto a white patient. Following this event, at 600AC, skin transplantation was introduced and used until the end of XV century. Further, at the XVIII century, John Hunter performed a transplant of human teeth and autotransplantation of cock's spurs into their combs. Naturally, the lack of understanding of the allogeneicity concept led to graft rejection in patients and deception to physicians. The first successful transplantation was achieved in 1902 by Emerich Ullmann by performing a kidney autotransplant in dog. The particularity of this success was that the kidney remained functional during the following 5 days after surgery. After this event, Jaboulay performed two renal xenotransplantation from pig to human in 1906 and Ernst Unger from monkey to human in 1909. Similarly to their predecessors, these transplantations failed several days after. Always at the beginning of XX century, Alexis Carrel improved technical procedures of transplantation and performed different types of transplantations including kidney, thyroid, ovary, heart, lung and small bowel. However, the failing in grafts acceptance still remained (1). Finally, the first successful renal transplantation was achieved at the Peter Bent Brigham Hospital in Boston by the team of Joseph Murray's in 1956. This procedure demonstrated the surgical feasibility of solid organ transplantation between twins (2). Parallel to this achievement, other group demonstrated that 6-mercaptopurine (6-MP), already prescribed for lymphocytic leukemia patients, it was indeed an immunosuppressive drug (3). These two concepts of surgical feasibility of solid organ transplantation and immunosuppressive drugs led to a novel approach to transplant unrelated organs. Due to the high toxicity of 6-MP, over the following years, the research on immunosuppressive drugs led to his replacement for a less toxic drug, the cyclosporine. Additionally, the cyclosporine led to an improvement in one-year graft survival (4). Nowadays, other more specific drugs are currently used in post-transplantation therapy such as the mophetil mycophenolate, a B and T-cell proliferation inhibitor, tacrolimus, a B and T-cell activation inhibitor (5), as well than monoclonal antibodies such as basiliximab, an IL2Ra (CD25) blocking

antibody (6). These new therapies have improved the post-transplantation therapy becoming safer and efficient in terms of allograft survival.

1.2. Mechanisms of rejection

Nowadays, organ transplantation is the only alternative for patients suffering for end-stage organs failure. However, the vascularized inclusion of a foreign organ can lead to the activation of the recipient immune response in order to eliminate the allograft. This immune reaction against the allograft is named rejection. There are three types of rejection, the hyperacute, acute and chronic rejection. The hyperacute rejection is the quickest response and is driven by pre-existing anti-donor antibodies. These antibodies induce to complement activation and endothelial cell stimulation leading to an intravascular thrombosis. This type of rejection is rare having considered previously the ABO compatibility and excluding the presence of donor anti-HLA antibodies in the recipient. The acute rejection appears usually between the first week and several months after the transplantation, and is lead by T-cells (cellular response) and B-cells (Humoral response). This type of rejection is usually avoided by the use of immunosuppressive drugs. Finally, the chronic rejection is difficult to predict. In this rejection different elements from the cellular and humoral response as well as other non-immune cell types are involved such as memory, plasma cells and endothelial cells among others. Additionally the presence of tertiary lymphoid organs in the allograft is characteristic of this type of rejection (7).

Due to the invasiveness of the surgical procedure, innate immune cells are able to sense this trauma through different receptors such as the pattern recognition receptors (PRR), complement receptors and scavenger receptors among other molecules. In fact, innate cells from both receptor and donor can sense the cellular stress derived from transplantation. Among the different types of PRR, immune cells can sense damage-associated molecular patterns (DAMPs) derived from plasma membrane of stressed cells, dying cells and apoptotic bodies. Following DAMP sensing, innate cells such as macrophages and dendritic cells (DC) respond through pro-inflammatory activation by secreting cytokines such as IL-1β and IL-18. Moreover, DAMPs and alloantigens intake also lead to DC maturation resulting in an increased capacity to migrate to lymphoid organs and present alloantigens to T-cells in order to stimulate adaptive response (7,8).

The adaptive response is mainly initiated by the interaction of DC and T-cells. In the case of $CD8^+$, the activation can be initiated by any cell that displays HLA class I molecule, meanwhile $CD4^+$ are stimulated by HLA class II (9). There are three different types of allorecognition depending on the interaction between T-cells and antigen presenting cells (APC); direct, indirect and semidirect pathways **(Figure 1)**. In direct pathway T-cells react against foreign MHC molecules. This type of rejection is usually related to acute rejection. In the indirect pathway, autologous DC uptake, process and present allopeptides to T-cells. This type of rejection is related to chronic rejection. Finally, in the semi-direct pathway, T-cell alloresponse is initiated by the interaction with autologous DC that previously have assimilated an intact foreign HLA class II molecules through cell-to-cell contact (10). About B-cells response in transplantation, it has been shown during the last decades that these cells are able to sustain alloimmune response through different mechanisms such as by the secretion of anti-donor antibodies and sustaining long-term humoral response, acquiring the role of APC, forming tertiary lymphoid organs and by the secretion of pro-inflammatory molecules (11).

In order to abrogate immune response against allograft, IS therapy is used after transplantation. Nowadays, this therapy is mostly focused on T-cell response in order to inhibit acute rejection. However, despite that IS drugs increase allograft survival, the immunosuppressive state have an associated risk of the development of cancer and infections. Moreover, the IS drug primarily prevents acute rejection meanwhile chronic rejection is hard to predict (12). For this reason, it urges to find other alternatives to avoid or at least minimize the use of IS drugs. In this purpose, cell based therapy alone or in combination with minimal immunosuppression appears as novel and promising strategy in order control chronic rejection.

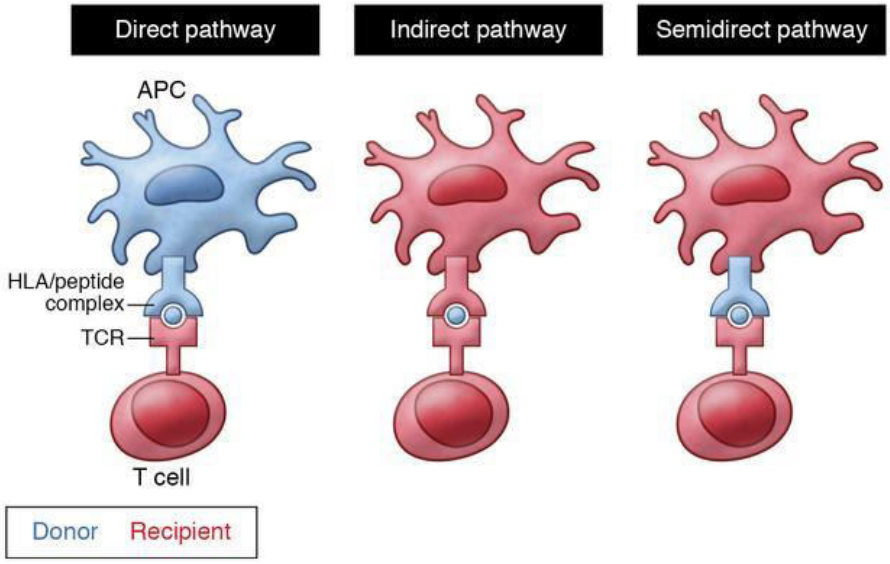


Figure 1. **Types of allorecognitions (From DeWolf and Sykes. 2017).** Alloresponse can be induced by three different pathways. In the direct pathway, recipient T-cells interact with allogeneic HLA molecules of donor APCs. In the indirect pathway, recipient APCs process and present donor peptides to recipient T-cells. In the semidirect pathway recipient, APCs adopt fully unprocessed HLA molecules from donor cells and interact with T-cells**.**

2. Cell based therapy

2.1. Cell based therapy and purposes

Cell based therapy was born as an approach to treat different types of diseases using natural cell mechanisms. In this purpose, different types of cells with different mechanisms have been considered appropriated depending on the therapeutic approach. For example, lymphoid cells, among other potential uses, they have been considered for cancer therapy in order to improve the anti-tumour response. Moreover, for T-cells in anti-tumour therapy context, there are many approaches that have been considered such as, the adoptive transfer of expanded circulating T-cells from tumour patients as well as the expansion of tumour-infiltrating T-cells (TIL). Additionally, T-cells can be modified by genetic engineering by generating chimeric antigen receptors T-cells (CAR-T cells). In this approach Ig variable domain is fused to constant TCR domain leading to T-cells able to detect and react against target antigens in a HLA-independent context (13). On the other hand, regulatory T-cell (Treg) expansion and regulatory CAR-T cells

approaches has also been considered to be used as immunoregulatory therapies in other contexts such as transplantation, autoimmune diseases and even allergic diseases (14,15). Also, NK cells have been considered to use in cancer therapy. These cells differently from T-cells do not react to specific antigens. In fact NK cells are specialized on the elimination of cells that downregulate antigen presenting molecules such as tumour cells and virally infected cells (16,17).

Another approach for cell based therapy consists on the re-education of immune system. In this approach APC are used to presents antigens to T-cells and polarize immune response in vivo. This therapy have been tested by two approaches: by injecting unmodified DC or in contrast by injecting DC previously loaded with concrete peptides or a mix of different proteins (Serum, synovial fluid, etc…). The advantage of this therapy is that DC can be polarized ex vivo to induce an immunogenic or a tolerogenic response. This functional plasticity is a powerful therapeutic tool for both immunogenic requiring therapies such as in cancer as well for tolerogenic requiring therapy such as in transplantation or autoimmune diseases (18,19).

Finally, the last approach for cell based therapy is focused on the use of cells with potent properties in the context of tissue regeneration or immunoregulation. For example, mesenchymal stem cells (MSC) have been demonstrated to improve tissue regeneration and to display a tolerogenic potential by inhibiting immune response (20). Additionally using similar mechanisms and displaying the capacity to present antigens, regulatory macrophages (Mreg) have been also considered in cell based therapy (21).

There are different approaches to treat different diseases, however in our group, we are focused on the approaches related to immunosuppression in transplantation.

2.2. Cell-based therapy in solid organ transplantation

During the last decade, several clinical trials using cell-based therapy in solid organ transplantation have been performed. For these clinical trials, different types of cells have been tested such as MSC, Mreg and regulatory T-cells. MSC have been used in liver and kidney transplantation (22). In this work, the authors aimed to compare the benefits of MSC to anti-IL2Rα alone therapy. The author showed that MSC therapy, comparing to anti-IL-2Rα, led to a lower incidence of acute rejection and better

estimated renal function at 1 year (23). About Mreg, they have been tested in a clinical trial in living donor renal transplantation. For this study, two patients received Mreg previously to transplantation and further treated with low dose of tacrolimus. The output of this study showed a stable graft function after tacrolimus weaning (24). Further, the same authors evaluated Mreg therapy in a phaseI/II clinical trial in living-donor kidney transplantation. In this trial patients were treated with Mreg 7 days before transplantation. After injection, authors showed an increase of $CD4^+CD25^+CD127^{int}$ Treg frequency in peripheral blood in five patients (25). Finaly, regulatory T-cells have been tested in a clinical trial in living donor liver transplantation. This study proved that Treg were safe and efficient leading to the arrest of immunosuppressive therapy for 6 of 10 patients (26).

Apart from these clinical trials, there are many others ongoing. In the context of the ONE study consortium, there are several types of cells that are being tested in living donor kidney transplantation clinical trial such as Treg, Mreg, Type 1 regulatory T-cells (Tr1), and Tolerogenic monocyte-derived DC (Tol-MoDC) in order to evaluate their safety and feasibility (www.onestudy.org) (27). In our group we are focused on Tol-MoDC therapeutic use.

3. Tolerogenic dendritic cells

3.1. Dendritic cells overview

DC were discovered and characterized from peripheral lymphoid organs by Steinman and Cohn back in the year 1973 (28,29). This discovery brought a new perspective of immunology clarifying the link between the innate and adaptive response. Despite that macrophages and B-cells are also able to present antigens, DC are known as the major APC. DC are capable to intake different types of extracellular structures such as microbial pathogens, apoptotic cells and immune complexes through different mechanisms such as phagocytosis, endocytosis and pinocytosis. After internalization, proteins are degraded to peptides and usually presented by HLA class II molecules to $CD4^+$T-cell. Apart to link the adaptive response, DC are also able to polarize the immune response to immunogenic or tolerogenic state (30). Over the following years, different dendritic cell subsets have been identified and classified depending on

phenotype, function and ontogeny as conventional DC (cDC), plasmacytoid DC (pDC), Langerhans cells and monocyte derived-DC (MoDC), also known as inflammatory DC (31). cDC are commonly located in lymphoid and non lymphoid organs and present antigens through MHC II. Interestingly, cDC can also cross-present antigens through MHC I. This characteristic is essential for the induction of tolerance (32). pDC, are usually located in peripheral organs and related to the anti-viral response due to the high secretion of type I Interferons upon viral infection. MoDC are derived from monocytes and infiltrate lymphoid and non lymphoid tissues upon inflammatory signals. Finally, Langerhans cells are skin-resident DC able to migrate upon stimulation to skin-draining lymph nodes. Curiously, even if Langerhans cells share different characteristics with pDC, cDC and MoDC that derived from a common precursor, their origin is prenatal (33).

DC are then responsible to detect harmful and foreign organisms and toxins, to initiate immune response against them and at the same time responsible to maintain tolerance to self. These tolerance mechanisms involve lymphocyte maturation and can be distinguished in terms of central and peripheral tolerance. In central tolerance, occurring in the thymus, the interaction between thymocytes and APC lead to a clonal deletion to self-reactive thymocytes and natural regulatory T-cell (nTreg) generation. On the other side, peripheral tolerance driven by DC is related to anergy and apoptosis induction to self-reactive T-cells but also to the generation of induced regulatory T-cells (iTreg) (34). The correct function of these cells is essential for the regulation of immune response during inflammation and homeostasis. In fact, it has been shown that the ablation of cDC, pDC and Langerhans cells in mice leads to spontaneous fatal autoimmunity (35).

There are many studies that describe different DC populations related to the tolerance maintaining such as in immune-privileged and mucosal tissues. For example, mucosal DC from the intestine are continuously exposed to food antigens and its correct regulation is necessary to avoid inflammation against foreign molecules (36). Also, in other immune privileged tissue such as the brain, it has been shown that DC presents a protective role by inhibiting T-cell proliferation (37). Apart from DC role in steady state, other studies have demonstrated that cDC can be manipulated in vivo to adopt a tolerogenic profile. In a study performed in Non obese diabetic (NOD) mice treated with granulocyte-macrophage colony-stimulating factor (GM-CSF), the authors

observed an increase in splenic $CD8\alpha^{-}CD11c^{+}$ DC. After purification, the authors demonstrated that this population impaired antigen-specific $CD8^{+}$ proliferation (38).

Altogether, DC have an essential role by linking innate and adaptive response and by polarizing the immune response. For this reason, the ex vivo manipulation appears as a potent strategy to treat different types of diseases or conditions. In fact, ex vivo immunogenic DC can be used to direct immune response against tumours, virus or in immunization meanwhile ex vivo tolerogenic-DC (Tol-DC) can be used to induce tolerance in transplantation, autoimmune disease and allergy.

3.2. Tol-DC differentiation

DC can be differentiated starting from monocytes or bone marrow. However, there are many differences between immunogenic DC and Tol-DC **(Figure 2)**. Immunogenic DC have a high expression of costimulatory molecules such as CD80, CD86 and CD40, high expression of presentation molecule HLA/MHC class II, they produce pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, tumor necrosis factor-α (TNFα) and they stimulate T-cell proliferation. On the other hand, Tol-DC display a low expression of costimulatory molecules, they are resistant to maturation, they secrete immunomodulatory cytokines such as IL-10 and transforming growth factor-β (TGFβ) and they are hypostimulatory cells toward T-cells. Nevertheless, there are common markers between immunogenic and Tol-DC such as the expression of CD11c, CD11b and the coexpression of MHC class I and MCH class II (39).

The generation of Tol-DC depends on the protocol. However, the vast majority of differentiation protocols contain GM-CSF and IL-4. GM-CSF is a growth factor well known to be a bone marrow precursor mobilization but also to induce DC differentiation (40). Interestingly, GM-CSF has a dual role between immunogenicity and tolerance. It has been shown that GM-CSF administration improves myasthenia gravis and type 1 diabetes in rodent models. On the other hand, the depletion of GM-CSF is beneficial in experimental autoimmune encephalomyelitis (EAE), arthritis, nephritis and psoriasis in rodent models (41). Despite the concrete role of GM-CSF in different diseases, this cytokine is required for DC generation in vitro. Interestingly, the

differentiation potential of GM-CSF is strongly related to its concentration. In fact, high concentrations of GM-CSF are associated to immunogenic DC meanwhile low doses to Tol-DC (42,43). IL-4 is a cytokine related to T helper cell 2 (Th2) T-cells differentiation. Primarily, IL-4 is secreted by mast cells, Th2 T-cells, basophils and eosinophils (44,45). However, this cytokine can also be released by macrophages polarized to a M2 phenotype and by DC (46). About the effects of IL-4, it has been shown that IL-4 has a protective role in EAE but a known inflammatory role in allergic inflammation (44,47). Independently from the effect, IL-4 is required in the most part of DC differentiation protocols.

There is not a unique standardized protocol to generate Tol-DC apart from the use of GM-CSF and IL-4. In fact different groups have reported the use of several others molecules to induce these cells such as cytokines, organic molecules, drugs and even genetic tools **(Table 1)** (48). The cytokines usually used to generate Tol-DC are IL-10 and TGFβ. These immunomodulatory cytokines are required in order to maintain the immature phenotype of DC (49,50). Concretely, IL-10 has been shown to induce human Tol-MoDC which spontaneously secrete high amounts of IL-10, display the capacity to impair T-cell proliferation and induce regulatory Tr1 cells (51). On the same way, the use of IL-10 and TGFβ during Tol-MoDC differentiation from type 1 diabetes (T1D) monocytes patients, have been shown to induce tolerance to insulin antigens. Phenotypically, these cells express CD83, CD1a, MHC II but not CD14 (52). Apart from cytokines, the use of small organic molecules have been shown to induce Tol-DC such as 1a,25dihydroxyvitamin D3 (VitD3) and prostaglandin E2 (PGE2) (48). VitD3 blocks maturation of immature DC upon lipopolysaccharide (LPS) stimulation. Tol-MoDC differentiated with VitD3 are unable to stimulate allogeneic T-cell proliferation. Phenotypically, these cells do not express CD1a nor CD14 (53). On the other hand, another study focused on Tol-MoDC differentiated with VitD3 showed that this differentiation lead to DC expressing DC-SIGN (CD209), CD14 but no CD1a (54). About the role of PGE_2 in Tol-DC differentiation, it has been shown that this molecule induce the expression of indoleamine 2,3 dioxygenase (IDO) in immature DC. This enzyme catalyze the conversion of tryptophan into kynurenine, a molecule related to regulatory T-cell induction and T-cell allogeneic response inhibition (55). Tol-DC can also be generated with drugs such as dexamethasone (Dex) and rapamycin (Rapa). A study focused on comparing Dex-DC and Rapa-DC demonstrated that both cells were

able to impair T-cell proliferation. However, they demonstrated that despite that both cells have immunomodulatory functions, Rapa-DC displayed a mature DC phenotype and were not able to produce IL-10 under LPS stimulation contrary to Dex-DC (56). Additionally, these inductor molecules can be used in a combination as it has been described in a clinical trial using Tol-MoDC in refractory Crohn's disease patients. In this clinical trial Dex-DC were treated with IL-6, TNFα, IL-1β and PGE_2 prior to injection. The outputs of this study showed that patients treated with Tol-MoDC displayed an increase of regulatory T-cells and a decrease in IFNγ in blood (57). Finally, Tol-DC can also be generated with genetic tools such as the use of antisense oligonucleotides (AS-ODN). A study demonstrated that the injection of Tol-DC modified with AS-ODN anti CD40, CD80 and CD86 in NOD-mice led to a delay in diabetes onset (58). Moreover, this method has been translated to human, by the same authors, in a clinical trial in T1D. Human Tol-DC generated with AS-ODN display similar characteristics than in rodents in terms of immunosuppressive function (59).

Apart from the protocols cited below, Tol-DC can also be generated with low dose of GM-CSF in absence of IL-4. In fact this protocol adopted by our group was firstly described by Lutz in 2000 in mice. Tol-BMDC derived with this protocol lead to the differentiation of Tol-DC expressing low levels of MHC II, CD40, CD80 and CD86. Moreover these cells are resistant to maturation under LPS stimuli and impaired T-cell proliferation in MLR. In the context of cell based therapy, it have been shown that tolerogenic bone marrow-derived dendritic cells (Tol-BMDC) generated with low dose of GM-CSF, increased fully allogeneic vascularized heterotopic cardiac allograft survival and minor antigen skin survival (42). This protocol has been also adapted to human from blood monocytes and highlighted a similar profile than the observed in Tol-BMDC(60). Nowadays, these Tol-MoDC generated with low dose of GM-CSF are being used in a first phase I/II clinical trial in kidney transplantation supervised by our team (61).

Altogether, even if there is not a single standardized method to generate Tol-DC we observe functional and phenotypical similarities between all these cells.

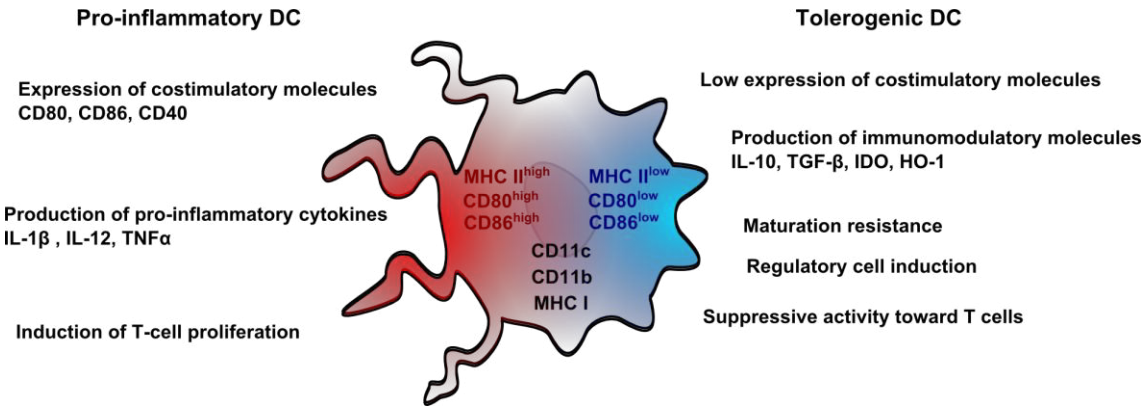


Figure 2. Differential characteristics of pro-inflammatory and Tol-DC. Pro-inflammatory DC express costimulatory molecules such as CD80, CD86 and CD40, produce pro-inflammatory cytokines such as IL1β, IL-12 and TNFα and induce T-cell proliferation. On the other hand, Tol-DC display a low expression of costimulatory molecules, produce immunoregulatory cytokines such as IL-10, TGFβ, IDO and HO-1, they are resistant to maturation stimuli, induce regulatory T-cells and impair T-cell proliferation (19).

Cell type	Differentiation protocol	Characteristics	Refference
DC10	GM-CSF, IL4 and IL-10	• Secrete high amounts of IL-10 • Impair T-cell proliferation • Induce Tr-1	Gregory et al. 2010
Tol-MoDC from T1D patients	GM-CSF, IL-4, TGFβ and IL-10	• Induce tolerance to insulin antigens • Express CD83, CD1a but no CD14	Torres-Aguilar et al. 2010
MoDC	GM-CSF, IL-4 and VitD3	• Impairs T-cell proliferation • Maturation resistance • CD1a downregulated	Penna and Adorini et al. 2000
Tol-MoDC	VitD3	• Semi mature phenotype • No expression of IL-12p70 • Express IL-10 and TNFα • Induce Treg • Impair T-cell response	Unger et al. 2009
Tol-MoDC	Dexamethasone	• Semi mature phenotype • No expression of IL-12p70 • Express IL-10 and TNFα • Induce non specific Treg • Impair T-cell response	Unger et al. 2009
Tol-MoDC	Dexamethasone	• Immature phenotype • Impair T-cell alloproliferation	Naranjo-Gomez et al. 2011
Tol-MoDC	VitD3	• Produce IL-10 • Immature phenotype • Impair T-cell response	Naranjo-Gomez et al. 2011
Tol-MoDC	Rapamycin	• Impair T-cell alloproliferation • Mature DC phenotype • No IL-10 production • Promote Treg	Naranjo-Gomez et al. 2011
Tol-MoDC	GM-CSF, IL-4, IL-6, TNFα, IL1β, PGE_2	• Expand Treg • Reduce IFNγ in blood	Jauregui-Amezaga et al. 2015
Tol-BMDC and Tol-MoDC	GM-CSF, IL-4, AS-ODN (CD40, CD80, CD86)	• Maturation resistance • Low production ol IL-12p70, TNFα and nitrites. • Delay diabetes onset	Manchen et al. 2004 Giannoukakis et al. 2011
Tol-BMDC	Low dose of GM-CSF	• Low expression of costimulatory molecules (CD40, CD80 and CD86) • Maturation resistant	Lutz et al. 2000

		• Impair T-cell response • Induce Treg	
Tol-MoDC	Low dose of GM-CSF	• Low expression of costimulatory molecules (CD40, CD80 and CD86) • Maturation resistant • Impair T-cell response • Induce Treg	Chitta et al. 2008 and Moreau et al. 2012

Table 1. Types of Tol-DC differentiation and characteristics.

3.3. Source for Tol-DC

In order to move Tol-DC to clinic in the context of transplantation, there is a dilemma about from which source Tol-DC must be generated. Donor or recipient? Differently to autoimmune diseases, transplantation involves antigens from the donor. This implies an exposure of allopeptides and foreign MHC molecules that can potentially start an allogeneic response leading to organ rejection (62). Therefore, in order to avoid rejection, two strategies have been considered: to infuse donor or recipient cells.

Infusion of donor antigens is a practice currently used in clinic in kidney transplantation. This infusion, named donor specific transfusion (DST), consists in the transfusion of donor blood to the recipient in order to stimulate tolerance to alloantigens. This strategy has been proved to increase allograft survival. In fact, a study performed in living donor kidney transplantation aimed to compare DST therapy combined with immunosuppressive drugs, demonstrated that in DST group there was a decrease in patients with acute rejection and an increase in patients with optimal renal function at 1 and 10 years after transplantation (63). However, the dissemination of allogeneic proteins after transplantation is unavoidable, and the sensitization against HLA molecules can become a risk factor for allograft rejection (64).

In the same context than DST, the use of Tol-BMDC initially was studied using the donor source. Lutz et al. demonstrated that, 7 days pretreatment with donor Tol-BMDC from B10 mice differentiated with low dose of GM-CSF, increased B10 allograft survival in CBA recipient mice. The 70% of treated mice achieved allograft survival for 100 days meanwhile those receiving Tol-BMDC from B10 and cardiac allograft from NZW mice or receiving BMDC generated with GM-CSF and IL-4 increased graft survival only in a 20%. Moreover in this study the authors demonstrated that T-cells were unresponsive to allogeneic Tol-BMDC in vitro after restimulation with APC from the same source but not to polyclonal restimulation, suggesting an antigen-specific

unresponsiveness (42). Similarly, another study demonstrated that donor Tol-BMDC generated with low dose of GM-CSF in combination with antilymphocyte serum (ALS), were able to increase rat cardiac allograft until 200 days on the 50% of treated individuals. The authors also confirmed in rats what it was shown in mice, T-cells purified from rats treated with donor Tol-BMDC and ALS, were unresponsive to donor antigens. Both models coincide on the induction of a antigen specific tolerance (65). Nevertheless, a later study demonstrated that the antigen specific tolerance was mediated by the presentation of donor Tol-BMDC allogeneic peptides, from apoptotic bodies or donor cells, by recipient APC. In this study, using apoptotic bodies from Tol-BMDC and Tol-BMDC from donors, the authors showed the same characteristics observed in previous works, a $CD4^{+}$T-cell hyporesponsiveness and an increase in regulatory T-cells (66). Moreover, another study demonstrated that, in vitro, immunosuppressive properties of allogeneic Tol-BMDC differs from in vivo. In this work, the authors demonstrated that allogeneic Tol-BMDC, failed to induce tolerance and even accelerate rejection in mice model of skin and heart transplantation. On the other hand, these donor Tol-BMDC were able to induce regulatory T-cells and impairing of T-cell proliferation in vitro. In vivo, this increase of allograft rejection was mediated by recipient APC that present alloantigens from donor-BMDC alloantigens (67). In order to avoid the risk of sensitization, and due to their efficacy demonstrated in vivo, the use of autologous Tol-DC appears to be a better strategy in terms of safety.

In order to determine the efficacy of autologous Tol-DC several studies have been performed. The first one in 2005, a study performed by our team demonstrated that the adherent fraction of recipient BMDC generated with GM-CSF and IL-4 was able to prolong cardiac allograft survival. Moreover, the authors demonstrated that this adherent fraction displayed an immature phenotype and was resistant to maturation. Comparing to donor adherent BMDC, autologous BMDC were more efficient delaying allograft rejection. Additionally in this work, the authors demonstrated that cell injection, 1 day before the procedure was enough to induce tolerance, comparing to the 7 days observed in previous works (68). After this study, our group demonstrated also that the treatment with autologous-Tol-BMDC in combination with suboptimal doses of nuclear factor-kappa-ligh-chain-enhancer of activated B-cells (NF-κB) inhibitor LF15-0195, achieved definitive allograft acceptance. Moreover, as it was shown in donor Tol-BMDC studies, the tolerance was donor-specific (69). As it will be described in the

following section, we confirm the efficacy of Tol-BMDC in mice using combined therapy. These results altogether demonstrate the superiority of autologous Tol-DC comparing to donor Tol-DC in terms of safety and efficacy in animal models.

3.4. Tol-DC in combined therapy

As it was previously described, Tol-DC therapy can be improved by combining with immunosuppressive drugs such as ALS or LF15-0195 but also anti-CD3 and rapamycin **(Table 2)**. The NF-κB blocking agent LF15-0195 administered alone, increases cardiac allograft survival in rats (70). In combination with autologous BMDC, low dose of LF15-0195 led to an increase in cardiac allograft survival in 92% of treated rats compared to the groups treated with Tol-BMDC alone or rapamycin alone or in combination with Tol-BMDC (69). Another specific target for combined therapy is CD3. Indeed, the use of monoclonal antibody anti-CD3 alone in transplantation models in mice lead to an increased survival of pancreatic islet, skin and cardiac allograft (71,72). In our group, we previously demonstrated that combined therapy using anti-CD3 and autologous-BMDC led to an increase in pancreatic islet allograft survival. Additionally, we demonstrated that the increase of allograft survival was related to a decrease in $CD4^+/CD8^+$ T-cell frequency and an increase in regulatory T-cells. In this model, regulatory $CD4^+CD25^+FoxP3^{hi}$ T-cells are essential for the immunosuppressive function and their depletion with anti-CD25 antibody lead to a reduced allograft survival (73). Additionally, we confirmed that anti-CD3 and autologous Tol-BMDC combined therapy increased allograft survival in minor antigen mismatch skin transplantation. Interestingly, in this study, our group found an increase in $CD8^+CD11c^+$T-cells, a population with regulatory functions (74). Another molecule with synergistic potential with Tol-BMDC is the rapamycin. In combination, Tol-BMDC generated with rapamycin and pulsed with donor antigens and the post-operative use of low dose of rapamycin increased heart allograft survival in mice. In this model, an increase of $CD4^+CD25^+FoxP3^{hi}$ T-cells was observed. Moreover the adoptive transfer of this regulatory T-cells to naïve mice receiving heart allograft led to an increase in allograft survival, demonstrating that the tolerance induced by Tol-BMDC generated with rapamycin was donor specific (75). Altogether these results demonstrated that combined therapy increase efficacy of Tol-DC.

Combined drug	Model	Output	Refference
LF15-0195	Cardiac transplantation in rat	• Increase of allograft survival comparing to rats treated only with Tol-BMDC	Bériou et al. 2002
Anti-CD3	Pancreatic islet, skin and cardiac allograft in mice	• Increase allograft survival • Decrease $CD4^+$ and $CD8^+$T-cell frequency • Increase Treg	Baas et al. 2014, Segovia et al.2014
Rapamycin	Heart allograft in mice	• Increase heart allograft survival • Increase of Treg	Turnquist et al. 2007

Table 2. **Drugs used in combination with Tol-DC generated with low dose of GM-CSF and characteristics.**

3.5. Tol-DC administration route and NHP assays

Autolgous Tol-DC have been shown to be efficient and safe in rodent models. However, before to move to clinic, there are two practical questions. Are they safe in non-human primate model (NHP)? Which administration route is the most convenient? To ensure the safety of these cells, several works have been performed in NHP. A first study performed in rhesus macaques in kidney transplantation model demonstrated that donor Tol-BMDC generated with VitD3 and IL-10, injected 7 days before the transplantation and cotreated with cytotoxic T-lymphocyte associated protein 4 (CTLA4)-Ig increased the median graft survival compared to control group. In this study, Tol-BMDC were injected intravenously (IV) (76). Several years later, the same authors demonstrated in NHP model that autologous Tol-MoDC pulsed with allogeneic cell membranes from donor monocytes increased kidney allograft survival comparing to unpulsed group. The authors demonstrated also that this increase of allograft survival was associated to antigen specific T-cell hyporesponsiveness resulting in a decrease in systemic IL-17 (77). Similarly, other studies confirmed the safety and efficacy of Tol-BMDC in NHP. In fact, in our group we have been demonstrated that autolgous Tol-BMDC can be beneficial in gene therapy. Concretely, we demonstrated that Tol-BMDC reduce immune response against transgene product in NHP. In this study Tol-BMDC were injected IV or intradermally (ID). The results demonstrated that IV administration route favors immune tolerance against transgene product (78). Additionally, the already performed clinical trials using Tol-MoDC demonstrated the safety of other

administration routes such as ID (59,79), intraperitoneal (IP) (57), IV and intra-articular (IA) (80).

3.6. Tol-DC migratory skills

In order to understand the multiple mechanisms described in Tol-DC, it is first essential to conceive the migratory skills of these cells. It is already well established that DC are cell with highly migratory capacities. In fact, at the immature state DC express chemokine receptors such as CCR2, CCR5, CCR6, CXCR4 and CXCR3 that allow them to migrate to inflammatory sites expressing chemokines such as, CCL2, CCL5 and CCL20. Once in the inflammatory site, DC uptake antigen and maturate leading to the expression of CCR7. This chemokine receptor allows DC to migrate to the lymph nodes through CCL19 and CCL21 chemoattraction. However, not all DC migrate to lymph nodes, several migrate to other lymphoid organs such as thymus, bone marrow and spleen (81). Interestingly, Tol-DC display similar migratory skills than classical DC. Recently, a study performed in experimental autoimmune encephalomyelitis (EAE) model demonstrated that after IV injection, myelin oligodendrocyte glycoprotein (MOG) pulsed-Tol-BMDC generated with GM-CSF and VitD3 migrated preferentially to liver and spleen. Moreover, Tol-BMDC remained stable in liver and spleen during the next 7 days. In a minor quantity, Tol-BMDC were found also in bone marrow, lymph nodes and thymus (82). Another study, using lentiviral vectors coding for IL-10 and CCR7 demonstrated that only donor BMDC transduced with both vectors were able to migrate to lymph nodes and spleen and prolong cardiac allograft. DC transduced only with CCR7 or IL-10 were unable to increase allograft survival (83). Altogether, these studies demonstrated that Tol-DC have similar migratory skills such as inflammatory DC.

3.7. Tol-DC mechanisms

As it was previously described, Tol-DC have the ability to impair T-cell proliferation and to induce regulatory T-cells in order to regulate the immune response and prolong allograft survival. However, due to the different protocols to generate these cells, and depending on the origin, bone marrow or monocytes, there is not a general consensus for molecular mechanisms that lead to tolerance. Usually, Tol-DC impair T-cell

response by inducing apoptosis, anergy or hyporesponsiveness. However, there are many proposed mechanism that can drive T-cells to these states. These mechanisms could be separate in contact-dependent and contact-independent mechanism. Contact-dependent mechanisms include several molecules such as programmed death-ligand 1 (PD-L1), Fas-L and inducible T-cell costimulator-ligand (ICOS-L) among others. Contact-independent mechanism include cytokines such as IL-10 and TGFβ, small molecules such as PGE_2 but also other mechanism related to nutrient deprivation such as inducible nitric oxide synthase (iNOS), arginase-1 (Arg-1) and IDO (84). However, it is essential to note that the following mechanisms required usually other stimuli or surface markers to drive the tolerogenic effect (85). In this part different mechanisms observed in different Tol-DC both from humans and rodent generated with different protocols will be described **(Figure 3)**.

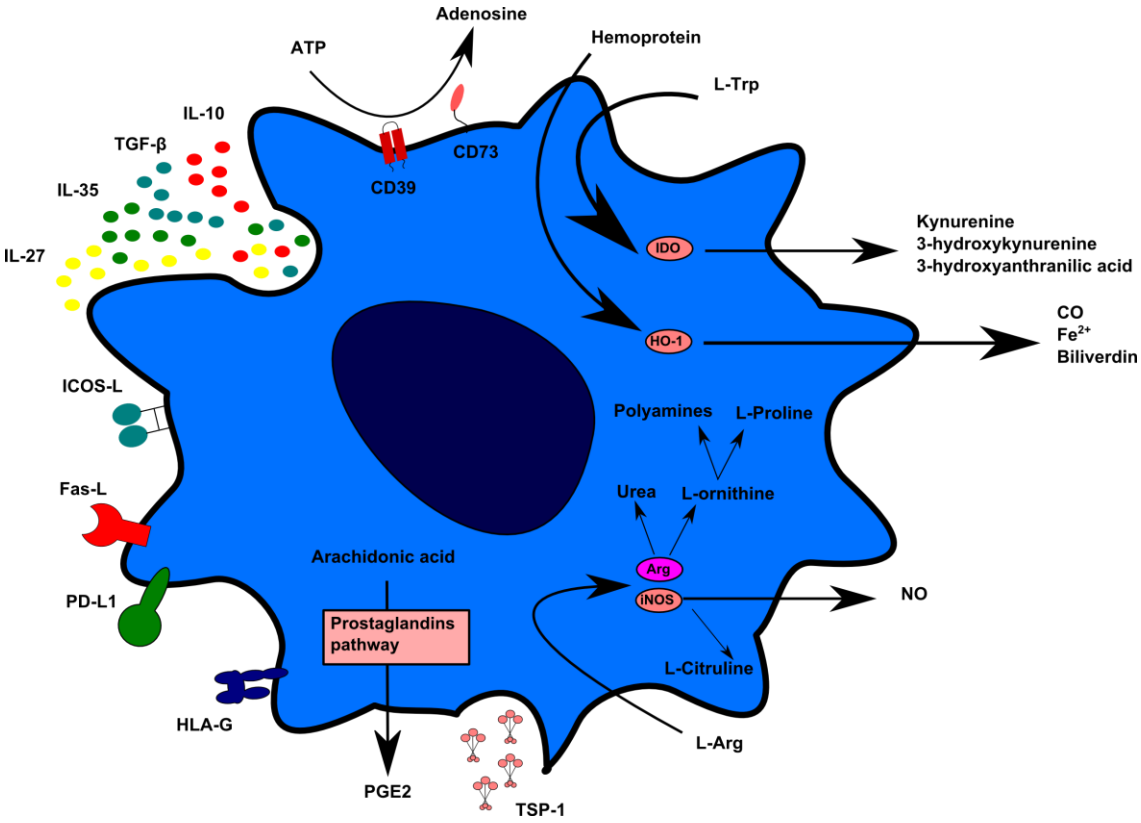


Figure 3. Tol-DC immunosuppressive mechanisms. Tol-DC can drive their immunosuppressive potential through different types of mechanisms. Tol-DC can impair T-cell proliferation through contact dependent mechanisms such as Fas-L, ICOS-L, PD-L1. On the other hand they can act through contact-independent mechanisms such as by the expression of immunomodulatory cytokines or proteins (IL-10, TGFβ, IL-35, IL-27 and TSP-1), small molecules (Adenosine, PGE_2, CO and Kynurenines and

their derivates) or by the nutrient deprivation mechanisms (Arginine or tryptophan deprivation) (19).

3.7.1. Contact-dependent mechanisms

Contact-dependent mechanisms are those who require cell-to-cell contact. This type of mechanism includes molecules such as ICOS-L, PD-L1, Fas-L, Ig-like transcript-2 (ILT2), ILT3, ILT-4 and HLA-G. ICOS-L is a protein expressed by immature DC. This ligand can interact with his receptor ICOS, expressed by T-cells, leading to an hyporesponsiveness not recovered after restimulation (86). Another molecule related to allograft tolerance and Tol-DC is PD-L1. In fact, the blockade of PD-L1 has been associated to an accelerated skin allograft rejection and cardiac allograft rejection (87,88). About the role of PD-L1 in Tol-BMDC, a recent study demonstrated that DC transfected with PD-L1 were able to induce an increase of kidney allograft survival. Interestingly, the authors showed that this improvement was related to a decrease in $CD8^{+}$T-cell proliferation and a decrease in pro-inflammatory cytokines production (89). Fas-L, is a contact-dependent mechanism usually related to apoptosis induction. A study performed using BMDC transfected with pBK-CMV coding for Fas-L demonstrated an increase of cardiac allograft survival in mice. Moreover, in this work the authors showed in vitro, that BMDC transfected with Fas-L impaired T-cell proliferation through apoptosis induction (90). Finally, ILT-4 in association with HLA-G are related to regulatory T-cells induction. Moreover, it has been demonstrated that ILT2/ILT4 and HLA-G in Tol-DC impaired T-cell proliferation as well (91).

3.7.2. Immunomodulatory cytokines

Several studies have described cytokines associated to the immunomodulatory effects of Tol-DC such as IL-10, TGFβ, IL35 and IL27. These cytokines have been related not only to T-cell impairment and regulatory T-cell induction, but also to DC maturation inhibition.

IL-10 is a regulatory cytokines essential for the induction of several regulatory populations in vitro and in vivo. This cytokines has been detected in different Tol-DC populations. For example, MoDC generated with IL-10 has been shown to secrete spontaneously IL-10 (92). However, other Tol-DC do not secrete spontaneously IL-10 but need to be stimulated with a pro-inflammatory stimuli such as Dex- and VitD3-Tol-MoDC (54). About the effect, it has been shown that IL-10 impairs T-cell proliferation by leading to a state of anergy and by inducing Tr1 cells (93).

TGFβ, is another well known immunoregulatory cytokine. This cytokine has been related to $CD4^+$ and $CD8^+$ T-cell inhibition and also as an essential factor for regulatory T-cell induction. The relevance of TGFβ in immunology is present in different studies. For example, one of them demonstrated that the lack of TGFβ signaling led to the development of autoimmune inflammatory disease driven by an uncontrolled $CD4^+$ T-cell proliferation (94). TGFβ has been also observed in Tol-DC, resulting essential for the role of tolerance induction (95). Moreover, another study, performed by our group in rat model, demonstrated that the induction of tolerance by LF15-0195 is associated to an increase in *tgfb* expression in allograft from tolerant rats. The adoptive transfer of splenocytes from tolerant rats to syngeneic rats receiving cardiac allograft, treated with rapamycin and blocking TGFβ demonstrated that this cytokine is partially responsible for this tolerance induction (96).

Apart from IL-10 and TGFβ, there are other cytokines that have been related to Tol-DC mechanisms such as IL-27 and IL-35. These two cytokines share the same subunity, the Epstein-Barr virus-induced gene 3 (EBI3). IL-35 is a heterodimeric protein formed by EBI3 and IL12p35. This cytokine is mainly produced by Treg but several studies have been demonstrated that can also being secreted by several APCs. In fact, a study demonstrated that IL-35 but not the others IL-12 members is produced by Dex-MoDC. Moreover, in this study the authors demonstrated that the silencing of *il12a* (IL-12p35) partially abrogated the effect of Tol-BMDC toward $CD4^+$T-cells (97). On the other hand, IL-27 is an heterodimer formed by EBI3 and IL27p28. It has been shown that IL-27 display multiple immunoregulatory functions such as impairing effector T-cell response, controlling neutrophil migration and impairing oxidative burst (98). However, IL-27 have been also associated to enhanced antitumour activity toward $CD8^+$T-cells, suggesting a dual role in immunogenicity and immunoregulation (99). In

transplantation, the role of IL-27 in association with TGFβ has been shown to increase cardiac allograft survival (96). Additionally to the studies about IL-35 and IL-27, it has been shown by our group that Tol-BMDC express EBI3, and this expression is related to an increased cardiac allograft survival. In this work we showed that rat treated with Tol-BMDC and low dose of IS displayed an increased population of splenic $TCR\alpha\beta^{+}CD3^{-}CD4^{-}NKRP1^{-}$DN T-cells expressing IFNγ. Moreover, the blockade of EBI3 or IFNγ abrogated the suppressive effect of Tol-BMDC suggesting an immunoregulatory network between Tol-BMDC and DN T-cells, essential for tolerance induction in this model (100).

3.7.3. Nutrient deprivation and other mechanisms

Apart from contact-dependent mechanisms and cytokines, there are many other molecules and mechanisms involved in immunoregulation such as the synthesis of small molecules and nutrient deprivation. These mechanisms have been also observed in different types of Tol-DC.

About nutrient deprivation mechanisms, there are several type of deprivation related to immunosupression and Tol-DC such as Arg-1, iNOS and IDO. iNOS and Arg-1 are two enzymes commonly associated to macrophage polarization. Concretely, iNOS is related to M1 polarization meanwhile Arg-1 is associated to M2 macrophages. The function of iNOS is to generate nitric oxide (NO) and citruline from L-Arginine. Usually, iNOS is associated to pro-inflammatory response, due to reactive nitrogen species (RNS) such as NO are able to peroxidize membrane lipids in order to eliminate inflammatory agent. Arg-1 on his part, metabolize L-arginine to ornithine and urea. This enzyme is usually associated to anti-inflammatory response and is also related to healing phase after inflammation. In fact, the products of Arg-1 lead to the synthesis of L-Proline, an aminoacid necessary for collagen synthesis in tissue healing (101,102). In transplantation models, Tol-BMDC iNOS expression has been shown to impair T-cell proliferation in mice. In a study performed by our group, we showed that splenic T-cells purified from grafted and Tol-BMDC treated mice displayed a low proliferation in vitro. To demonstrate the role of iNOS, an inhibitor of iNOS (L-NMMA) was injected in grafted mice receiving Tol-BMDC. The use of L-NMMA allowed the recovery of T-cell

proliferation in treated mice (68). On the same way, another study demonstrated the implication of iNOS in tolerance and T-cell proliferation. In this study, Tol-BMDC from control and inos$^{-/-}$ mice were differentiated with retinoic acid (RA) and pulsed with OVA peptide in order to induce a lymphoproliferative response in OT-II T-cells. The authors showed that inos$^{-/-}$ Tol-BMDC were unable to impair T-cell proliferation comparing to control Tol-BMDC. This work demonstrated the immunosuppressive role of Tol-BMDC expressing Arg-1 and iNOS (103).

Another enzyme related to nutrient deprivation is IDO. This enzyme metabolizes tryptophan to kynuernine. IDO has been related to T, B and NK proliferation and regulatory T-cell induction. On the other hand, IDO expression has been also associated to DC differentiation, suggesting a dual role in immunogenicity and immunoregulation (104). However, the suppressive mechanism mediated by IDO has been associated to the products of tryptophan metabolism. In fact, a study performed in DC transfected with adenovirus coding for IDO demonstrated that T-cell hypoproliferation was associated to the IDO products 3-hydroxykynurenine, 3-hydroxyanthranilic acid but no anthranilic acid nor quinolinic acid (105). In transplantation model, a recent study demonstrated that IDO^{+}BMDC improved cardiac allograft survival in rodents by impairing CD4^{+}T-cell proliferation through the induction of apoptosis (106).

Similarly to IDO the synthesis product of Heme oxygenase-1 (HO-1) has been also associated to immunoregulation and Tol-BMDC. HO-1 catalyzes the reaction Fe-Protoporphyrin-IX (Heme group) to biliverdin, ferrous iron and carbon monoxide (CO) (107). CO has been related to a protective and anti-apoptotic role in several types of cells. However, in lymphocytes it has been associated to proliferation and activation inhibition (108,109). This immunomodulatory effect was already demonstrated in several studies by the use of HO-1 inductor, the cobalt protoporphyrin (CoPP). In fact, a study performed in pancreatic islet transplantation in mice demonstrated that the pretreatment of allograft or mice recipient with CoPP or with CO improved allograft survival. Interestingly, the treatment of both, allograft and recipient, results in an increased rejection delay (110). In DC, HO-1 has been detected in immature DC. Our group, we demonstrated that immature DC stimulated with HO-1 inductor CoPP, preserved the immature DC state and leads to DC with low production of IL-12p70, increased production of IL-10 and the ability to impair T-cell proliferation in human and rats (111). In transplantation model, our group demonstrated that HO-1 was involved in

tolerance induced by Tol-BMDC. In this study we demonstrated that heart allograft survival increased by the use of Tol-BMDC was abrogated by the use of HO-1 inhibitor tin protoporphyrin IX (SnPP) (112).

Alternatively, other molecules have been associated to Tol-DC and immunoregulation such as thrombospondin-1 (TSP-1), PGE_2 and adenosine. In fact, a study aimed to compare Tol-MoDC differentiated with different cytokines such as IL-10, IL-10/TGFβ and IL-10/IL-6 demonstrated the role of these molecules in vitro. The outputs of this study demonstrated that IL-10/TGFβ generated Tol-MoDC lost its suppressive potential in presence of ARL67156 (CD39 inhibitor, enzyme owing to the extracellular pathway of adenosine synthesis) or indomethacin (PG inhibitor synthesis). On the other hand, the suppressive potential of IL-10 and IL-10/IL-6 differentiated Tol-MoDC was abrogated by IL-10 and TSP-1 inhibition (113).

Altogether, Tol-DC can act through different mechanism including those that are dependent or independent from contact. Moreover, tolerance induced by Tol-DC can also be associated to combined mechanisms and even other still unknown.

3.8. Tol-DC immunosuppressive cellular mechanisms

As it was already described in different transplantation models, Tol-DC are able to prolong allograft survival. On one hand, the delay of graft rejection is driven bythe inhibition of allograft-specific T-cell proliferation. On the other hand, the long term survival of the graft is controlled by allograft-specific regulatory T-cells. Transplantation models using Tol-DC have been demonstrated that they are able to induce different types of regulatory T-cells such as classical $CD4^+CD25^+FoxP3^{hi}$ as well Tr1 cells, $CD8^+$Treg, DN Treg (114) and Breg (59) **(Figure 4)**

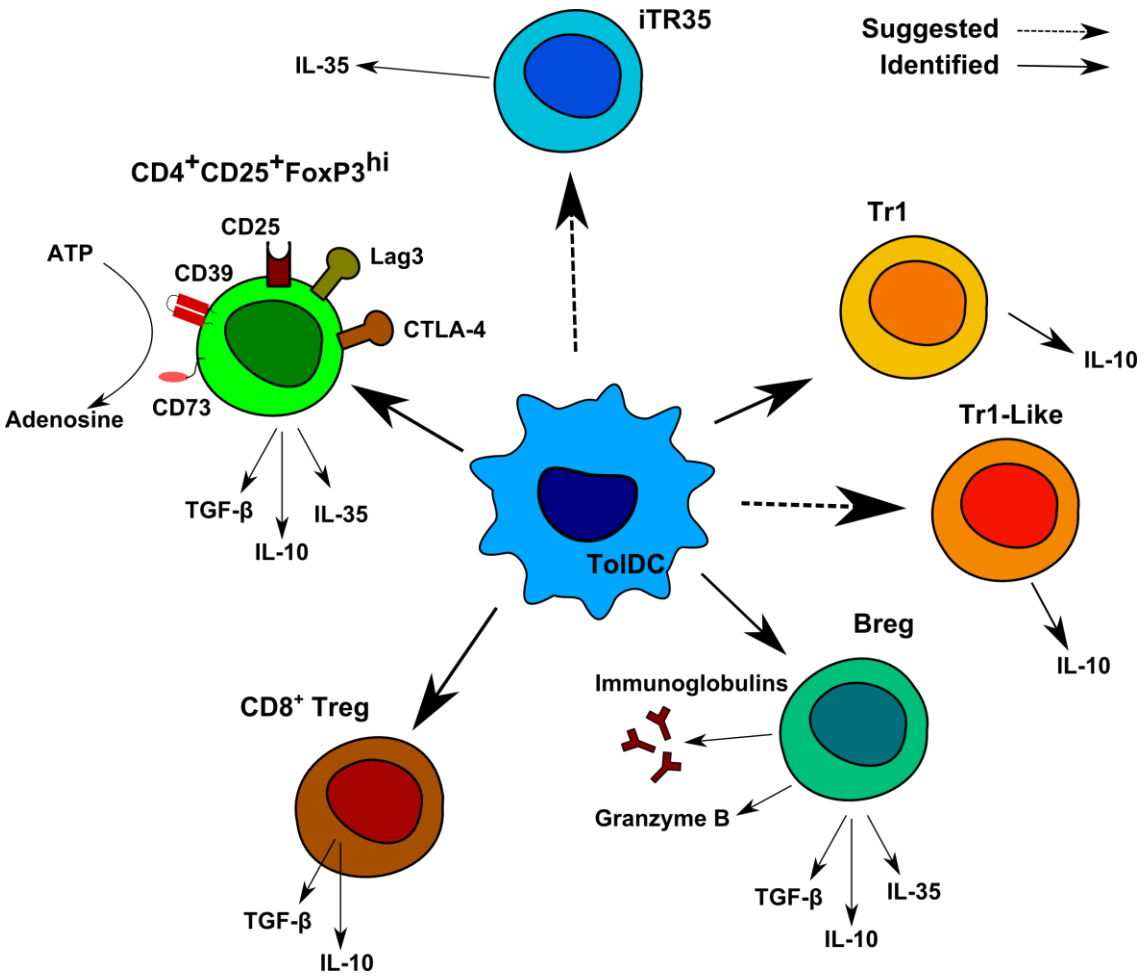


Figure 4. Regulatory cells induced by Tol-DC. Tol-DC can induce regulatory cells such as $CD4^+CD25^+FoxP3^{hi}$ T-cells, Tr-1, $CD8^+$Treg and Breg. The immunosuppressive potential of these induced-cells contribute to the regulation of inflammation. The induction of iTr35 through Tol-DC remains unclear(19)**.**

3.8.1. $CD4^+$ regulatory T-cell induction

The potential of allograft-specific $CD4^+CD25^+FoxP3^{hi}$ T-cells in transplantation has been already demonstrated. In fact, it has been demonstrated that the transplantation of skin allograft from tolerant mice onto new recipient receiving donor skin allograft lead to transfer of tolerance. This effect was not observed when third party skin allograft was used. This experiment suggest, a donor specific tolerance transferred through regulatory T-cells (115). Classical regulatory $CD4^+CD25^+FoxP3^{hi}$ T-cells usually display several immunosuppressive mechanisms. For example, they express immunosuppressive-related molecules such as CTLA-4 and Lag3. These molecules have been related to the impairment of dendritic cell antigen presentation to T-cells. Treg also secrete

immunomodulatory cytokines such as IL-10, TGFβ and IL-35. Moreover, the high expression of CD25 also leads Treg to compete with effector T-cells for IL-2. IL-2 deprivation in effector T-cells impairs proliferation and induce apoptosis (116,117). Finally, several studies have shown that Treg synthesize adenosine, an immunomodulatory molecule, through the ectoenzymes CD39 and CD73 (118). In transplantation models, the induction of $CD4^{+}CD25^{+}FoxP3^{hi}$ T-cells has been described by several groups using Tol-DC therapy. For example, it has been shown in heart allograft model in mice that, the treatment with Tol-BMDC generated with rapamycin and pulsed with donor antigens increased were able to increase $CD4^{+}CD25^{+}FoxP3^{hi}$ population. Additionally, the adoptive transfer from tolerant mice to syngeneic mice transplantated with heart allograft from the same source improved heart allograft survival, suggesting that this induced $CD4^{+}CD25^{+}FoxP3^{hi}$ population was donor-specific (75). In addition, a recent study performed by our group demonstrated that, the treatment with Tol-BMDC and anti-CD3 in pancreatic islet allograft model lead to an increase in $CD4^{+}CD25^{+}FoxP3^{hi}$ population in lymph nodes, spleen and graft (73).

Another regulatory $CD4^{+}$T-cell population observed in Tol-DC models were Tr1 and Tr1-like cells (114). Tr1 cells are known to express CD49b and Lag3 but no FoxP3 and secrete high amounts of IL-10 (119). About its generation, it has been shown that Tol-MoDC generated with IL-10 induce Tr1 cells. Moreover, this induction is driven by HLA-G/ILT4 pathway (51). In vivo, Tr1 have been related to an important role by sustaining graft $CD4^{+}CD25^{+}FoxP3^{hi}$ from the spleen in pancreatic islet allograft model (120). Tol-MoDC generated with VitD3 has been shown to induce Tr1-like cells leading to the inhibition of allogeneic T-cell proliferation (121). Interestingly, the induction of this Tr1-like is driven by PD1/PD-L1 pathway (54). On the other hand, another study demonstrated that Tr1-like cells ($IL\text{-}10^{+}FoxP3^{-}CTLA4^{+}CD25^{hi}Egr2^{+}$) can be induced from anergic $IL\text{-}10^{-}FoxP3^{-}CTLA4^{+}CD25^{+}Egr2^{+}$ T-cells following immature DC interaction. These Tr1-like cells are able to inhibit T-cell proliferation in vivo and in vitro in a antigen-specific manner (122).

Finally, another regulatory $CD4^{+}$T-cell population potentially associated to Tol-DC therapy is iTR35. iTR35 are a regulatory population that lead his immunoregulatory potential through IL-35 but not IL-10 or TGFβ production. Similarly to Tr1, these cells do not express FoxP3. About iTR35 induction, they can be generated in vitro by addition of IL-10 and IL-35. In vivo these cells has been observed in intestine infection

and cancer (123). About IL-35 expression, this cytokine is also highly expressed by Tol-MoDC generated with dexamethasone and stimulated with IFNγ, CD40-L or LPS (97). However, today the induction of iTR35 by Tol-DC in vivo remains a conjecture.

3.8.2. Non $CD4^+$ regulatory T-cell induction

Tol-DC have been also related to the induction of other non $CD4^+$ T-cell population such as regulatory $CD8^+$ T-cells and regulatory B-cells. Regulatory $CD8^+$T-cell have been less characterized than $CD4^+$ T-cells, however their contribution in immunosuppression is not trivial. In mice and humans, the splenic population $CD8^+CD122^+PD\text{-}1^+$, a regulatory population, has been related to an increase in allograft survival (124). Moreover this population has been also associated to an anti-inflammatory function in other models (Dai et al., 2010). $CD8^+$Treg have been observed in several transplantation models using Tol-BMDC therapy. For example, a recent study performed in NHP treated with CTLA4-Ig and donor-Tol-BMDC prior to kidney transplantation showed that treated NHP presented an increased proportion of $Eomesodermin^{lo}CTLA4^{hi}CD8^+$T-cells. This $CD8^+$Treg population was associated to an improvement in allograft survival (126). Moreover, in our group we demonstrated in allograft skin transplantation model that, autologous Tol-BMDC and low dose of anti-CD3 an increase in $CD8^+CD11c^+$T-cells in treated mice. Interestingly, the adoptive transfer of this $CD8^+$Treg population was able to increase allograft survival in new transplanted mice. These results suggest that $CD8^+CD11c^+$T-cells induced by Tol-BMDC can display an important role in donor-specific tolerance (74). Finally, in humans it has been demonstrated in a clinical trial aiming to use immature pulsed DC to immunize against influenza matrix peptide resulted in an induction of CD8+T-cells with antigen-specific suppressive activity (127).

Regulatory B-cell for its part have been also observed in Tol-DC in transplantation models. Different types of Breg have been decribed from immature state to plasma cells. Similarly to T-cells, their suppressive role has also been associated to contact-dependent mechanisms, IL-10, TGFβ and IL-35 expression and cytotoxic activity through Granzyme B (GrzB). Also, contrary to Treg, Breg immunosuppressive potential have been also related to immunoglobulin secretion (128). In transplantation, the tolerogenic effect of B-cells have been already demonstrated in several studies in

rodents (129,130). In fact, a study demonstrated that the adoptive transfer of splenic B-cells from tolerant animals were able to delay rejection in both heart allograft in rats and skin allograft in mice (131,132). In humans, it has been reported an increase in peripheral B-cells in transplatanted patients with stable kidney graft and without immunosuppression(133,134). Interestingly, B-cells stimulated with CpG and CD40 are able to inhibits $CD4^+CD25^-$ polyclonal proliferation through contact-dependent mechanisms involving GrzB production(135). In the same context than tolerant patients, an increase in $B220^+CD11c^-$ B-cells in blood patients during the first 6 weeks of treatmentin was found in a clinical trial performed in T1D patients treated with Tol-MoDC(59). Later, the same group already demonstrated in NOD mice models after Tol-BMDC treatment an expansion of pre-existing IL-10^+B-cells. The authors suggested that this expansion of IL-10^+B-cells could be related to the retinoic acid production by Tol-BMDC (136). However, the role of Tol-DC induced Breg in allograft tolerance remains unclear.

Altogether, these results demonstrated that Tol-DC therapy is able to induce a large spectrum of regulatory cells in order to control the long term tolerance of transplanted organ in animal and humans.

3.9. Tol-DC in clinical trials

Apart from all the works cited above, the use of DC for therapy has a background of more than 20 years. In fact, the first trial with DC dates back at 1995. In this trial, three immunocompentent patients with advanced melanoma were treated with autologous DC pulsed with MAGE-1 nonapeptide. The rational of this work was based on the fact that DC could present MAGE-1 peptides to T-cells and enhance the anti-tumour response. However, the outputs of this work do not reveal any therapeutic response. Nevertheless, the intradermal injection of autologous DC seemed reasonably safe (137). Further, in 1999, it was observed for the first time a biological effect lead by DC therapy. In this work, nine healthy individuals were injected subcutaneously with DC pulsed with keyhole limpet hemocyanin (KLH), HLA-A*0201-positive restricted influenza matrix peptide (MP) and tetanus toxoid (TT). The results showed a sensitization against these antigens observed by antigen-specific proliferation of $CD4^+$ T-cells against KLH, TT and $CD8^+$ T-cell specific response against MP in most individuals (138). After this

work, the same team demonstrated that the injection of immature DC pulsed with MP and KLH lead to a $CD8^+$ antigen specific hyporesponsiveness. Moreover, they observed an increase in IL-10 producing cells among MP specific stimulation. These results suggested for the first time the immunoregulatory potential of DC for therapy (139). During the following decade, DC therapies were mainly focused on cancer and anti-viral immunotherapy rather than in autoimmune diseases and transplantation **(Table 3)**. It was not until 2011 that the first clinical trial using Tol-MoDC in T1D patients was performed. In this double-blind study, 10 healthy insulin-requiring type I diabetic patients were treated with Tol-MoDC and then monitored during one year. In this study, Tol-MoDC were generated by genetic modification using ODN anti-CD40, anti-CD80 and anti-CD86, injected intradermally and at biweekly frequency for a total of 4 administrations. Groups were separate between those who received ODN modified Tol-MoDC and those who receive control Tol-MoDC. Any adverse effect was observed during the trial. On the other hand, the only parameter suggesting biological effect was an increase of $B220^+CD11c^-$ B-cells during the first 6 weeks (59). After this work, another clinical trial was performed, this time in rheumatoid arthritis (RA) patients. In this study, 18 HLA-postive RA patients with minimal disease activity were treated with Tol-MoDC. In this trial, patients were treated with a single dose containing 1 or 5 millions Tol-MoDC. Tol-MoDC were generated with Bay11-7082, an NF-κB inhibitor, and pulsed with 4 citrulinated peptides antigens named "rheumavax". The outputs of this study showed, on one side the safety of therapy and on the other side, a biological effect. The authors observed an increase in circulating regulatory T-cells, a decrease of IL-15, IL-29, CX3CL1 and CXCL11 in serum and a decrease of IL-6 production by T-cell response to $vimentin_{447-455}$-Cit450 (79). A third clinical trial was performed in refractory Crohn's disease patients. In this study patients received Tol-MoDC generated with dexamethasone and stimulated with cytokine cocktail containing IL-1β, IL-6, TNFα and PGE_2. The administration route was intraperitoneal and cohorts were split by patients receiving 2, 5 or 10 millions cells in a single dose or biweekly. This clinical trial was monitored for one year. The outputs of this study showed an increase in circulating regulatory T-cells and a decrease in IFNγ. However, the authors do not demonstrated any correlation with the clinical response (57). Finally, the most recent study was performed in rheumatoid and inflammatory arthritis patients. In this clinical trial, 13 patients were divided in 4 cohorts, 3 receiving 1, 3 and 10 million Tol-MoDC

and a control cohort receiving saline solution. Tol-MoDC used in this study were generated with dexamethasone and VitD3 and loaded with synovial fluid. The outputs of this work showed the feasibility and safety of the therapy. About biological effect, the scores of hypertrophy, vascularity and synovitis were stable in all patients. Nevertheless, two individuals receiving 10 million Tol-MoDC displayed a decrease in synovitis score (80). Finally, two ongoing clinical trials using Tol-DC has published preliminary results, one in the context of liver transplantation and the other in the context of multiple sclerosis (MS) and neuromyelitis optica (NMO). In liver transplantation clinical trial, one patient HLA-B12^{+} was treated 7 days before the transplantation with donor HLA-A3^{+} regulatory DC (DCreg). These DCreg were generated using GM-CSF, IL-4, VitD3 and IL-10. The injection was intravenous at a concentration of 5 million cells/Kg. No adverses effects were observed after infusion. Interestingly, at the time of transplantation cross dressed DC has been observed displaying HLA molecules from both donor and recipient. Moreover, these cross dressed DC exhibit low levels of IRF4, and high levels of PD-L1 and FoxP3. The authors suggest that these cross dressed DC could lead to immunoregulation in vivo (140). The second ongoing clinical trial, in the context of of MS and NMO demonstrated no adverses effects of Tol-DC infusion. This trial was performed using Tol-DC differentiated with dexamethasone and loaded with diseases relevant peptides. Tol-DC were administered every 2 weeks for a total of three injections. Interestingly, ELISPOT assay showed an increase in IL-10 and a decrease in IFNγ production (141). Apart from these studies there are many other ongoing in different pathologies and conditions such as in asthma (NCT01711593), multiple sclerosis (NCT02618902, NCT02283671, NCT02903537), type 1 diabetes (NCT02354911), Crohn's disease (NCT02622763), Rehumatoid arthritis (NCT03337165) and liver transplantation (NCT03164265). Among the ongoing studies, we supervised a phase I/II clinical trial in kidney transplantation (NCT02252055) as a part of the ONE study consortium (27). In this trial, autologous-Tol-MoDC generated with low dose of GM-CSF are being injected in patients the day before transplantation by intravenous route at a dose of one million/Kg.

Altogether, these results constitute the basis of the feasibility and safety of Tol-MoDC therapy. For the moment, no toxicity have been related to the therapy. However, due to the short list of clinical trials performed until today is hard to evaluate the efficiency. It

is necessary to evaluate the adequate cell doses and combinatory drugs to improve this therapy in further phases of clinical trials.

Clinical trial	Number of patient	Conditions	Injection	Cells injected	Protocol	Properties	Safety profile
Type 1-Diabetes	10 healthy insulin-requiring type I diabetic patients	3 patients injected with TolDC non manipulated 7 patients injected with TolDC manipulated	Biweekly	10 millions	Recipient TolDC modified with ODN anti-CD40/80/86	Increase in $B220^{+}$ $CD11c^{-}$ B-cells during the firsts 6 weeks of treatment	No adverse effects observed
Rheumatoid arthritis	18 HLA positive RA with minimal disease activity patients	9 patients treated with a high dose and 9 with a low dose of TolDC	Single dose	Low dose: 1 million TolDC; High dose: 5 millions TolDC	TolDC modified with NF-κB inhibitor and pulsed with 4 citrulinated peptides	Increase in circulating regulatory T cells Decrease in serum cytokines IL-15, IL-29, CX3CL1 and CXCL11 Decrease in T-cell IL-6 response against the $vimentin_{447-455}$-Cit450	No adverse effects observed
Refractory Crohn's disease	12 refractory Crohn's disease patients	3 cohorts receiving 2, 5 or 10 million TolDC in a single dose and 3 cohorts biweekly. Two patients in each cohort	Single dose or biweekly	2, 5 or 10 millions	TolDC stimulated with dexamethasone and cytokine cocktail (IL-6, TNF-α, IL-1β, Prostaglandin E_2)	Increase in circulating regulatory T-cell Decrease in IFNγ in blood	Three patients withdrew because of a disease worsening. No adverse effects observed in the other six patients
Rheumatoid arthritis and inflammatory arthritis	12 patients	3 cohorts receiving 1, 3 or 10 million Tol-DC and 1 receiving saline solution	Single dose	1, 3, 10 million cells	Tol-MoDC differentiated with Dex and loaded with autologous synovial fluid	No biological effect in blood Decrease in synovitis score in 2 patients receiving 10 million cells	No adverse effects
Liver transplantation (NCT03164265)	Ongoing	Patient receive donor DCreg	Single dose at day 7 before transplantation	5 million/kg	Donor Tol-MoDC differentiated with VitD3 and IL-10	Presence of cross dressed DC in blood with HLA from recipient and donor.	No infusion reaction or cytokine release post injection
Multiple sclerosis (MS) and neuromyelitis optica (NMO) (Ongoing) (NCT02283671)	6 MS and 3 NMO patients (Ongoing)	Single group receiving Tol-DC	Every two weeks for a total of three administrations	Not listed	Tol-DC generated with dexamethasone and loaded with diseases relevant peptides	Increase of IL-10 and decrease of IFNγ in ELISPOT assay	No adverses effects were observed
Asthma (NCT01711593)	Estimated enrolment of 20 allergic asthmatic patients and 10 healthy controls	2 cohorts (Allergic asthmatics and healthy controls	In vitro	In vitro	Immune tolerant DC (it-DC)	No results yet	Completed, not published yet.
Multiple sclerosis (NCT02618902)	Estimated enrolment of 9 Patients with MS	Not listed	Not listed	5, 10 or 15 million cells	Myelin-derived peptide pulsed-TolDC generated with VitD3	Study not yet open	Study not yet opened
Kidney transplantation (NCT02252055)	10 enroled and 8 treated	Single group receiving Tol-DC	Once before the intervention	1 million cells/Kg body weight	Low dose GM-CSF-TolDC	No results yet	Ongoing
Multiple sclerosis (TOLERVIT-MS) (NCT02903537)	16 estimated patients enrolled	Three cohorts receiving 5, 10 or 15 million cells.	4 injections at two weeks of interval and 2 final injections at the interval of 4 weeks	5, 10 or 15 million cells	Autologous Tol-DC tolerized with VitD3 and pulsed with myelin peptides	No results yet	Ongoing
Cronh's disease (TolDecCDintra) (NCT02622763)	20 estimated patients enrolled	Not listed	Not listed	10-100 million cells	MoDC generated with dexamethasone	No results yet	Ongoing
Rehumatoid arthritis (NCT03337165)	15 estimated patients enrolled	Five cohorts receiving one dose of 1, 3, 5, 8 or 10 million cells	Single intra articular injection	1, 3, 5, 8 or 10 million cells	Tol-DC generatied with IFNα and dexamethasone	No results yet	Ongoing
Type 1 diabetes (NCT02354911)	24 estimated patients enrolled	12 patients receiving TolDC and 12 receiving shams injection as control.	4 injections at two weeks of interval 2 weeks	10 million cells	Recipient TolDC modified with ODN anti-CD40/80/86	No results yet	Ongoing

Table 3. Clinical trials using Tol-DC.

4. Metabolism and immunometabolism

Since the last decades, there is an accumulation of evidences implying that cell metabolic alterations and metabolic networks have an impact on immune cells functions. Initially, the interest on metabolic implications on immune cells was a topic mainly related to tumour microenvironment and metabolic alterations such as obesity and diabetes. Nowadays, the recent growing interest in immunometabolism has allowed to understand deeply the immune response and to discover new targets and approaches for new therapies. However, the research on immunometabolism is still embryonic due to the complexity of metabolic fluxes, and the differences between the regulations of metabolism in immune cells.

.

4.1. General metabolism

The Embden-Meyerhoff-Parnas pathway (Glycolytic pathway), Krebs cycle (Tricarboxylic acid cycle) and the electron transport chain (ETC) are the main pathways that allow to generate energy in animal cells. This energy is stored as ATP, NADH and NADPH. In order to regenerate these molecules and survive, cells adapt their own metabolism depending of their function, activation state and nutrients and oxygen availability. These adaptations can consist on the use of different substrates such as glucose, lipids and amino-acids as well as to favor a metabolic pathway instead to another such as, for example, the oxidative to glycolytic metabolic shift during T-cell activation (142). Furthermore, intracellular and extracellular events can modify cell metabolism. For example, it is well known that insulin induce a rapid glucose intake in skeletal muscle cells by stimulating the translocation of glucose transporter type 4 (GLUT4) (143). On the other hand, at intracellular level, differences in AMP/ATP ratio can be sensed by AMP-activated protein kinase (AMPK), a metabolic regulator that senses the excess of AMP, leading to an increase in ATP generation and inactivation of pathways that consume ATP (144). In fact, the metabolism is tightly regulated by different signaling pathways, enzymatic substrates and cofactors availability, allosteric modifications and transcription (142). In order to simplify these events we will develop metabolism in terms of nutrients and metabolic regulators.

4.1.1. Nutrients and bioenergetics

In general, animal cells can use a large spectrum of nutrients such as monosaccharides, lipids and amino-acids **(Figure 5)**. Monosaccharides such as glucose can be uptaked by cells, usually by two transporter types, SGLT and GLUT. SGLT are sodium-glucose linked contransporters. These transporters are commonly associated to the glucose intake in the intestine and glucose reabsorption in the kidney. GLUT are facilitated diffusion glucose transporters and there are three classes: Class I (GLUT1 to 4), Class II (GLUT5, 7, 9 and 11) (also related to fructose transport) and Class III (GLUT6, 8, 10, 19). The expression of these transporters is variable between tissues (145). Once in the cell, glucose can be phosphorylated by Hexokinase (HK) in most part of cells or by Glucokinase (GCK), mainly in hepatocytes (146). Once at the state of glucose-6-phosphate (G6P), the metabolite can be derived to the glycolytic pathway or on the other hand, to the pentose phosphate pathway (PPP). The PPP have several functions in cells related to carbon homeostasis, amino-acids (Histidine, Tyrosine, Phenylalanine, Tryptophan) and nucleotide precursors synthesis, regeneration of NADPH, and oxidative stress reduction (147). Through the glycolytic pathway, G6P is metabolized into two molecules of pyruvate. Through this catabolic pathway, several metabolic intermediates can be used to synthesize amino-acids such as glycine, serine, cysteine, valine, alanine and lysine. Glycerol can also be generated from dihydroxyacetone phosphate (148). At this point, pyruvate can be transformed in lactate in the cytoplasm or in acetyl-CoA in mitochondria. Once in mitochondria acetyl-CoA is oxidized in Krebs cycle. In the same way that all previous steps, different metabolites from cell cycle can be used as substrate for amino-acid synthesis. Moreover, through the citrate shuttle, citrate can leave the mitochondria and be used as fatty acid and cholesterol precursor. It is important to note that depending on the pyruvate fate the energetic balance will be different. From 1 molecule of glucose through the glycolytic pathway, 2 molecules of pyruvate, 2 molecules of ATP and 2 molecules of NADH are synthesized. In the mitochondria, from the two molecules of pyruvate, in the oxidative decarboxilation 2 molecules of NADH are synthesized, and during the Krebs cycle, 2 molecules of GTP, 6 molecules of NADH and 2 of $FADH_2$. The oxidation of NADH through the electron transporter chain leads to the production of 28 molecules of ATP. In total, from one molecule of glucose, 32 molecules of ATP and 2 cytosolic molecules

of NADH are produced. Whereas oxidative glycolysis just produce 2 molecules of ATP and no NADH. However, even if oxidative glycolysis appears as a waste of energy, the kinetic of this pathway is faster than oxidize glucose through mitochondria. This metabolism it is essential in cells that need large amounts of ATP very quickly such as skeletal muscle during exercise or during T-cell activation (149).

On the other hand, cells can use other substrates in order to generate energy. Fatty acids (FA) are highly energetic molecules. For example, the mitochondrial oxidation of one molecule of the palmitic acid (Short fatty acid), lead to the production of 129 molecules of ATP. Furthermore, the transition from acyl-CoA to acetyl-CoA liberates $FADH_2$ and NADH in addition to those derivate from Krebs cycle. Transport of lipids across the cell membrane can be directed by different mechanisms such as passive diffusion, active transport, translocation or endocytosis (150,151). Once in cell, lipids can reach and merge different structures such as ER, they can also accumulate as lipid droplets, be oxidized through beta-oxidation or even act as ligands for transcription factors such as peroxisome proliferator activated receptor (PPAR) family (152). Amino-acids are less energetic substrates whom catabolic derivates can join glycolysis or Krebs cycle at different reaction point (Depending on the amino-acid). Amino acids catabolism is essential for several cell types in particular conditions, such as glutamine during T-cell activation (153). Finally, other metabolites such as ketonic bodies and lactate can also be oxidized by cells. From ketonic bodies (β-hydroxybutirate and acetoacetate), the yield of acetyl-CoA synthesized is 2 molecules (154). On the other hand, lactate oxidation leads only to one molecule of acetyl-CoA.

Interestingly, different cells in different conditions, can use different combined catabolic routes. Moreover, through metabolic sensors, cells are able to inhibit some routes in order to favor others. The priority is to staying alive.

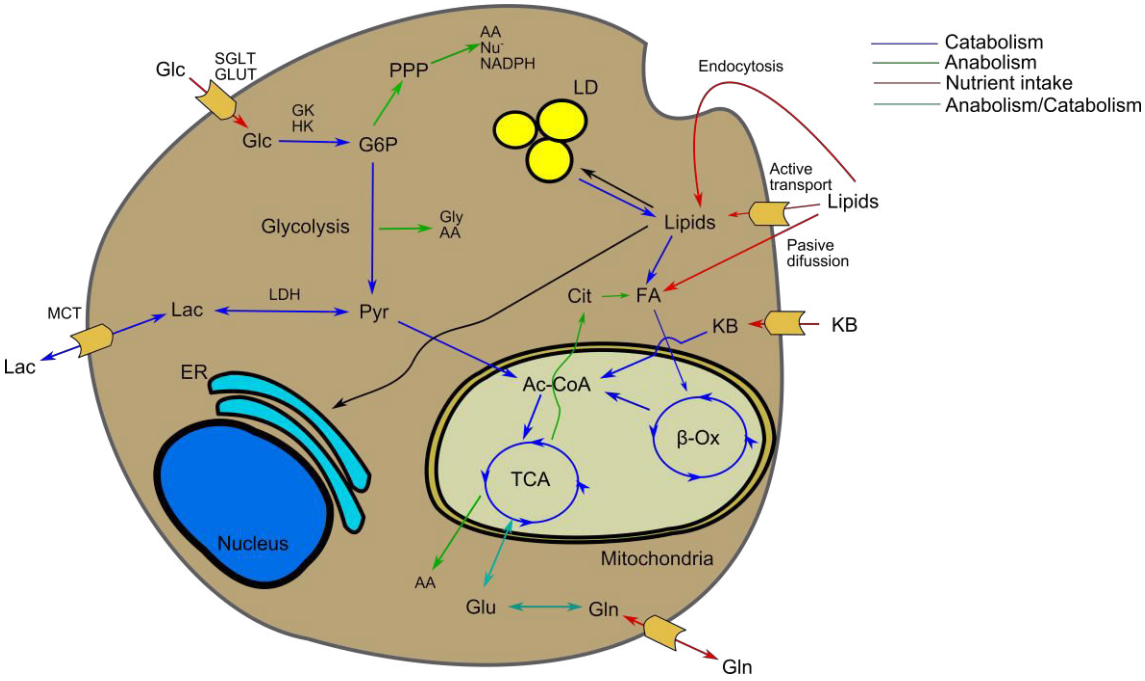


Figure 5. Main metabolic pathways. Cells can use a large spectrum of metabolites in order to obtain energy. Glucose can be uptaked by SGLT and GLUT transporters and then be metabolized to lactate or oxidized through the tricarboxylic acid cycle (TCA) at the mitochondria or on the other hand be metabolized at the PPP. Lipids can be uptaked by endocytosis, active transport or simple diffusion. Once in the cell, lipids can accumulate in lipid droplets or can be oxidized at the TCA. Other metabolites such as ketonic bodies, lactate or amino-acids are also oxidized at the mitochondria. On the other hand, the intermediates of all these metabolic pathways can be use to synthesize amino-acids, nucleotides, lipids, glucose and other molecules. Glucose (Glc), Glycerol (Gly), Amino acids (AA), Lactate (Lac), Ac-CoA (Acetyl-CoA), Ketonic bodies (KB), Lipid droplets (LD), Glutamate (Glu), Glutamine (Gln), β-oxidation (β-OX).

4.1.2. Metabolism regulation

As it was previously mentioned, functional metabolic networks are fixed by cell lineage and microenvironment. Additionally, metabolic adaptations can be modulated at short and long term. These adaptations can be led by de novo synthesis of transcription factors, specific enzymes isoforms and regulatory factors, post-translational enzyme modifications or even through the allosteric effect of metabolites in specific reactions.

However, at long term, there are several regulatory proteins that have been suggested as master regulators of metabolism such as AMPK and mammalian target of rapamycin 1 (mTOR1) **(Figure 6)** (142). AMPK is a trimeric heteroprotein formed by one catalytic α subunit and two regulatory β and γ subunits. AMPK is a highly conserved master regulator of metabolism able to senses the increase in AMP/ATP ratio. The increase in AMP/ATP is usually the consequence of a decrease in energy production or an excess in ATP use, which in terms of homeostasis means lack of energy for his current purpose. AMPK is regulated in different ways apart from the sensing of AMP/ATP ratio. In fact, AMPK can be activated by phosphorylation by other proteins such as calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) in response to calcium increase and also by liver kinase B1 (LKB1) in response to oxidative stress or genetic damage. Once activated, AMPK function is essentially focused on the inhibition of ATP consuming processes and the enhancement of ATP producing processes. In order to increase ATP, AMPK phosphorylate proteins related to fatty acids oxidation, glycolysis, glucose uptake, autophagy, mitophagy and mitochondrial fusion. On the other hand, AMPK also drive the inhibition of lipogenesis, gluconeogenesis and protein synthesis (155). Additionally, AMPK also activates other metabolism regulators related to oxidative metabolism such as sirtuin type 1 (SIRT1), responsible of the expression of PPARγ-coactivator 1-α (PGC1α) and carnitine palmytoiltransferase 1 (CPT1), related to mitochondrial biogenesis and fatty acid oxidation. Also FoxO is a transcription factor related to AMPK activation. FoxO, is related to the increase of anti-oxidative response and the increase of oxidative metabolism (156). On the other hand, AMPK is responsible of the inactivation of mTOR1, another metabolic sensor related to oxidative glycolysis and cell growth (157).

AMPK can regulate metabolism by sensing energy levels in cells, however, other proteins can also modulate metabolism by cell signaling in response to inner and outer stimuli such as PPAR family, mTOR1 and mTOR2, hypoxia-inducible factor 1-α (HIF1α) and Myc among others. PPAR family is related to lipid metabolism in response to different types of lipids (fatty acids, eicosanoids, prostaglandins, etc…). In a simplistic view, PPARα induce lipid intake and oxidation, PPARβ/δ are related to fatty acid and glucose oxidation and PPARγ is related to lipogenesis (158). mTOR is a protein kinase related to cell proliferation and growth. mTOR1 is formed by mTOR, Raptor, mLST8 and the inhibitory subunits DEPTOR, PRAS40 and FKBP12-rapa

meanwhile mTOR2 own Rictor instead of Raptor and is associated to inhibitory molecule DEPTOR and regulatory molecules Proto1/2 and mSin1. About the activation, mTOR2 is usually activated by phosphatidylinositiol (3,4,5)-triphosphate (PIP3), synthesized by phosphoinositide 3-kinase (PI3K), an enzyme downstream the signaling of growing factor receptors. On the other hand, mTOR1 can be activated by a large spectrum of stimuli such as by downstream signaling of growing factor receptors or through protein kinase B (Akt) signaling. Interestingly, mTOR1 is very sensitive to the metabolic state and can be inhibited by the lack of glucose or amino-acids. Due to the implications in cell growth and cell division, mTOR1 activation lead to an increase of anabolic routes such as nucleotides and lipid biosynthesis, increase of glucose metabolism through HIF1α synthesis, protein turnover and mRNA translation. mTOR2 for his part, is related to apoptosis inhibition, cell migration, cytoskeleton rearrangement and ion transport (159). Other metabolic regulators such as Myc and HIF1α are related with oxidative glycolysis and will be discussed further.

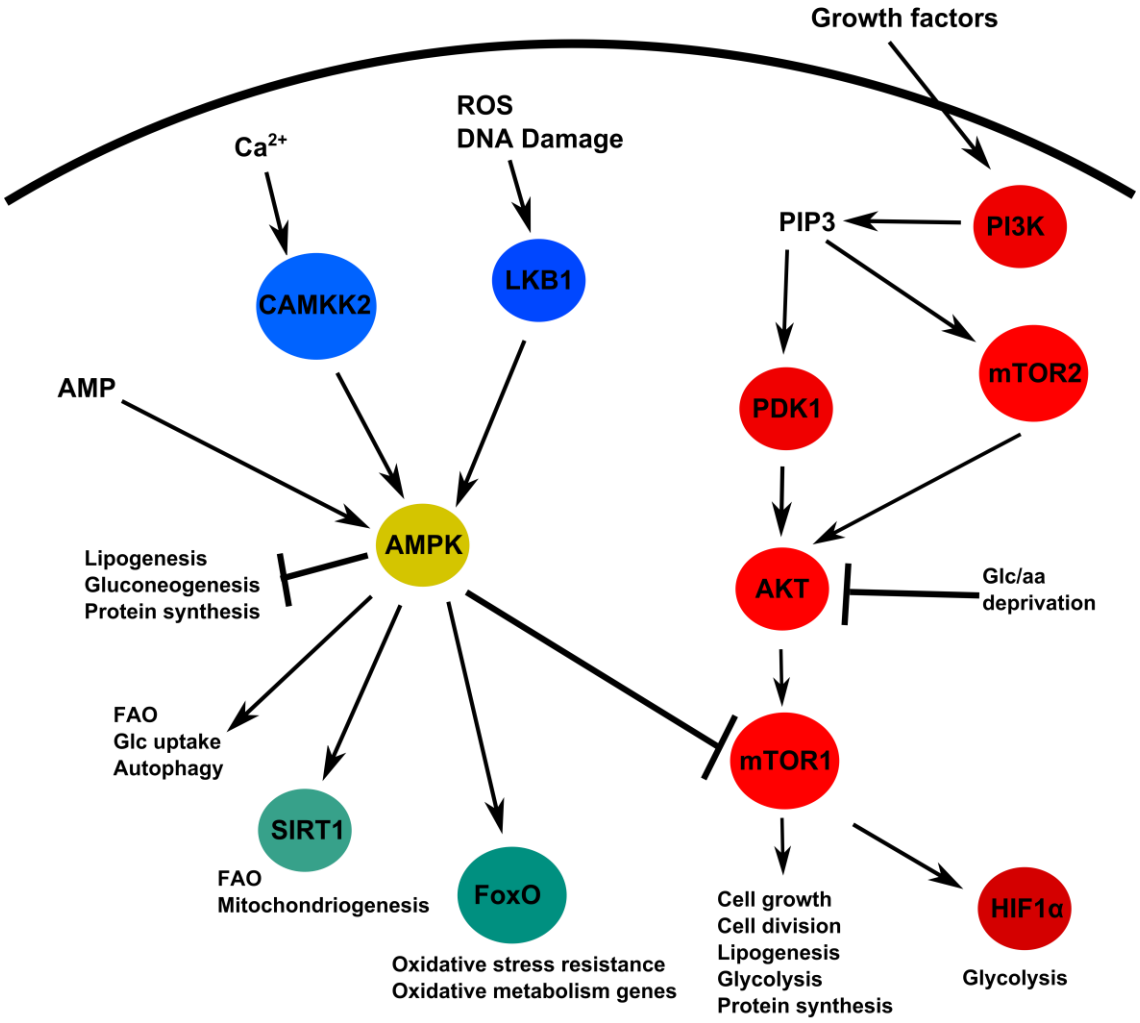


Figure 6. Metabolic regulation by AMPK and mTOR1. Different master regulators such as AMPK and mTOR1 control cell metabolism. AMPK can be activated by phosphorilation through LKB1 or CAMKK2 or through the sensing of AMP excess. After activation, AMPK phosphorilates different regulators such as FoxO or SIRT1, among others. AMPK activation lead to a decrease in energy spending by favoring the energy generation. mTOR1 can be activated by different pathways such as through mTOR2 or AKT among others. The activation of mTOR1 lead to an increase of catabolism by favoring glycolysis and also the increase of anabolic routes in order to prepare cell for protein synthesis, growth and cell division.

Apart from these metabolic sensors other enzymes can be affected by the accumulation of metabolites or energetic molecules (**Figure 7**). In mitochondria, the activity of pyruvate dehydrogenase (PDH) is controlled by inhibitory phosphorylation by the

pyruvate dehydrogenase kinases (PDKs) and the pryruvate dehydrogenase phospatases (PDPs). The inhibitory phosphorilation by the different isoforms of PDK lead to a decrease in pyruvate to acetyl-CoA conversion, resulting in a decrease in pyruvate flux to the mitochondria. PDK isoenzymes are activated by the sensing of ATP, Acetyl-CoA and NADH, and negatively regulated by pyruvate. This mechanism impairs the accumulation of Krebs cycle intermediates from different sources. In fact, this regulation inhibits glucose oxidative when lipid oxidation is the main oxidative substrate (160,161). Additionally, enzymes from the glycolytic pathway can also be regulated by metabolites. The inhibition or reduced kinetic lead by these metabolites at precise points of certain pathways can reduce drastically the catabolic flux. Phosphofructokinase 1 (PFK-1) is an enzyme that regulate the first irreversible reaction of glycolysis and it is a critical enzyme for the determination of glycolytic flux. It has been shown that excess of ATP and citrate generated through the lipolytic pathway can inhibits PFK-1 activity. On the other hand, AMP and fructose 2,6-bis phosphate are allosteric activators. This type of regulation suggests that during lipid oxidation, glycolytic pathway can be reduced at PFK-1 level (162). PDK and PFK-1 regulation are just few examples among others from the extremely complex metabolic regulation.

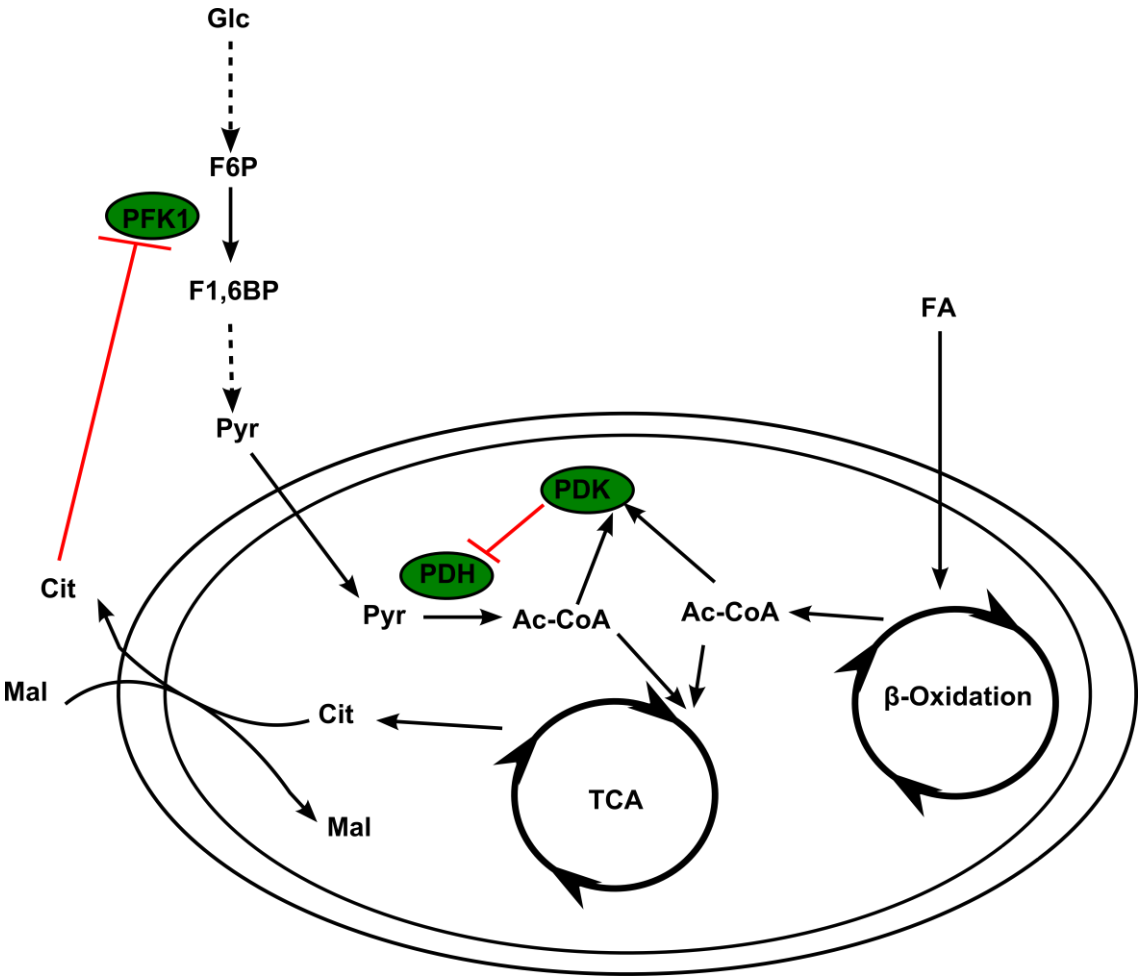


Figure 7. Negative regulators of glycolytic flux and pyruvate mitochondrial oxidation. Oxidative use of pyruvate can be impaired by metabolic intermediates. In mitochondria, the excess of acetyl-CoA lead to the inhibition of PDH and in consequence the inhibition of pyruvate to acetyl-CoA reaction. This inhibition occurs through the acetyl-CoA, ATP and NADH sensing by the PDK, the negative regulator of PDH. On the other hand, the TCA intermediate citrate can leave the mitochondria through the Citrate/Malate shuttle and inhibits PFK-1. PFK-1 catalyzes the first irreversible reaction of glycolysis and it is essential to control the gycolytic flux. Both mechanisms reduce glucose oxidation.

In conclusion, metabolism can be regulated depending of cell type and microenvironment by different mechanisms. Additionally to the mentioned mechanisms, there are many others such as the enzyme modification or inhibition or activation by metabolites accumulation.

4.2. Immunometabolism

The most part of immune cells require a highly plastic metabolism in order to maintain their functions. Indeed, immune cells need to adapt rapidly their metabolism to the modifications induced by chemoattraction, activation and differentiation among others. Interestingly, among the different types of immune cells, it happens that the same stimuli can induce two different responses in ontologically closed cells displaying different metabolisms. For example, effector T-cells and regulatory T-cells can induce immunogenicity or tolerance, concretely, to the same antigens, and both cells display a different metabolism. Effector T-cells are usually glycolytic cells meanwhile regulatory T-cells are highly oxidative (163). On the other hand, immune cells are subjected to the same regulators than other cells. In this part, only macrophage, DC and $CD4^+$T-cell metabolisms will be discussed.

4.2.1. Macrophage metabolism

Macrophages are a perfect example of metabolic and functional shift model in myeloid cells. On one hand, macrophages can polarize to a pro-inflammatory (M1) phenotype characterized by the production of pro-inflammatory cytokines such as IL-1β, TNFα, IL-6 and other molecules such as NO. This differentiation can be induced in presence of danger signals such as PAMPs and DAMPs among other stimuli. Funtionally, M1 macrophages are specialized on the elimination of harmfull agents. On the other hand, macrophages can also differentiate to an anti-inflammatory phenotype (M2) characterized by the production of vascular endothelial groth factor (VEGF), IL-10, TGFβ and poly amines. This phenotype can be induced in the presence of IL-4 or apoptotic bodies among other stimuli. Functionally, M2 macrophages are specialized in wound healing and tissue repairing (164). Interestingly, M1 and M2 macrophages display opposit metabolisms. M1 macrophages display a highly glycolytic metabolism meanwhile M2 macrophages display an oxidative metabolism. Both types of metabolisms are essentials to maintain M1 and M2 functions. For example, after pro-inflammatory stimulation, M1 macrophages increase the glycolytic flux resulting in an increase in pyruvate formation. This pyruvate is metabolized to lactate via the lactate dehydrogenase (LDH) but also is metabolized to acetyl-CoA at mitochondria. It is important to note that M1 macrophages repress PDK1 and in consequence acetyl-CoA

synthesis from pyruvate can not be inhibited. Moreover, M1 macrophages TCA is truncated at several reaction points. TCA truncation leads to the accumulation of several metabolic intermediates and a decrease in oxidative capacity. Once in mitochondria, acetyl-CoA is metabolized to citrate which it will acumulate due to the lack of isocitrate dehydrogenase (IDH). Citrate can be then metabolized to itaconate (anti-baterial metabolite and inhibitor of succinate dehydrogenase (SDH)) or can be used as carbon source for "de novo" lipogenesis. On the other hand, M1 macrophages consume high amounts of glutamine. In M1 macropahes glutamine is used to fill TCA metabolites pool. However, SDH is also reduced such as IDH. The accumulation of succinate has been shown to stabilize HIF1α leading to the expression of pro-inflammatory cytokines. Finally, M1 macrophages display also a highly active PPP in order to support lipid biogenesis and NO production through iNOS. On the other hand, M2 macrophages display an oxidative metabolism mainly based in lipids and amino acids oxidation and a low glycolysis. Differently from M1, M2 macrophages do not have a truncated TCA. Functionally, M2 macrophages metabolize arginine through Arg1 in order to generate polyamines and L-Proline, essentials for colagen synthesis and wound healing (165).

Interestingly, metabolic landscape of macrophages share several characteristics with DC.

4.2.2. Dendritic cells metabolism

Monocytes, resting DC, pro-inflammatory DC and Tol-DC display different types of metabolisms **(Figure 8)**. During the differentiation of monocyte to MoDC with IL-4 and GM-CSF, it has been shown that lipid biosynthesis regulated by PPARγ is necessary for the correct development. In fact, during differentiation, PPARγ upregulates glucose oxidative metabolism, also through mitochondriogenesis induced by PGC1α, leading to an increase in Krebs cycle activity and citrate release for lipogenesis (166). Once differentiated, resting MoDC display a low and oxidative metabolism based mainly on lipids and amino-acids oxidation. On the other hand, the use of glucose by resting DC has been suggested as a source for lipid biosynthesis in order to oxidize them and serving as a futile cycle to maintain mitochondrial health (167). Once activated by TLR signaling, MoDC shift from an oxidative metabolism to a highly glycolytic metabolism, in part, by producing NO through iNOS and leading to an inhibition of electron

transport chain. This increase in glycolytic pathway serves to DC mainly to produce ATP but also to synthesize lipids by increasing citrate export from mitochondria in order to expand ER and Golgi. PPP is also increased in activated DC in order to regenerate NADPH to use them for lipid biosynthesis. De novo lipid synthesis is essential for DC maturation, migration, function and cell priming. In fact, it has been suggested that in part, the immunogenicity of DC is driven by their lipogenic anabolism (168). Interestingly, the use of drugs related to lipid oxidation induction, such as resveratrol, a PPARγ agonist, on MoDC differentiation lead to DC with tolerogenic proprieties. These resveratrol induced Tol-DC have a low expression of costimulatory molecules CD40, CD80 and CD86, produce IL-10 but no IL-12p70 and they are able to inhibit $CD4^+$T-cell proliferation and migration. Interestingly, contrary to other Tol-DC, these cells were unable to induce $CD4^+FoxP3^{hi}$ Treg (169). On the same context, as it was previously described, the Tol-MoDC can be also generated with another metabolic-related drug, the rapamycin. Rapamycin is an inhibitor of mTOR1, the regulatory protein that lead to cells growth. It is interesting to note that rapamycin effect on mature MoDC lead to an increase in pro-inflammatory activity meanwhile immature DC treated with rapamycin and maturated with TLR ligands lead to MoDC with tolerogenic potential (170). This dual role of mTOR1 depending of the context suggest that metabolic requirements for differentiation are essentials for MoDC function (170). Interestingly, it has been shown that resveratrol also affects mTOR1 activation through AMPK, suggesting that both mechanisms could be related by similar mechanisms in MoDC metabolism (171). In the case of other Tol-DC, it has been shown that Tol-MoDC induced with dexamethasone and VitD3 overexpresses genes related to lipidic oxidative metabolism (172,173). Altogether, researches on Tol-DC metabolism converge on similar evidences. Tol-DC display an lipid oxidative metabolism.

About glucose metabolism, several studies have reported a high activity of aerobic glycolysis in Tol-DC. In a recent study aiming to depict the metabolic role related to tolerance in Tol-MoDC differentiated with dexamethasone and VitD3, the authors demonstrated that high glycolytic rate during differentiation is essential for the tolerogenic function in this cells. In fact, the authors demonstrated that the inhibition of PDK, the regulatory protein that inhibits pyruvate oxidation in mitochondria, led to a lower hypostimulatory potential and a decrease in IL-10/IL-12p70 ratio in Tol-MoDC. These results were no observed in addition of etomoxir, an inhibitor of lipid transport

into mitochondria. These results suggest that Tol-MoDC tolerogenic profile is regulated by glucose oxidation rather than lipid oxidation during differentiation. Additionally, the authors demonstrated that the axis PI3K-Akt-mTOR is essential for the maintaining of glycolytic rate and tolerogenic function during differentiation (174). Another study using also Tol-MoDC generated with dexamethasone and VitD3 confirmed the previous results, showing that these cells present a highly glycolytic metabolism. The authors also demonstrated that Tol-MoDC comparing to MoDC, displayed a higher basal oxygen consumption rate and spare respiratory capacity. Additionally, and confirming lipid data from the previous works, Tol-MoDC induced with VitD3 displayed a higher fatty acid oxidation index comparing to mature MoDC and an enhanced expression of lipid oxidation metabolism genes. Finally the authors demonstrated that Tol-MoDC activated with LPS and incubated with etomoxir abrogate partially allogeneic T-cell activation. suggesting that lipid oxidation is necessary to keep their function (175).

Altogether, these results showed that Tol-DC display a highly functional metabolism in terms of oxidative metabolism and aerobic glycolysis in order to maintain their functions.

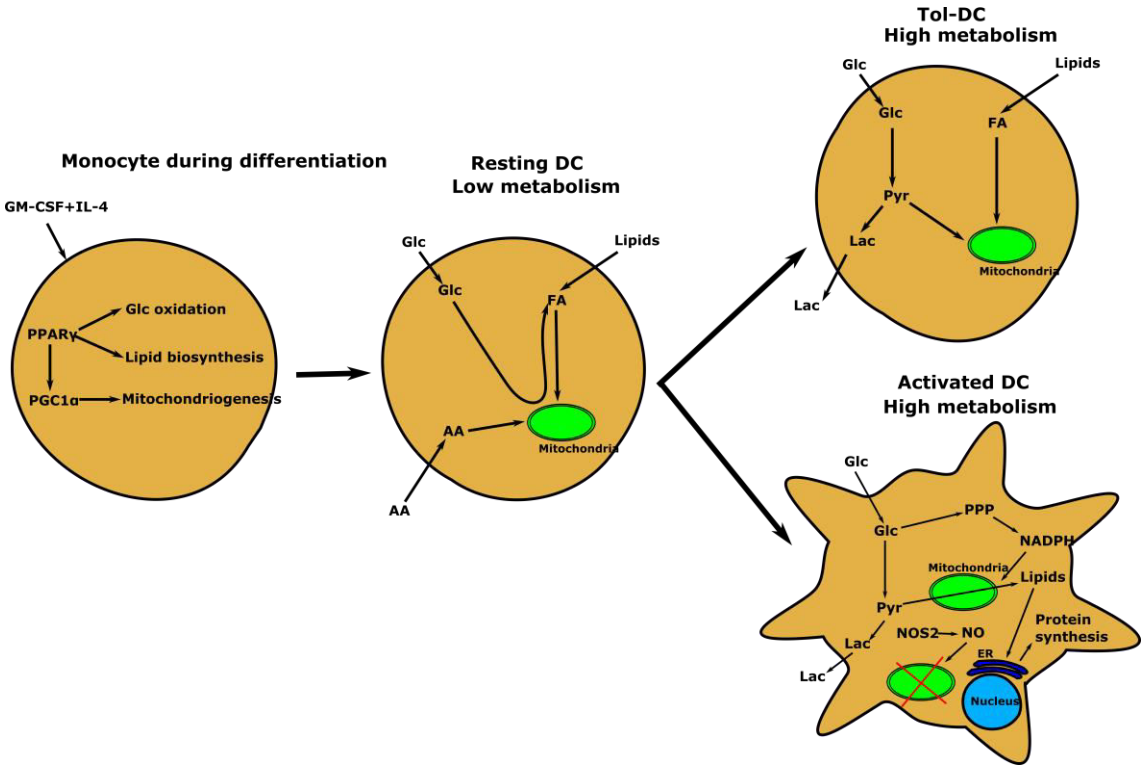


Figure 8. DC metabolism during and after differentiation. DC display a variable metabolism depending on the maturation and activation state. During differentiation to DC, monocytes require a highly glycolytic metabolism in order to synthesize lipids. In

this purpose PPARγ represents an essential transcription factor to maintain the oxidative glucose metabolism and lipid biosynthesis. Once differentiated, DC display a low metabolism based mainly in lipids and glucose oxidation. After activation by TLR stimuli, DC increase aerobic glycolysis and PPP in order to synthesize lipids and proteins. Additionally, activated DC reduce their oxidative capacity by the production of NO through iNOS. Finally, Tol-DC display a highly active metabolism by using aerobic glycolysis and lipid oxidation.

4.2.3. General features of CD4$^+$T-cell metabolism during activation

Nowadays it is not possible to generalize about T-cell metabolism. Different subsets of CD4$^+$T-cells such as Th1, Th2, Th17 and Treg display different metabolism. Additionally activation state and microenvironment also have an impact on their metabolism. The regulation of metabolism is essential to maintain their function and survival. In fact the lack of a concrete nutrient during activation can lead to apoptosis in the same way that lack of regulation can drive to over-activation and uncontroled inflammation (176). Despite the complexity, a classical metabolic model has been established representing the basis of T-cell activation **(Figure 9)**. This model explains the metabolic shift from resting T-cells to activated T-cells. Resting T-cells required very small amounts of energy in order to maintain homeostasis. The most part of this energy is obtained from glucose, glutamine and lipids through the oxidative metabolism (163). Following TCR stimulation by MHC peptide presentation several signaling pathways are activated. On one hand, activated phospholipase C-γ (PLCγ), processes phosphatidylinositol (4,5) bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 is then sensed by IP3 receptor at the ER leading to the release of calcium and activation of calmodulin and NFAT. DAG activates Ras and PKC leading to the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways. On the other hand, TCR activation lead to the activation of a critical regulator factor related to T-cell activation, the protein kinase B also named Akt. Akt has a central role on the T-cell metabolic shift due to its control on mTOR1 activation. On the other hand, Akt also controls other functions such as apoptosis repression and cell cycle progression. Interestingly, same as for Tol-DC generated with VitD3, the axis PI3K-Akt-mTOR1 is essential for the induction and maintaining of glycolysis in T-cells. In fact, TCR activation drives T-cell metabolism to the preferential pathway of aerobic glycolysis.

This activation is sustained by the enhancement of glucose intake through Akt signaling and GLUT1 translocation to the membrane, but also through the uptake of glutamine. Glucose is mainly metabolized through the aerobic glycolysis and PPP meanwhile glutamine serves as substrate for lipid biosynthesis. The classical model of metabolic shift is useful to understand T-cell metabolism during activation. However, apart from TCR signaling, there are other signals that must be considered such as those coming from costimulatory molecules or cytokine receptors.

The different subsets of $CD4^{+}$T-cells add an additional complexity to the understanding of T-cell metabolism. However, years of research focused on T-cell metabolism have allowed finding some singularities on T-cell metabolism among the different subsets. In fact, it is well accepted that effector T-cells are relied to glycolytic metabolism with increased mitochondriogenesis and reactive oxygen species (ROS) production and increased biosynthesis of lipid, nucleotides and aminoacids. On the other hand, regulatory and memory T-cells are associated to an oxidative metabolism mainly supported by lipid oxidation (163,177). Interestingly, modification in metabolism can alter T-cell functions. For example, it has been shown that the inhibition of Acetyl-CoA Carboxylase, an essential enzyme for lipid biosynthesis can impair Th17 differentiation leading to cells with regulatory functions (178).

In order to sustain metabolism during and after differentiation, several molecules and organelles are involved. For example, several studies have demonstrated the role of ROS in T-cell activation. In fact, a study demonstrated that mitochondrial ROS (mROS) is essential for NFAT signaling. In this work, the authors used T-cell specific KO mice for Rieske iron sulphur protein (RISP), a subunit of complex III from the respiratory chain. These T-cells were unable to produce mROS leading to a decrease in calcium release and nuclear factor of activated T-cells (NFAT) activation and impairing T-cell activation (179). Moreover, another study demonstrated that ROS was involved in mTOR1 phosphorylation. In this study the authors demonstrated, using a potent antioxidant, manganese metalloporphyrin, that the decrease in T-cell proliferation and glucose intake are related to a decrease in mTOR1 and an impairment of glycolytic shift. On the other hand, the authors suggest in this work that the origin of ROS was derived from NADPH oxidase (NOX) activation (180). On the other hand, mitochondrial dynamics also plays an important role on T-cell metabolism. In fact, it has been shown that between effector T-cells and memory T-cells there are several

difference in mitochondrial architecture that favors their metabolism. Effector T-cells present a fractioned mitochondrial phenotype lead by the phosphorylation of mitochondrial fission protein p-Drp1^{S616} by extracellular signal-regulated kinase 1/2 (Erk1/2). This fissioned organization favors effector T-cell aerobic glycolysis. On the other hand, memory T-cells display a mitochondrial fused network that favors fatty acid oxidation (FAO) metabolism (181). In a recent work, the authors showed that the forcing of mitochondrial fusion in effector T-cells led to the induction of T-cells with memory characteristics (182). Additionally to these mechanisms, the metabolic shift can also induce epigenetic modification to maintain effector T-cell state and function. In fact, a recent study demonstrated that metabolic shift in effector T-cells enhance acetyl-CoA production leading to an increased (hystone 3 lysine 29) H3K9 acetylation profile at the *Ifng* enhancer and stabilizing Th1 phenotype (183).

.

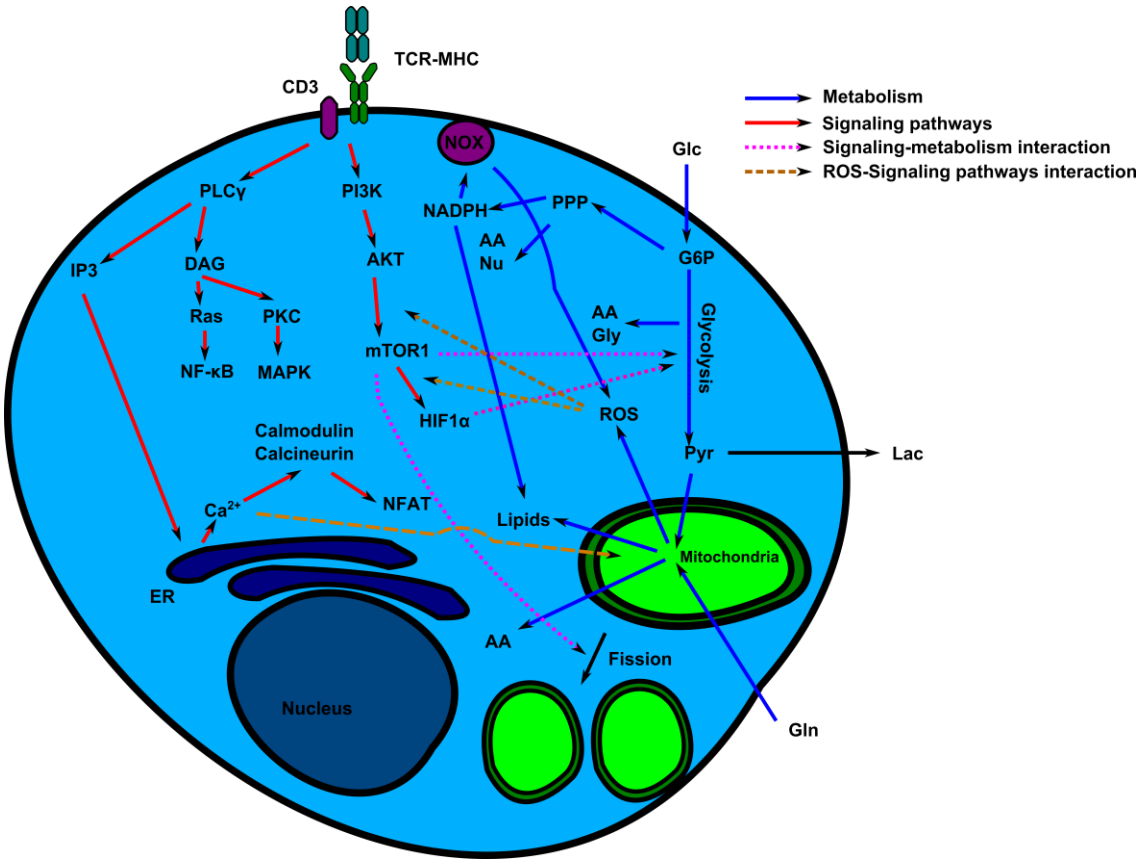


Figure 9. Classic model of T-cell metabolic shift. Once stimulated through the TCR complex, different signaling pathways take place. PLCγ processes phospholipids to IP3 and DAG. IP3 induce calcium release leading to the activation of calmodulin and

calcineurin. The calcium released also activates several enzymes of Krebs cycle in order to increase mitochondrial oxidative kinetic. On the other hand DAG, activates signaling pathways related to NF-κB and MAPK. Also PI3K is activated upon TCR stimulation. PI3K is related to the activation of NOX resulting in an increase in ROS, essential for the correct activation of several signaling pathways. On the other hand, PI3K also activates Akt-mTOR1 pathway. mTOR1 lead to an increase of aerobic glycolysis, lipids biosynthesis and mitochondrial fission in order to prepare T-cell to growing, proliferate and synthesize cytokines.

Altogether, these datas demonstrated that $CD4^+$T-cell metabolism during activation and differentiation is tighly regulated. On the other hand, this regulation can be broken by the effect of the microenvironment.

4.2.4. $CD4^+$Treg metabolism

It is well accepted that $CD4^+$Treg display an oxidative metabolism mainly supported by FAO. However, the reality is far more complex. It has been shown that mouse Treg differentiated in vitro display a low glycolytic metabolism and enhanced FAO. This metabolic preference in this cells was directed by mTOR1 inhibition by AMPK (184). On the other hand, and compromising the classical metabolic model for Treg, a recent study demonstrated that ex vivo isolated Treg are mainly glycolytic **(Figure 10)**. After culturing in vitro, these Treg engages also FAO metabolism. Interestingly, the inhibition of glycolysis with 2-deoxyglucose (2-DG) and lipolysis with etomoxir, impaired Treg function toward $CD4^+$T-cell inhibition and additionally impaired FoxP3 and CTLA-4 expression (185). Moreover, another recent study demonstrated that glycolytic metabolism is essential for migratory skills of Treg in mice. In this article, the authors showed that migratory skills from ex vivo expanded Treg were affected in presence of 2-DG. On the other hand, migratory skills were increased in presence of metformine and unaffected in presence of etomoxir. These results suggest that Treg migration is lead by glycolytic metabolism and not through lipid oxidation. The authors also demonstrated that the increased glycolytic metabolism was mediated by GCK association to actin filaments. GCK expression was directed by PI3K-mTOR2-akt pathway. Moreover, an increase Treg motility front CCL19 and CCL21 was observed in

humans overexpressing GCK due to a loss-of-function allele of GCK regulatory protein (186). These results suggest that Treg metabolism is different between isolated and in vitro Treg. In fact, it has been observed that in vivo Treg display a high metabolic rate due to an hyperactivation of mTOR1. About this subject, a study focused on mouse Treg demonstrated that mTOR1 and high glycolysis and oxidative metabolism are essential for differentiation and function in Treg. The authors also showed that mTOR1 activation in Treg is necessary for lipid biosynthesis (187). Paradoxiaclly, mTOR1 inhibitor rapamycin has been related to Treg induction and expansion in vivo as well as in vitro (188). Supporting the favored role of mTOR1 inhibition, AMPK is also related to Treg differentiation and function (189). Moreover, another metabolic sensor has been described as essential for Treg differentiation and function, Lkb1. Lkb1 was believed to be upstream of AMPK, however recent evidences demonstrated that Lkb1 can bypass AMPK but can activate MAP/Microtubule affinity-regulating kinases (MARKs) and salt-inducible kinases (SIKs). In this article, the authors demonstrated that Treg lacking of Lkb1 gene display a decreased oxygen consumption rate (OCR), extracellular acidification rate (ECAR), ATP production and mitochondrial content. Moreover, adoptive transfer of these allogeneic effector T-cells into Rag1$^{-/-}$ mice result in a uncotroled alloreaction suggesting that Lkb1 is essential for metabolism and function in vivo (190). Altogether, these results imply that Treg metabolism is not yet totally understood but differently from effector T-cells, can be relied to FAO.

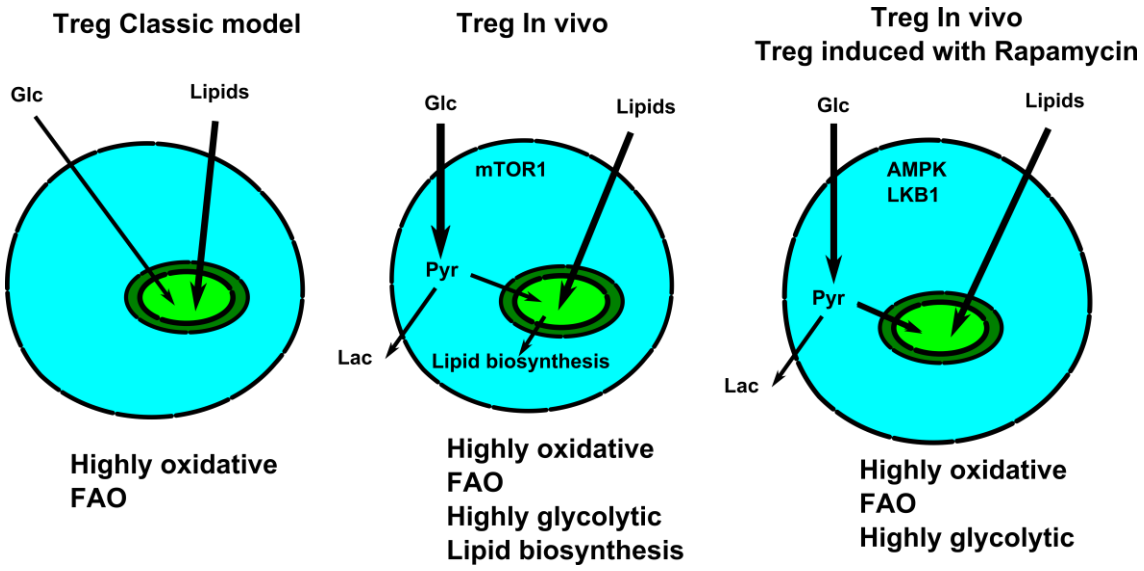


Figure 10. Treg metabolism. Treg display different types of metabolism. In vitro (Classical model), Treg adopt a highly oxidative metabolism based mainly in lipids degradation. In vivo, Treg display a highly glycolytic metabolism but also an enhanced capacity to oxidize lipids and synthesized them. mTOR1 is essential to maintain their metabolism. Finally, Treg differentiated with rapamycin display a highly oxidative and glycolytic metabolism. In this case, it seems like AMPK an essential factor for their metabolism.

4.2.5. $CD4^+$T-cell sensitivity to microenvironment

Finally, another complexity level controls T-cell metabolism and function, the microenvironment. Into tumour microenvironment, T-cells must to face a large spectrum of stimuli such as pH acidification, nutrient deprivation, signaling molecules release by tumour and the excess of metabolic waste such as lactate. It has been shown that tumour cells with high glycolytic metabolism can lead to a decrease in microenvironment glucose impairing T-cell anti-tumour activity. Also, it has been suggested that glucose deprivation lead to a decrease in T-cell glycolytic flux reducing the formation of phosphoenol pyruvate and limiting intracellular calcium release and in consequence T-cell activation. Moreover, in several models, the blockade of glucose intake in tumours lead to a better activation profile in T-cells (191,192). Interestingly, a recent work demonstrated that in microenvironments with low glucose and high lactate, T-cells can induce FoxP3 and oxidative metabolism leading to Treg survival and effector T-cell impairment (193). Amino acid deprivation such as tryptophan, glutamine, and arginine among others, can also impair T-cell activation in tumour microenvironment. Tryptophan is a metabolite essential for protein synthesis (194). In fact, the lack of tryptophan inhibits the transition G1 to S phase during cell cycle leading to T-cell apoptosis (195). Arginine deprivation has been shown to impair aerobic glycolysis without affecting oxidative metabolism in T-cells. This reduction of glycolytic flux was associated to an increase in serine biosynthesis, glutamine anaplerosis and an increase in oxidative metabolism lead by the downregulation of pyruvate kinase M2 (PKM2) expression (196,197).

Similarly, in metabolic diseases, it has been reported differences in T-cell behavior. For example, in T1D, it has been suggested that the exposure of increased glucose

concentration due to the lack of glycemia control lead to an over-activation of T-cells as a consequence of increased glycolysis. This over-activation results in a hyperprolfieration and increased expression of pro-inflammatory cytokines (198). Also in obesity, it has been shown that in adipose tissue T-cells develop a pro-inflammatory phenotype leading to a high expression of pro-inflammatory cytokines. On the other hand, malnutrition lead to an immune dysfunction due to essential amino-acid deprivation (199).

Altogether, these studies demonstrated the fragility of T-cell metabolism that results in loss of function or increased function.

5. Lactic acid

5.1. Lactic acid overview

Lactic acid (2-Hydroxypropanoic acid) is an organic compound with a molecular weight of 90.078g/mol. The founding of this molecule initially named "Mjölksyra" was attributed to the Swedish chemist Karl Wilhelm Scheele in 1780, who isolate it from sour milk. Further, during the first half of the 19th century, Jöns Jakob Berzelius and Justus von Liebig found that lactic acid was present in meat and dead organisms. From this discovery three major facts on lactic acid history in physiology were followed. The first one, discovered by Emil Heinrich du Bois-Reymond was that muscles could produce lactic acid. The second discovered by Johann Joseph Scherer and after by Carl Folwarczny, was that lactic acid could be detected in human blood under septic and hemorrhagic shock. Finally, at the end of 19th century, Araki and Zillessen observed that under oxygen deprivation, muscles increased the production of lactic acid, showing for the first time the relation between hypoxia and anaerobic metabolism (200). During the 19th century, other scientists were interested by lactic acid. In fact Pelouze, Boutron, Frémy, Liebig and Pasteur were interested on the production process of lactic acid and fermentation. The advances of these chemists on lactic acid production and the recently adopted microbiology led to the first time clinical use of lactic acid and derivates in 1871. These lactic acid derivates, according to the pharmacopoeia of new German Empire and French pharmacopoeia, were indicated to improve digestion, as a diphtheria treatment, for tooth cleaning, as a laxative, as an antispasmodic and for osseous affection among others uses (201).

However, history of lactate starts 4000 years ago. During the pre-scientific era, humans have been using lactic acid bacteria without knowing it to produce fermented products in order to improve food preservation and modify texture and organoleptic proprieties of the products (202). Nowadays, even if it is known that lactic acid bacteria impair the growing of several potentially harmful microorganisms such as Salmonella Enteritidis, Escherichia Coli and Listeria Monocytogenes, the exact mechanisms are still not yet fully understood. On one side, the end-product of lactic acid bacteria fermentation is lactic acid. As a weak acid with a pKa=3,86, lactic acid induces environment acidification. This acidification inhibits the growth different microorganisms growing.

On the other side, many other inhibitory systems depending on the microenvironment could take place. Among different proposal mechanisms, one of them is the protein leakage through damaging bacterial membranes (203,204). This historical approach shows us, that lactate is more than the end-product of glycolysis.

5.2. Lactic acid and lactate dehydrogenase

Lactic acid is the end-product of the anaerobic glycolysis. This metabolite is present in a large number of members of the three domains: Bacteria, Archaea (205) and Eukarya. However, those individuals able to produce lactate are not necessarily producing them for bioenergetic purposes. For example, in Arabidopsis Thaliana, it has been shown that the LDH activity in roots, is necessary to stimulate ethanol fermentation in hypoxia conditions for plant survival (206). In bacteria, lactic acid is on one side the consequence of the metabolism, but at the same time it acts as a gram negative antibacterial agent by increasing outermembrane permeability and also by lowering the pH, avoiding the optimal growth of pH sensitive species (207). Even in animal cells, the role of lactate goes beyond what at the beginning was considered, as an end-product or an oxidizable substrate. In fact lactic acid in animals presents an immunosuppressive activity (208).

5.2.1. LDH quaternary conformation

The lactate is a metabolite derived from the reduction of lactate to pyruvate. In this reaction also participates the NADH, that is oxidized to NAD^+. This reaction but also the reverse reaction is catalyzed by the enzyme LDH. The LDH is a tetramer formed, in animals, by the subunits LDH-M, LDH-H and LDH-C coded respectively by *Ldh-a*, *Ldh-b* and *Ldh-c* (209). *Ldh-a* and *Ldh-b* have a ubiquitous expression meanwhile *Ldh-c* is restricted to sexual organs. Ldh-c homotetramer is essential for spermatogenesis, oogenesis and for functional activity of spermatozoids and egg (210). On the other side, the LDH in the most part of tissues can be detected as homotetramer or heterotetramer formed by the subunits LDH-M and LDH-H. Depending of the subunits forming the tetramer, the LDH is named as, LDH-1 (4H), LDH-2 (3H,1M), LDH-3 (2H, 2M), LDH-4 (1H, 3M) and LDH-5 (4M). The final form of the tetramer defines the reaction

kinetics. For example, LDH-5 is commonly found in muscles, a highly glycolytic tissue, and this isoform have a Km(pyruvate->lactate) around 0,1 and 3,4mM meanwhile the Km(lactate->pyruvate) is around 20mM. On the other side, LDH-1, commonly found in heart, a highly oxidative tissue, the Km(pyruvate->lactate) is around 0,1mM and for the reverse reaction Km(lactate->pyruvate) is around 9mM. However, Vmax in LDH-5 is higher than LDH-1. These enzymatic characteristics show that LDH isoenzymes have similar affinity for pyruvate, but at saturating concentration, LDH-5 catalyze more conversion of pyruvate to lactate. On the other side, LDH-1 catalyzes more efficiently the reverse reaction (lactate to pyruvate) (211–213)

.

5.2.2. LDH allosteric regulation and post-translational modifications

Apart from differences on kinetic based on the quaternary conformation, the LDH is subjected to allosteric regulation and inhibition by several molecules. In fact, LDH activity is impaired by both, substrate and product. It has been observed that at higher concentrations of pyruvate the reaction activity in both senses is reduced. This is due to a ternary and binary complexion of pyruvate with the coenzyme NAD^+ (214). Additionally, another study showed that this inhibition by excess of substrate affects more the LDH-1 than the LDH-5, suggesting that isoforms related with lactate use as substrate are more sensitive to excess of product (212). On the other hand, lactate can act also as an inhibitor of LDH. This lactate inhibition appears to be more important to LDH-5 than LDH-1, suggesting that isoforms related with pyruvate use as substrate are more sensitive to excess of product (214,215). However these results showed a mechanistic approach of LDH in vitro, and there is not data about how these inhibition could take place under physiological condition.

LDH allosteric regulation is controversial. This controversial rise from the fact that several allosteric mechanisms found in some species are not presents in others. For example, the first allosteric regulation observed in LDH was found in lactic acid bacteria. This regulation was mediated by Fructose-1,6 bisphosphate, inorganic phosphate and ionic strength, and affected differently LDH activity depending of lactic acid bacteria. The reason for these different LDH regulation is related to habitat adaptations (216). However, this regulation is not observed in vertebrates (217). In

vertebrates, the approaches on allosteric regulation of LDH are based especially in cancer. For example, it have been shown that the oncogenic receptor tyrosine kinase fibroblast growth factor receptor 1 (FGFR1) directly phosphorylates LDH-A tyrosines Y10 and Y83 leading to an enhancement of LDH activity (218). Also, another study demonstrated that the acetylation on Lysine K5 inhibits LDH-A activity by inducing his recognition by the chaperone HSC70 and leading to degradation at lysosomes (219). Interestingly, acetylation has already been detected as a LDH activity modulator in winkle. However, in this study the authors showed that the reduction in LDH acetylation impaired enzyme activity (220). Additionally to phosphorylations and acetylations, another study performed in turtles, showed that LDH in these animals are subjected to several other post translational modification such as ubiquitinations, SUMOylations, nitrosylations and methylations (221). However, much more research must be performed in the field of LDH allosteric regulation in humans.

5.2.3. Regulation of LDH expression

Despite the scarce information on LDH allosteric regulation, there is a vast knowledge about genetic control of this enzyme in mammals. In the case of *Ldha* promoter, there are several well known transcription factors related to the enhancement of the expression such as HIF, Myc, FoxM1, cAMP response element-binding protein (CREB) and Jumonji C Domain 2A (JMJD2A) among others (209,222). HIF is a transcription factor related to the metabolic control under lack of oxygen. HIF is an heterodimeric protein composed of one HIF1β and one HIF1α or HIF2α subunits. Under normoxia condition, HIF1α subunit is first hydroxilated via the hemeproteins prolyl hydroxylase domain enzymes (PHD), recognized by von Hippel-Lindau tumour suppressor protein (pVHL), ubiquitinilated and finally degraded through proteasome. However, under hypoxic conditions, prolyl hydroxylation is impaired and HIF1α can translocate to the nucleus to form the functional HIF transcription factor. This transcription factor is related to the shift from oxidative metabolism to aerobic glycolysis. In fact, it is related to the expression of glycolytic enzymes such as phosphoglycerate kinase-1, GLUT-1, LDHA, and the lactate transporter MCT-4 among others. Apart from glycolytic enzymes upregulation, HIF is also related to the expression of PDK, an enzyme involved in pyruvate to acetyl-CoA inhibition in mitochondria. Therefore, HIF increases glycolysis and impairs pyruvate oxidation through mitochondria. Additionally, HIF also

controls the response to intracellular acidification by increasing the expression of Na^+/H^+ pumps such as NHE1 and the Carbonic anhydrase IX (CAIX) that convert CO_2 into carbonic acid in order to buffer intracellular pH (223). Another well known transcription factor related to *Ldh-a* expression is Myc. Myc is a transcription factor from the family of basic helix-loop-helix (bHLH) proteins. Myc can heterodimeryze with another member of bHLH superfamily such as Miz1 and Mix but more importantly with Max. However, it have been shown that there is interaction with the DNA in absence of Max, suggesting that there are others proteins that can heterodimerize with Myc (224,225). About his function, Myc is a master regulator that integrates multiple cellular signals and it is involved on cell growth, proliferation, apoptosis, differentiation, protein synthesis and metabolism among other functions. In fact it have been shown that Myc increases the expression of Ldh-a, *Glut-1* and phosphofructokinase-1 (*PFK-1*) leading to an increase of glucose intake and lactate production (226,227). Apart from HIF and Myc that are also related with the expression of other glycolytic enzymes, there are other transcription factors associated with *Ldh-a* expression but less with other metabolism related enzymes. Recently it have been demonstrated, in pancreatic cancer, that FoxM1 is involved on *Ldh-a* expression even if it apparently does not control other genes of glycolysis (228). Similarly, the histone demethylase JMJD2A has been shown to control *Ldh-a* expression but not the most part of glycolytic enzymes in nasopharyngeal carcinoma (222). Another study focused on early-stages breast neoplastic lesion demonstrated that the regulatory protein 14-3-3ζ overexpression increases *Ldh-a* expression through the signaling pathway MEK/ERK/CREB (229). To abbreviate, there are many transcription factors related to *Ldh-a* expression. In fact, according to GeneHancer data base, there are around 263 transcription factors and regulatory proteins potentially related to *Ldha* expression.

On the other side, the regulation of *Ldh-b* expression has been less investigated. However, the relevance of Ldh-b expression is not trivial due to the juxtaposition with *Ldh-a*. In fact it has been demonstrated in human breast cancer, that Ldh-b promoter methylation, and hence downregulation, is a frequent event (230). Also, despite the limited information about his regulation, *Ldh-b* has been related to an increase of oxidative metabolism in skeletal muscle. In this study the authors shown that exercise induced through PGC1α, estrogen-related receptor (ERR) and myocyte enhance factor 2 (MEF2), increases of *Ldh-b* expression. This increase of *Ldh-b* was also associated to a

decrease in extracellular pH and an increase in O2 use (231). Nevertheless, the knowledge about regulation of *Ldh-b* still limited despite that according to GeneHancer data base there are up to 134 transcription factors related to *Ldh-b* expression.

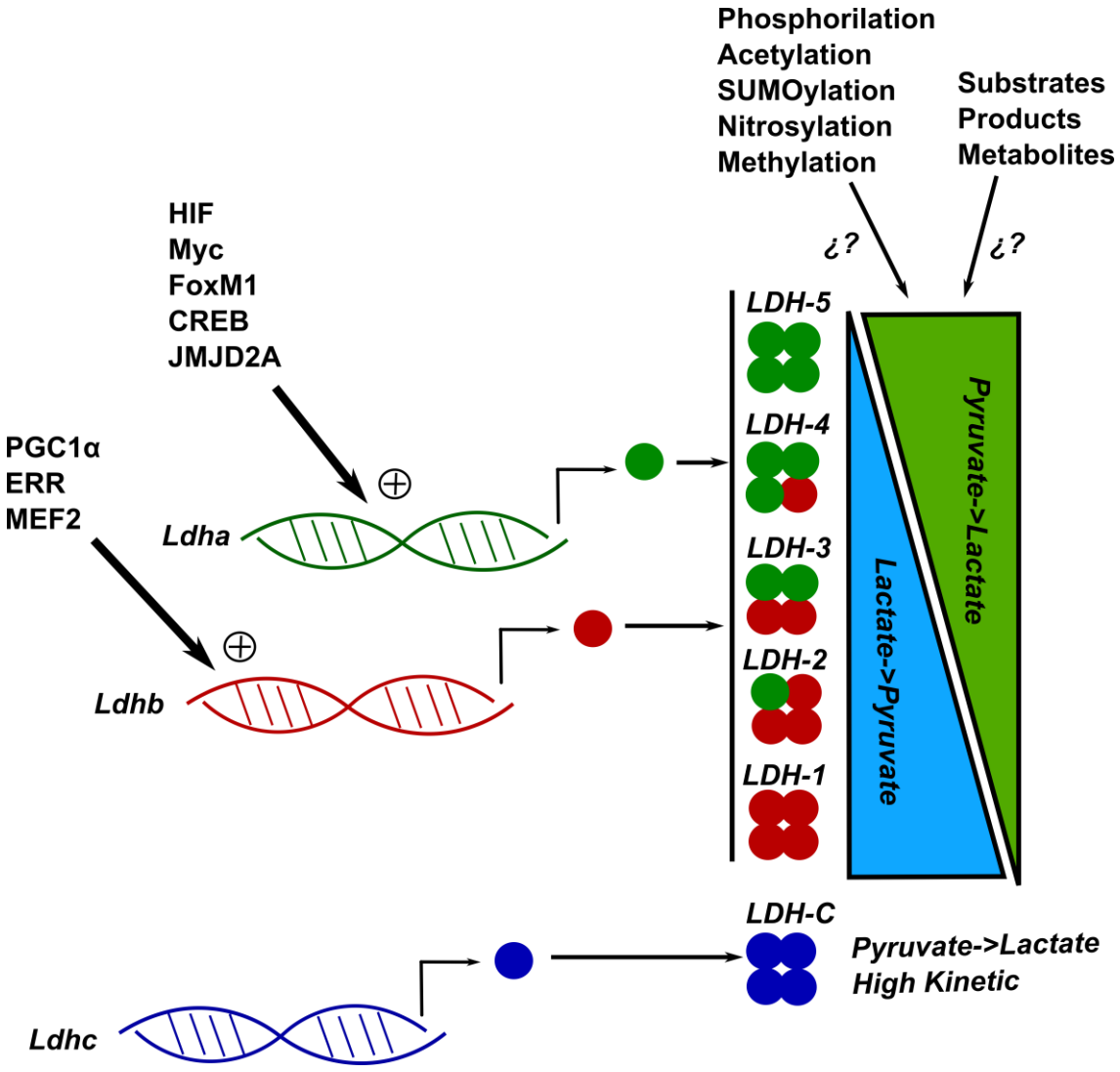


Figure 11. **LDH regulation.** In mammals, there a three different genes that code for LDH, Ldha, Ldhb and Ldhc. Ldha and Ldhb have an ubiquitous expression meanwhile Ldhc is restricted to sexual organs. LDH is regulated at different levels. *Ldha* expression is regulated by different transcription factors such as HIF, Myc, FoxM1, CREB and JMJD2A. On the oher hand *Ldhb* is regulated through other transcription factors such as PGC1α. Once translate, monomers of LDHa and LDHb can interact to form the quaternary structure of LDH, an homo or heterotetramer. Depending of the monomers forming the LDH, reaction kinetic will favor the lactate synthesis (mainly

LDHa) or pyruvate synthesis (mainly *LDHb*). Moreover, LDH can be modified by kinases leading to changes in his kinetic, and in the same way, different metabolites can act as negative regulators of LDH.

Taken together, lactate production is tightly regulated by monomers expression and by allosteric modification. Moreover, these monomers are regulated differently according to cellular type but also through the response to different stimuli **(Figure 11)**. The understanding of how lactate production is regulated at enzymatic and genetic levels is essential to conceive the physiological and cellular role of this metabolite in health and disease.

5.3. Lactic acid, transport and sensing

5.3.1. Monocarboxilic acid transporters

Once synthetized, lactate is thrown out of the cell through the Monocarboxylate transporters (MCT). MCT transporters belong to the SLC16 family of solute carriers that are related to the transport of thyroid hormone (MCT8, MCT10), aromatic aminoacids (MCT10), Carnitine (MCT9), lactate (MCT1, MCT2, MCT3 and MCT4), pyruvate (MCT1 and MCT2) and ketonic bodies (KB) (MCT1, MCT2, MCT4 and MCT7) (acetoacetate and β-hydroxybutyrate). On the other hand, the most part of transporters of this family are orphans (MCT5, MCT6, MCT11, MCT12, MCT13 and MCT14). The mechanism of activity depend on the MCT transporter but usually they act as symporters that contransport in the same direction a monocarboxylic acid and a proton (232,233). MCT1, apart from symporter mechanisms display a exchanger activity by translocating two monocarboxylic acids simultaneously (234). Focusing on lactate transporters, they are distributed differentially on tissues and their expressions are associated preferentially to the uptake or the release of lactate. For example, MCT1 has a high affinity for lactate, pyruvate and KB, and lead to a passive and bidirectional transport. MCT1 expression is related to tissues able to uptake and oxidizes lactate as a TCA substrate such as myocytes or neurons but also, it is expressed in gluconeogenic tissues such as liver and kidney which use lactate to produce glucose. On the other hand, MCT1 is also related to highly glycolytic cells such as red blood cells, T-cells and

tumour cells which throw out lactate in order to reduce intracellular acidification. MCT2 displays a similar profile than MCT1 leads a bidirectional passive transport and it has more affinity to lactate, pyruvate and KB. However, differently that MCT1 that own an ubiquitously expression, MCT2 is restricted to highly oxidative tissues such as brain and gluconeogenic tissues such as liver and kidney. MCT4 is largely express in most tissues, but especially on glycolytic cells such as skeletal muscle, astrocytes, adipocytes, chondriocytes and white blood cells. This transporter has a low affinity for lactate, pyruvate and KB. This characteristic avoids the lost of pyruvate to the extracellular environment and allows the throw out of lactate only when it is accumulated in the cells. Similarly, MCT3 is related to the release of lactate rather than the uptake. However, MCT3 expression is confined to retinal pigment epithelium and choroid plexus (233,235). These particularities between the different MCT proteins in addition to the metabolic preferences of different cells, set lactate as an essential molecule for precises physiological functions. These characteristics will be discussed on the following sections. About the regulation of these transporters, it has been shown that their expression and functional activities are regulated by other proteins that interact with MCT such as CD147 (Basigin), LAT1, CD98 and ASCT2 (L-Glutamine transporter) (236). In fact, the transmembrane glycoprotein belonging to Ig superfamily CD147, is as a chaperone that stabilize MCT1 at protein level (237). Additionally to kinetics characteristics of each isoform and stabilization by other proteins, MCT transporter activity also depends on physicochemicals properties of microenvironment. A study using L6 skeletal muscle cells, displaying MCT1, MCT2 and MCT4 transporters, showed that lactate uptake was enhanced when media pH dropped from 7,4 to 6. Interestingly, the authors also showed that acid extracellular pH impairs the kinetic of lactate efflux (238).

5.3.2. Sodium-linked monocarboxilate transporters

Another family of lactate uptake transporters is the sodium-linked monocarboxylate transporters (SMCT). The most known transporters related to lactate, pyruvate, KB and NAD transport are Slc5a8 with high affinity and the Slc5a12 with low affinity. Slc5a8 has been related to physiological functions such as short fatty acids absorption in the intestine, lactate and pyruvate reabsortion in kidney, and lactate and ketonic bodies uptake by neurons. Slc5a12 has been identified in intestine, kidney, astrocytes and

Müller cells (239). Moreover, Slc5a12 has been detected in $CD4^+$T-cells (240). The cotransport of monocarboxylate acid through SMCT is made by passive and unidirectional transport and is related to ion charge in the microenvironment, for example Chlore concentration (241,242).

5.3.3. GPR81

Apart from MCT and SMCT, lactate also can be sensed by cells through the G-protein coupled receptor 81 (GPR81). This receptor is expressed ubiquitously, but is enhanced in adipose tissue. In fact, the history of GPR81 is linked to the discovery in 1960 of the anti-lipolytic effect of lactate on adipocytes. However it was not until 2009 that this effect was associated to the orphan receptor GPR81. In this work, the authors showed that the addition of L-Lactate into a culture of human subcutaneous differentiated adipocytes inhibits glycerol and free fatty acids release. Additionally, the authors also characterized the receptor. They demonstrated that GPR81 is coupled to a Gi protein leading to a decrease in cAMP. This effect could be reverted by the addition of Gi inhibitor Pertussis toxin. Also, the authors showed the effective concentration (EC_{50}) of other potential ligands of this receptor. GPR81 is a receptor for α-hydroxybutyrate, γ-hydroxybutyrate, glycolate, trifluoroacetate, α-hydorxyisobutyrate, dichloroacetate and α-hydroxycaproic acid. However L-lactate displays the lower EC50 for GPR81 (~5mM). Finally, the authors demonstrated that GPR81 is internalized after L-lactate stimulation (243).

Till today, GPR81, SMCT and MCT are the only lactate sensing proteins known in animals (**Figure 12**). Nevertheless, these proteins are essential for several interactions in physiology.

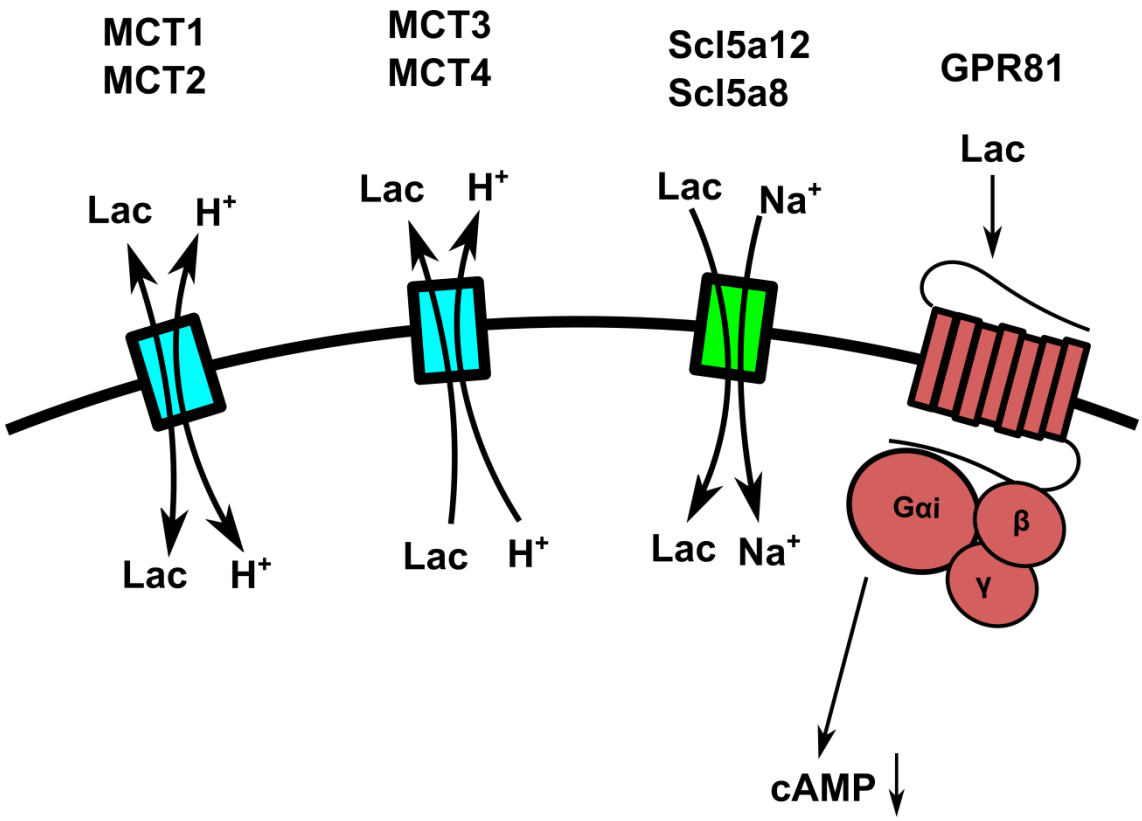


Figure 12. Lactate receptor and transporters. Lactate can be uptake, release or be sensed by cells through different mechanisms. Lactate can be uptake through the symporters MCT1, MCT2 with a proton cotransported, or as well through Scl5a12 and Scl5a8 with a sodium ion cotransported. These receptors display a passive mechanism, meaning that the flux of lactate depends mainly to the internal and external concentration. Lactate also can be sensed by cells through the G-protein coupled receptor GPR81. This GPCR is associated to an inhibitory Gαi protein, leading to an inhibition of AMP cyclase. Finally lactate is exported out of the cells through the symporters MCT3 and MCT4.

5.4. Intracellular lactate

Lactate can be generated and secreted or in contrast uptaked from the microenvironment. However, the lactate fate and role despite to be secreted, oxidize or used as anabolic substrate it still unclear. It is already known that the production of lactate is linked to the redox state of cells. The reduction of pyruvate to lactate regenerate one molecule of NAD^+ that will be used on glycolysis by the

glyceraldehydes-3-phosphate dehydrogenase (GAPDH). This NAD^+/NADH cycle allows to increase glycolytic flux in order to produce less amounts of ATP than through TCA but more quickly. This metabolic adaptation is essential in different conditions such as during exercise by skeletal muscle or T-cell activation (244,245). Also, it has been proposed that in order to sustain NAD^+ production and consequently the glycolytic flux, lactate can be used as an electron donor to sustain Malate-Aspartate shuttle (MAS) **(Figure 13)**. This shuttle together with the glycerol-phosphate shuttle are essential components to regerate NAD^+ in cytosol. In MAS, cytoplasmic lactate can be converted into pyruvate by LDH present in mitochondrial intermembrane space. The lactate to pyruvate reaction regenerate NADH that will be used by malate dehydrogenase (MDH) to transform oxalacetate (OAA) into malate and oxidizing NADH to NAD^+. This process also seems to be important in order to oxidize lactate (246). On the other hand, lactate has also been related to other functions such as histone deacetylation inhibitor. However mechanisms underlying this effects remains unknown (247).

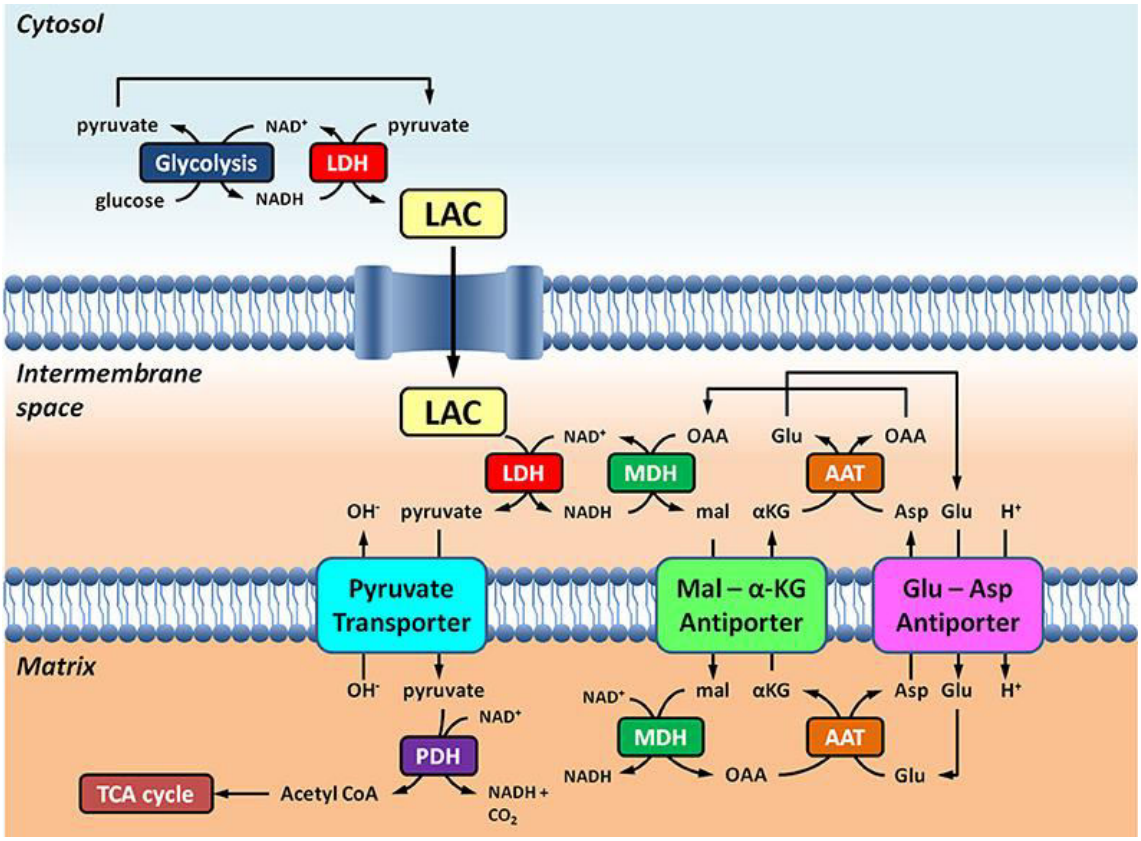


Figure 13: Malate-Aspartate shuttle (From Latham et al. 2012). Lactate-MAS shuttle nourish glycolytic flux by increasing intracellular NAD^+ and at the same time ATP synthesis by the increase of NADH production at mitochondria. Lactate derived

from glycolysis can be transformed into pyruvate by the LDH at the intermembrane space at mitochondria. Pyruvate is then imported to the matrix and joins the TCA. On the other hand, NADH produced during lactate to pyruvate reaction can be used for oxalacetate reduction to malate by the MDH. Malate is exchange by the antiporter Mal-αKG and is reduced to Oxalacetate at the matrix, additionally generating mitochondrial NADH. By this exchange, α-ketoglutarate reaches the intermembrane space and in presence of aspartate, they are transformed into glutamate and oxalacetate. Intermembrane oxalacetate will be used for the MDH reaction. Finally, matrix oxalacetate and glutamate are transformed into aspartate and α-ketoglutarate. Aspartate is then exported to the intermembrane by exchanging for a glutamate and a proton in order to nourish aspartate, α-ketoglutarate to oxalacetate, glutamate reaction. In this shuttle, NADH is generated into the mitochondria, meanwhile NAD^+ can be used to increase glycolytic flux. Lac (Lactate), OAA (Oxalacetate), Mal (Malate), a-KG (a-Ketoglutarate), Glu (Glutamate), Asp (Aspartate), MDH (Malate dehydrogenase), AAT (Aspartate Aminotransferase).

5.5. Lactate in physiology

Lactate physiological value in blood of healthy individuals is <2mM and it is very similar in arterial and venous blood, being a little increased in veins (248). This variability between arterial and venous lactate comes from the fact that there are tissues that produce lactate whereas other clear lactate. In homeostasis, there are tissues and cells that sustain the physiological production of lactate such as skeletal muscle (maximal contribution), heart, brain, adipocytes and erythrocytes. On the other hand other tissues contribute to the lactate clearance such as kidney and liver (majority), but also other organs and paradoxically, myocardium (Clears between 5 and 15%) and brain. These organs able to remove lactate from blood can be classed between those that use lactate as a bioenergetic source and those that using it to regenerate glucose, glycerol or other molecules **(Figure 14 and 15)** (249).

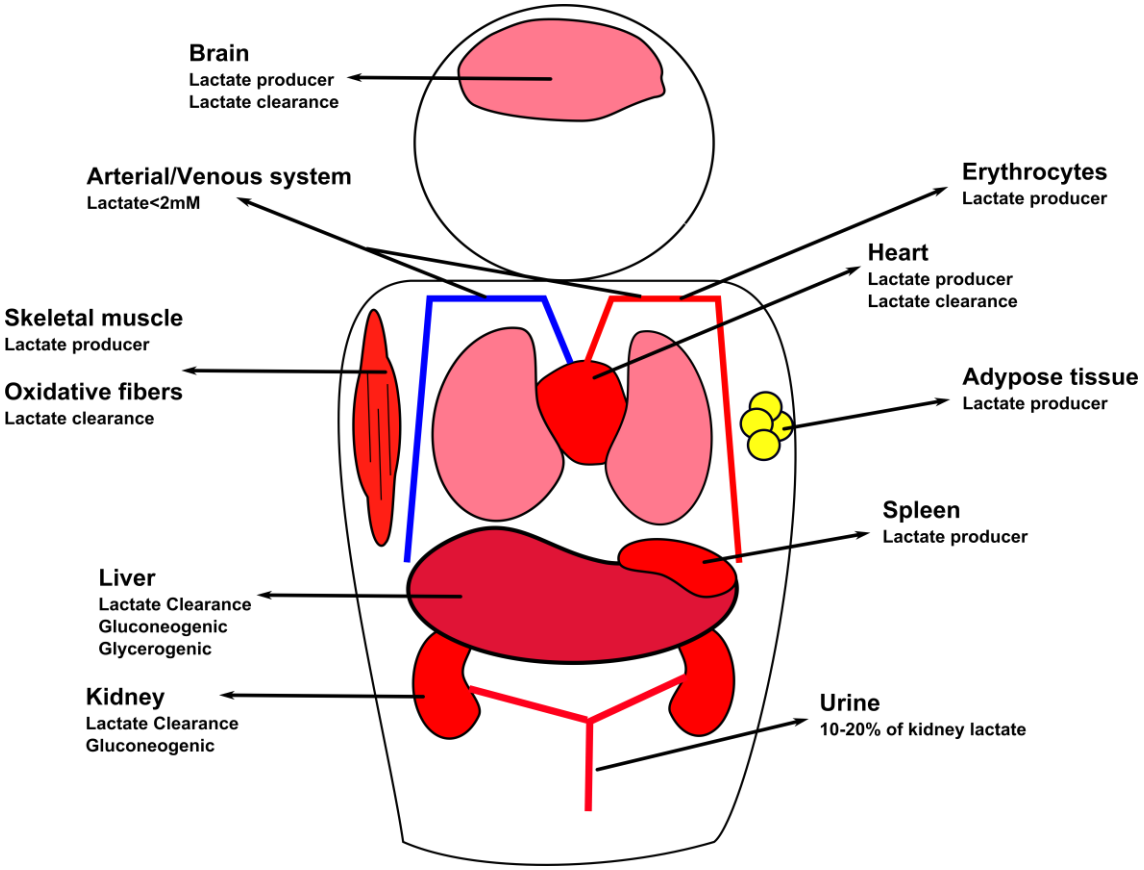


Figure 14. **Lactate consuming and producer organs.** In the human body, there are organs that produce and others that consume lactate. Skeletal muscle, adipose tissue, spleen and lymphnodes (during pro-inflammatory response), brain and heart are lactate producers. On the other hand, liver and kidney, the main organs that clear lactate, can use it to regenerate glucose. Also, lactate can be excreted through the urine. Lactate secretion represents just a small quantity of lactate clearance comparing to circulating and kidney lactate. Finally, several organs can do both, produce and oxidize lactate such as brain, heart and skeletal fibers.

Among the different tissues able to oxidize lactate, there are many shuttles that describe the way how lactate oxidative cells are nourished by, usually, proximal glycolytic cells. The most known example of this tissue relation is the Cori Cycle that occurs between the skeletal muscle and the liver. According to the Cori cycle, skeletal muscle use glucose and produce lactate and this lactate is uptake by liver to regenerate glucose that will be used for skeletal muscle (250). However, despite the contribution of Cori cycle during exercice (20%), it has been shown that lactate production by glycolytic muscles

is mainly uptake by adjacent oxidative fibers and heart (251). About the oxidative metabolism of cardiomyocytes, recently a group focused on proteomic analysis suggested a novel metabolic interconnection between cardiomyocytes and fibroblasts. In this work authors suggest that fibroblasts generates lactate through glycolytic metabolism and cardiomyocytes oxidizes the lactate (252). Another well known metabolic interconnection is observed in brain between astrocytes and neurons. In this lactate shuttle, the astrocytes metabolize glucose to lactate and this lactate is uptake by neurons. However, neurons are also able to metabolize glucose, that is uptake through the highly active transporter GLUT3 (253).

Apart from oxidative use of lactate, there are several tissues that can use carbon backbone from lactate to generate other molecules. As it was previously mentioned, in Cori cycle, liver is able to uptake lactate from the bloodstream and using it as a gluconeogenic substrate to regenerate glucose. However, liver is not the only tissue able to use lactate to regenerate glucose, kidney is also a gluconeogenic tissue. The relevance of these gluconeogenic organs remains on the fact that they are the main tissues that remove lactate. Additionally, kidney is responsible for the urinary secretion of lactate. On the other hand, urinary secretion of lactate is low, as it has been shown that lactate secretion is only the 10-20% of total kidney lactate (254). Another metabolite related to lactate is glycerol. It has been reported a small contribution of lactate as a glycerogenic substrate on hepatocytes during lipogenesis (255). Additionally, another study demonstrated that lactate is the major contributor as lipogenic substrate on periuterine adipose tissue in fed rats (256).

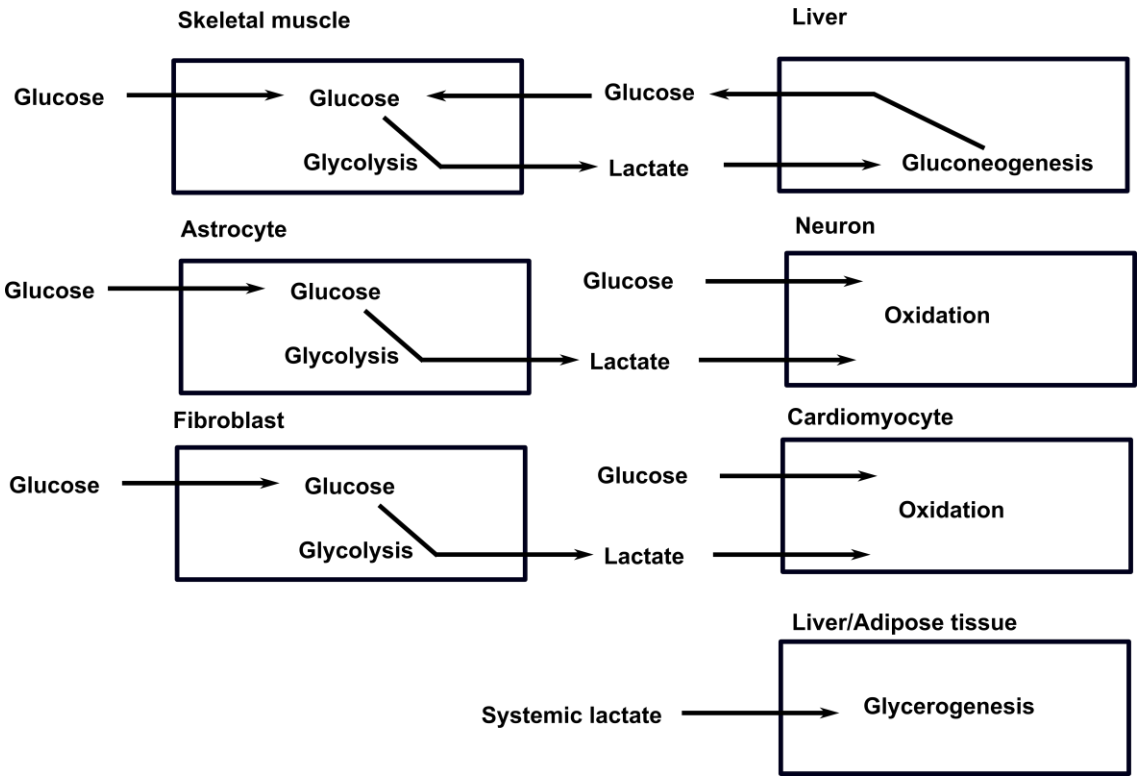


Figure 15. **Lactate shuttles between tissues.** There are several lactate shuttles described in physiology. In Cori cycle, muscles consume glucose and secrete lactate. This lactate is then uptaked by liver in order to regenerate glucose though the gluconeogenesis. This glucose is then secreted by the liver and again consumed by the muscles. In brain, it has been shown that neurons use both substrates, glucose and lactate. Lactate consumed by neurons is mainly derived through the astrocytes. This relationship has been also observed between fibroblasts and cardiomyocytes. In this case cardiomyocytes consume the lactate exported by fibroblasts. Finally, other tissues can uptake systemic lactate in order to regenerate glycerol, such as liver and adipose tissue.

About the effects of lactate in physiology, there are several examples that support the hypothesis that lactate sensing or uptake has an impact on cell metabolism **(Figure 16)**. In skeletal muscle, it has been shown that lactate infusion in rats induced insulin resistance in skeletal muscle. Interestingly, the authors showed that the glycolysis impairment appears before insulin-induced glycolysis, meaning that lactate impairs glycolysis resulting in a decreased stimulated glucose uptake (257). Moreover, as it was previously showed lactate impairs lipolysis on adypocytes (243). Another study demonstrated, using GPR81-deficient mice, that the anti-lipolytic effect of insulin was

mediated by lactate signaling. In this study the authors demonstrated, that white adipose tissue explants from GPR81$^{-/-}$ mice treated with isoproterenol (lipolytic stimulus), and insulin (anti-lipolytic stimulus) failed to inhibit lypolysis. These results suggested that insulin-induced glycolysis is required to generate lactate, which through an autocrine loop will induce lipolyisis impairment through GPR81 by reducing cAMP (258). Another study using myoblast cell line C2C12 differentiated myotubes demonstrated that lactate sensing induced triglycerides accumulation and an increase in MalonylCoa:ACP transferase (MCAT) and PDH proteins related to mitochondrial maintenance (259). Lactate also has been proved to have an impact on nervous cells. A recent study demonstrated, using GPR81$^{-/-}$, that lactate induced by exercise or subcutaneously injected, enhanced cerebral vascular endothelial growing factor A (VEGFA) and cerebral angiogenesis in the sensorimotor cortex (260). Additionally, another study demonstrated MCT1 is essential for Schwann cell function after nerve crush for nerve regeneration. This study was performed using heterozygous MCT1 mice (261).

Moreover, the effects of lactate on physiology have already been described on lactemia (No acid involved) and lactic acidosis (Acid involved). The increase of lactate on blood can be the consequence of different causes. Lactic acidemia type A usually is associated to septic shock, regional ischemia or cardiogenic shock among other causes. Lactic acidemia type B is usually associated to diseases and malignancies (262). Symptoms of this increase of lactic acid in blood are usually related to headaches, fatigue, and drowsiness among other. However, the most notorious effect of lactic acid in disease is related to the hemodynamic. In fact a study performed in rats and dogs using the agonist of GRP81 AZ2, demonstrated that the use of GPR81 agonists increase mean arterial pressure leaded by a decreased renal flow. This study also suggests that the decrease of renal flow is directed by the GPR81 dependent expression of endothelin-1 (ET-1). This peptide has a vasoconstrictor effect that could be related with the decrease of renal flow and consequently with the increase in mean arterial pressure (263).

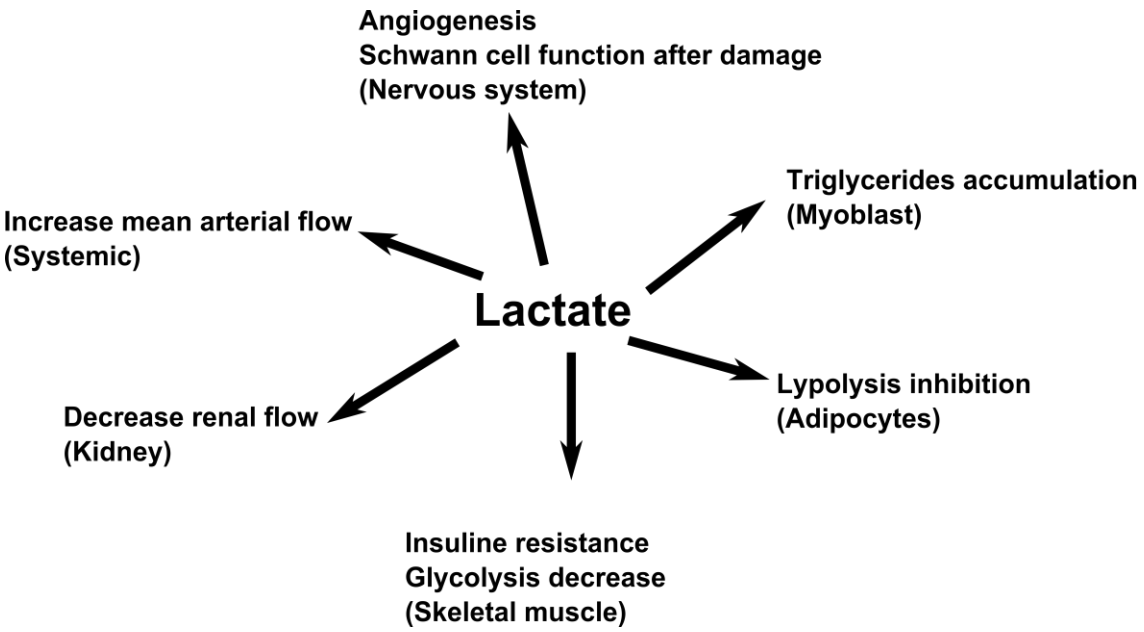


Figure 16. **Lactate effects in physiology.**

To sum up, lactate has differents role on physiology, and GPR81, SMCT and MCT make possible these functions.

5.6. Lactate in immunology

As expected, lactic acid has also an impact on immune system. However, it is important to distinguish between lactate effects on immune cells and lactate correlation with the disease severity. Indeed, as it was previously mentioned several activated immune cells have a highly glycolytic metabolism such as neutrophils or macrophages. In this part, we will focus specifically on the effect of lactate and lactic acid on immune cells.

One of first work suggesting the effect of lactate on immune cells was performed using peritoneal dyalisis (PD) solution. This solution was used in patient with end-stage failure in order to impair local inflammatory response. Interestingly, the authors demonstrated that PD also inhibited LPS-induced monocyte-derived DC maturation. The analysis of PD showed presence of sodium lactate and glucose-degradation products. In this article, authors demonstrated that PD solution and concretely, lactate, impaired both in MoDC, IL12p70 and TNFα production, CD83 expression and costimulatory activity in MLR context. Additionally, the authors demonstrated that this

inhibitory effect was associated to the impairment of NF-κB activity (264). However, the most part of the studies performed on immune cells and lactate are related to lactate derived-tumour cells. The effects of lactic acid on immune system have an enormous interest especially in the study of tumour microenvironment and immune escape. In fact lactate represents a risk factor and a predictor of tumour malignancy and metastases (265). However, similarly to the study mentioned before, the first work linking tumour-derived lactate and the immune system demonstrated that tumour-derived lactate impaired dendritic cell activation. In this work the authors confirmed what it was observed using PD solution in MoDC. Using spheroids or supernatant from MelIm (Melanoma) and J82 (Urothelial carcinoma) cell lines the authors observed a decrease in IL12p70 production and an impaired capacity of MoDC to stimulate $CD8^+$T-cells. Additionally, the authors showed a decreased of CD1a marker in presence of tumour supernatant and lactic acid without involvement of acidification (266). After this work, the same authors demonstrated that lactic acid also affects lymphoid cells. In this article, they showed that lactic acid but not his conjugate base, lactate, impairs cytotoxic lymphocyte (CTL) proliferation. They also observed an inhibition in CTL of IL-2 and IFNγ production and their cytotoxic activity. These results were observed in presence of lactate producing MelIm spheroids. Moreover, the authors showed that the inhibition of lactate production with oxamate, an LDH inhibitor, abrogated partially the suppressive effect of spheroid tumour. Another relevant point of this article is the first time linking of lactate, immunosuppression and MCT1. In fact, the authors showed that lactate can be uptake by CTL cells just in acid media (267). From these two studies, lactate taked place as an important factor in tumour microenvironment for immune escape. After these works, other teams have inquired on the immunosuppressive role of lactate in different immune cells both in myeloid as in lymphoid.

On the other hand, lactate has been also related to non-suppressive activities. For example, recently it has been shown that lactate is a neutrophil mobilization factor. In fact, lactate can be sensed by bone marrow endothelial cells through GPR81. After activation by lactate, bone marrow endothelial cells (BMEC) reduced VE-cadherin expression leading to an increase of vascular permeability and a release of bone marrow neutrophils (268).

5.6.1. Effect of lactate on Myeloid cells

The effect of lactate on myeloid cells has been reported to impair proinflammatory functions in different types of cells such as monocytes, DC and macrophages **(Figure 17)**. In monocytes, lactic acid impairs the expression and production of TNF-α. Moreover, in this work the authors showed that monocytes stimulated with LPS and lactic acid had a decreased glucose uptake and a decrease in ATP production. None of these effects was mediated by acidification. Interestingly, this effect was already reported in skeletal muscle by Choi et al. 2002. This work also confirmed the results observed by Fischer et al. demonstrating that medium acidification is needed to intake lactate (269). About lactate effect on DC, different studies have been performed showing impairment on production of pro-inflammatory cytokines, migration and differentiation. A study focused on the culture density of differentiated DC from monocytes demonstrated that at high cellular concentration lactate production is increased and it is related to a decrease in IL-12, TNFα and IL23 cytokines meanwhile they observed an increase in IL-10 production. The authors also showed a defect in CCL19 dependent migration and differentiation based on the decrease in CD1a marker (270). On the other hand, another study demonstrated that transient exposure of DC to acidic media (90 minutes) induced an increase of IL-12 production, a spontaneous maturation with over-expression of HLA-DR, CD40, CD83, CD86 and CCR7, an enhanced endocytosis and enhanced capacity to stimulate T-cell proliferation in MLR context. Additionally the authors showed an increased phosphorylation of p-AKT, p-ERK and p-p38 suggesting an activation induced by acidification. However, in this work it was evaluated just the effect of acidification but not lactate which still has an interest due to acidification derived from lactic acid export (271). As it was previously mentioned, most part of works linking lactate to immunosuppression has been focused from a tumour biology perspective. However, the effect of lactate has also been considered in other pathologies such as colonic inflammation. In fact, a recent work demonstrated the relevance of lactate receptor GPR81 in myeloid cells in a model of murine colitis by using GPR81 KO mice. In this article, the authors showed that GPR81 is essential to inhibit colonic inflammation and to restore colonic homeostasis. In $GPR81^{-/-}$ mice, the authors observed an increase of Th1/Th17 T-cells and a decrease in regulatory T-cells. In order to determine the cell mediator expressing GPR81 that was

or were involved in inflammatory suppression, the authors used a CD45RB^{hi}CD4 T-cell-transfer colitis model in Rag2$^{-/-}$ to eliminate T and B-cells and Rag2$^{-/-}$GPR81$^{-/-}$. As Rag2$^{-/-}$GPR81$^{-/-}$ displayed an exacerbated colitis, the authors deduced that innate cells such as macrophages and DC were responsible for inflammatory suppression. Additionally, the authors showed that GPR81$^{-/-}$ mice have an increased production of pro-inflammatory cytokines such as IL-6, IL-1β and TNFα and a decrease in immunoregulatory cytokines such as IL-10 in excised colon samples cultured ex vivo from DSS treated mice. (272). Another study using bone marrow-derived macrophages showed that the pro-inflammatory activation with LPS impairs *Il1b, Il6, Il12b* expression. In this article, the same effect that in muscle was observed, BMDM displayed a reduced glycolytic flux in presence of lactate after LPS treatment (273). Apart from impaired myeloid cells function and differentiation, lactic acid derived from tumours has been described as a differentiation factor for tumour associated macrophages (TAM), also called M2-like macrophages. TAM are resident cells in the tumour microenvironment with a key role in tumour progression and metastasis. These cells contribute to the chronic inflammation and angiogenesis (274). It has been shown that TAM can be induced by tumour-derived lactic acid. In fact, a study demonstrated that the culture of BMDM with lewis lung carcinoma (LLC) supernatant induce macrophages phenotypicaly similar to TAM expressing Arg1 and VEGF, that are factors that promote angiogenesis. In this work, the authors demonstrated that only the supernatant fraction under 3KDa was able to induce the expression of Arg-1 and VEGF. Moreover, the authors demonstrated that this expression was lead by HIFα activation, suggesting that lactic acid through HIFα induce TAM differentiation (275). Another recent study also demonstrated that tumours displaying a high expression of LDHa and GLUT1 produce more lactic acid that those that have low expression and this correlated with M2-like macrophages accumulation in human head and neck squamous cell carcinoma (HNSCC) (276).

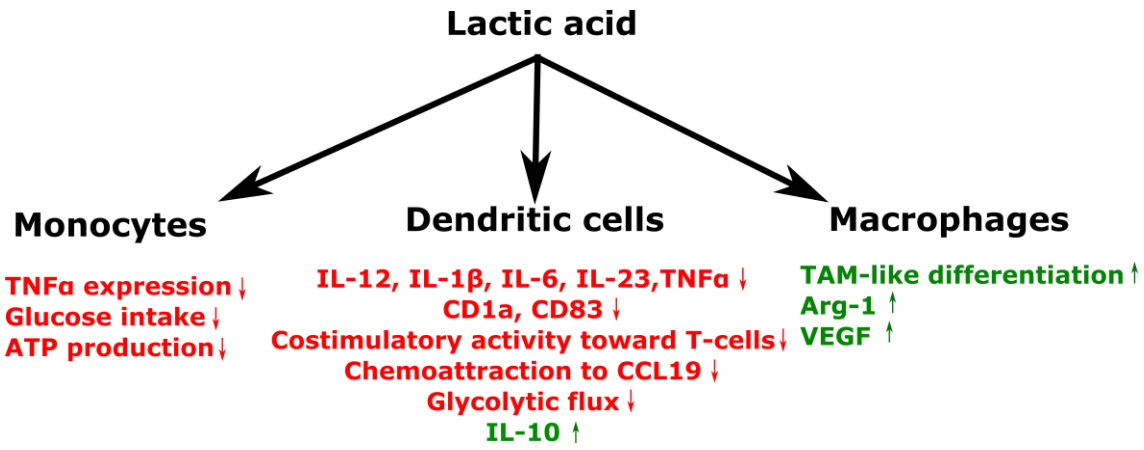


Figure 17. Lactate effects on myeloid cells. Lactate has an impact on myeloid cells. In monocytes, lactate impairs TNFα expression, glucose intake and ATP production. In DC, lactate impairs the expression of pro-inflammatory cytokines such as IL-12, IL-1β. IL-6, IL-23, TNFα, leads to a decrease in several markers such as CD1a and maturation marker CD83, and impairs costimulatory activity and motility directed by chemokines. Similarly to monocytes, lactate impairs glycolytic flux in DC. On the other hand, lactate increases the expression of the immunoregulatory cytokine IL-10. In macrophages, it has been shown that lactate induces TAM-like differentiation (Red: Decrease, Green: Increase).

5.6.2. Effect of lactate on lymphoid cells

Lactate also affects lymphoid cells (**Figure 8**). Several articles describe different effects on CD4, CD8 and NK cells regarding activation, polarization and migration. A recent study demonstrated that melanoma patients with a high LDH-A expression have a reduced survival comparing to LDH-A low patients. In order to corelate lactate production and loss of immunosurveillance, the authors generated a model of cancer in mice using pancreatic adenocarcinoma cell line (Panc) KO or with high expression for LDH-A. The authors showed a higher tumour growth in wild type and Panc-LDH-A^{high} comparing to Panc-LDH-A^{low}. Moreover, the authors showed that in mice bearing Panc-LDH-A^{low} tumour there was an increase in $CD8^+$ and $NK1.1^+$ cells with a higher expression of IFNγ and Granzyme B. Additionally the authors suggested that this impairment in NK and CD8 activation was led through lactic acid by a decrease in intracellular ATP and a decrease in NFATc1 expression. This article supports previous

works suggesting the immunosuppressive role of lactate on T-cells and confirms that lactate is internalized just in presence of acid (277). On the other side, the suppressive effect of lactate on NK was already reported. In the article, the authors showed that mice bearing Panc-LDH-$A^{-/-}$ have a lower MDSC in spleen and a NK improved cytolytic activity. The authors also showed that the in vitro pretreatment of NK cells with lactic acid display a lower expression of perforin and granzyme. In order to reduce lactate production in vivo to improve anti-tumour response, the authors used a ketogenic diet (KB substrates are uniquely oxidatives). The use of this diet resulted in a small tumour growth reduction. On the other hand, this diet induced a reduction of myeloid cells and $CD4^+FoxP3^+$ cells and an increase in $CD4^+$ and $CD8^+$ T-cells in spleen. Interestingly this diet reduces prostate and brain tumours (278). Alternatively, lactate not only affects functionality but also can affect motility. In fact it has been demonstrated that lactate affects differently migratory activity of $CD4^+$ and $CD8^+$ T-cells. The chemoattration by CXCL10 impairs $CD8^+$ migration in presence of lactic acid meanwhile $CD4^+$ T-cells in presence of lactate (240). Moreover, in this work, the authors showed that the inhibition of MCT1 with CHC, phloretin or Slc16a1 antibody leads to the recovering of migratory capacity of $CD8^+$ T-cells. On the other hand, the migratory skills induced by CXCL10 in $CD4^+$T-cells seem to be mediated by Scl5a12 transporter. The author showed also that lactate without acidification impairs aerobic glycolysis in $CD4^+$T-cells by decreasing glucose uptake and glycolytic flux. Same results were observed but with lactic acid in $CD8^+$T-cells. Moreover the authors showed that $CD4^+$T-cells treated with lactate enhanced IL17 and RORc expression without affecting other pro-inflammatory cytokines. On the other hand the presence of lactic acid impaired cytolytic activity of CTL. Finally, the authors showed in a mice model of peritonitis induced by zymosan, that the blockade of slc5a12 lead to the release of $CD4^+$T-cells from the inflamed peritonea meanwhile the blockade of MCT1 transporters lead to the release of $CD8^+$T-cells. Altogether these results suggest that lactate has an important role by the retention of lymphocytes into the inflamed tissues (240). Interestingly, the immunosuppressive role of lactate also benefits to regulatory T-cell function and differentiation. Recently, a group demonstrated that regulatory T-cells are favored in microenvironments with low concentration of glucose and high concentration of lactate. In their article, the authors demonstrated that in normal conditions, activated T-cells induced c-myc, that is responsible to the shift of oxidative to glycolytic metabolism. By this shift, NAD^+ is regenerated through LDH and lactate production in

order to give substrate to the GAPDH. In activated cells in low glucose and high lactate medium, the increase in intracellular lactate impairs NAD^+ regeneration leading to a decrease in glycolytic flux and the loss of effector T-cell function. On the other side, in normal condition regulatory T-cells express FoxP3, that is an inhibitor of c-myc expression and lead the cells to an oxidative metabolism. In low glucose and high lactate media, the oxidative metabolism of regulatory T-cells allows them to survive without loss of function. Interestingly, in this work, the authors showed that the addition of sodium lactate to C57BL/6 splenocytes stimulated with anti-CD3 antibody impaired $CD4^+$ and $CD8^+$ proliferation. This effect was strongest for $CD4^+$T-cells in low glucose condition. On the other hand, the authors also showed that lactate synergize with TGFβ enhancing regulatory T-cell induction in polyclonal stimulation (193)

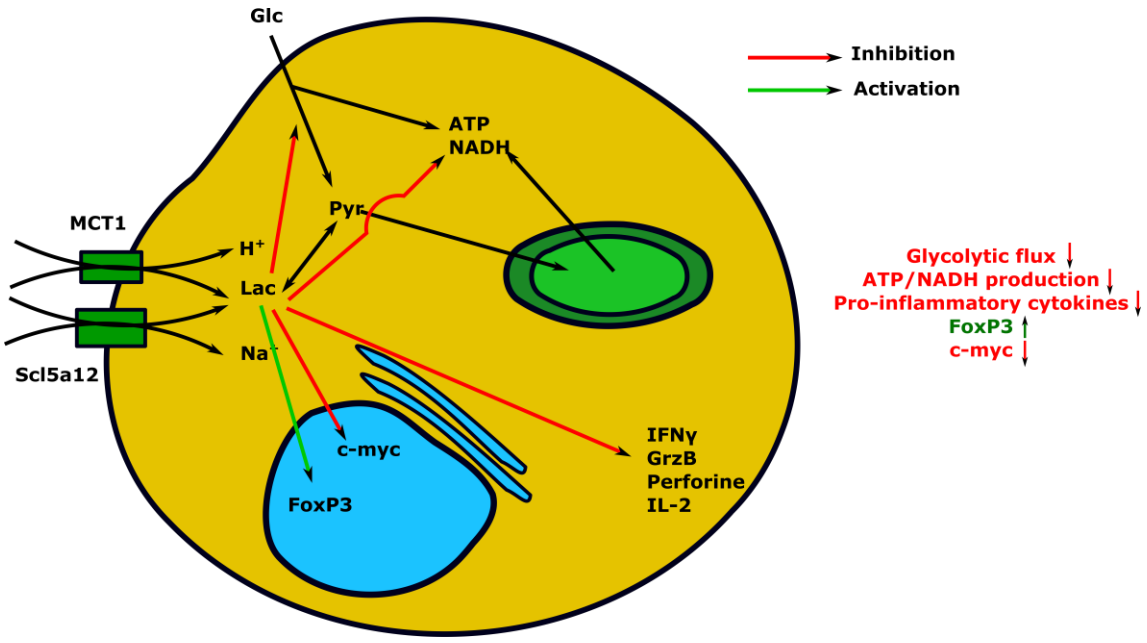


Figure 18. Lactate effects on lymphoid cells. Lactate affects metabolism and function of lymphoid cells. First, lactate can be uptake by lymphoid cells through MCT1 and Scl5a12 transporters. Once inside the cell, lactate impairs glycolytic flux, expression of cytokines, *Grzb* and Perforine and leads to a decrease in $NADH/NAD^+$ ratio. Also, the presence of high concentration of lactate has been related to the expression of *Foxp3* and the repression of *c-myc*.

Thesis context

Our team has been investigating tolerogenic dendiritc cells in the context of cell based therapy during more than 15 years. During this time, we demonstrated that Tol-BMDC were able to increase heart, skin and pancreatic islet allograft survival in rodent models. Due to their beneficial effect in transplantation models, these cells have been moved to clinic in a first phase I/II clinical trial in the context of kidney transplantation.

At my arrival, clinical trial was already started and hATDC were partially characterized in terms of function and phenotype. We knew that hATDC were IL-10 stable producing cells and were able to impair T-cell proliferation. Moreover their efficacy in vivo was also demonstrated. However, the mechanisms underlying hATDC were unknown. Several inhibitors of classically known Tol-DC mechanisms were tested in order to abrogate hATDC immunosuppressive effect without success. Therefore, the main objective of my thesis was to characterize in vitro, the immunosuppressive mechanisms of hATDC. Throughout the thesis results, we considered the requirement to characterize hATDC metabolism. On the other hand, as I demonstrated that hATDC mechanisms were independent from contact, we were interested in hATDC supernatant study in terms of effects toward T-cells and also in terms of composition. After different tests, we demonstrated that lactate present in hATDC supernatant was partially responsible for the immunosuppressive effects. However, other small molecules with immunosuppressive potentials were and are still being unidentified. Moreover, as the molecule of interest, lactate, is also an oxidative substrate and literature describes this metabolite as a metabolic modulator, we were interested on the study of $CD4^+$T-cell metabolism in presence of lactate and hATDC supernatant.

Results I: Thesis article

Title:Human tolerogenic dendritic cells regulate immune response through lactate synthesis

Authors: Eros Marin[1,2†], Laurence Bouchet-Delbos[1,2†], Cédric Louvet[1,2], Amandine Even[1,2,3], Matthieu Giraud[1,2], ThienPhong Vu Manh[4], Amy Managh[5], Véronique Daguin[1,2],Claire Pecqueur[6], Audrey Aguesse[7], Gaelle Bériou[1,2], Elise Chiffoleau[1,2], Brigitte Alliot-Licht[1,2,8], James A. Hutchinson[9], Natasa Obermajer[10], Barry Sharp[5], Edward Geissler[9], Bernard Vanhove[1,2], Gilles Blancho[1,2,11], Marc Dalod[4], Régis Josien[1,2,11,12], Maria-Cristina Cuturi[1,2,3#] andAurélie Moreau[1,2,3 #*]

Affiliations:

1. Centre de Recherche en Transplantation et Immunologie UMR1064, INSERM, Université de Nantes, Nantes, France.
2. Institut de Transplantation Urologie Nephrologie (ITUN), CHU Nantes, Nantes, France.
3. LabEx IGO "Immunotherapy, Graft, Oncology", Nantes, France.
4. Aix Marseille Univ, CNRS, INSERM, CIML, Centre d'Immunologie de Marseille-Luminy, Marseille, France.
5. Centre for Analytical Science, Department of Chemistry, Loughborough University, Loughborough, UK.
6. Centre de Recherche en Cancérologie et Immunologie Nantes-Angers UMR1232, INSERM, Université de Nantes, Nantes, France.
7. CRNHO, West Human Nutrition Research Center, Inra UMR 1280 PhAN, Mass Spectrometry Core Facility, Nantes, France

8. Faculté d'Odontologie, Université de Nantes, Nantes, France.

9. Department of Surgery, University Hospital Regensburg, Regensburg, Germany.

10. Division of Surgical Oncology, University of Pittsburgh, Hillman Cancer Center, Pittsburgh, PA 15232, USA.

11. Faculté de Médecine, Université de Nantes, Nantes, France.

12. Laboratoire d'Immunologie, CHU Nantes, Nantes, France.

[†]co-first authors

[#]co-senior authors

[*]To whom correspondence should be addressed:

Aurélie Moreau

CRTI INERM U1064

CHU Hotel-Dieu

30 bd Jean Monnet

44093 Nantes

France

Phone nb : +33.2.40.08.74.10.

aurelie.moreau@univ-nantes.fr

Competing interests: The authors have declared that no conflict of interest exists.

One sentence summary: ATDC promote immune tolerance through lactate synthesis and Treg induction

ABSTRACT

Cell therapy appears as a promising strategy to treat patients suffering from autoimmune or inflammatory diseases or receiving a graft. Our previous works on rodents and primates revealed that Autologous Tolerogenic Dendritic Cells (ATDCs) were safe in macaques and promoted graft tolerance in rodents. We recently derived human counterparts which are currently administered in a first-in-man phase I/II clinical trial to patients receiving a kidney transplantation. In this study, we highlight that human ATDCs display a unique profile based on phenotypic and transcriptomic analyses.The original feature of ATDCs is that these cellssuppress T cell proliferation and expand regulatory T cells by their secretome. ATDCs have a highly dynamic metabolism and their high production of lactate strongly contributes to shape T cell responses towards tolerance. Regarding their *in vivo* properties, ATDCs delay Graft-versus-Host-Disease developmentin a humanized mousemodel andpromote the expansion of human regulatory $CD4^{+}Foxp3^{+}$ T cells in association with elevated levels of lactate in blood. In light of their strong tolerogenic potential *in vitro* and *in vivo*, we believe that ATDC therapy should be extended to other clinical trials with the aim of regulating the immune response.

INTRODUCTION

Current therapies for autoimmune diseases and allograft rejection generally involve the continuous use of non-specific immunosuppressive drugs that are associated with increased risks of infections, certain types of cancer and toxicities. Cell-based immunotherapy with tolerogenic dendritic cells (DCs) has long been recognized as an efficient mean of promoting antigen specific tolerance (1, 2). Indeed, the administration of tolerogenic DCs in rodents potently prevents various autoimmune diseases (3-5) and rejection of allografts as previously reported by our group and others (6-10). In these models, the potency of tolerogenic DC therapy in the induction of transplantation tolerance was linked to an increase of regulatory T cells (Tregs) in lymphoid tissues (8-12). Strategies for the preparation of clinical-grade monocyte-derived tolerogenic DCs have emerged only within the last few years, which has enabled several groups to investigate the potential clinical benefit of their adoptive transfer into patients suffering from autoimmune, inflammatory or graft rejection diseases.The first phase I clinical trial using tolerogenic DCs was reported in 2011 in type 1 diabetic patients (13) and was then followed by other trials in rheumatoid arthritis (14, 15) and Crohn's disease (16). Injections of tolerogenic DCs were well tolerated as no adverse effects or toxicity were observed. In the field of transplantation, no clinical trials with tolerogenic DCs have been reported yet. We previously demonstrated the efficacy of Autologous Tolerogenic DCs (ATDCs) in rodent models of transplantation and their safety in non-human primates (6, 9-11, 17,18). Based on this expertise, we developed a manufacturing process to obtain a unique population of human ATDCs. These cells, studied in this paper, are currently being administered to recipients of a renal transplant from a living donor, in a first-in-man phase I/II clinical trial, in order to evaluate the safety and efficacy of this cellular immunotherapy in solid organ transplantation (NTC0225055).

In this article, we characterized ATDCs based on the analysis of their phenotype and transcriptome, their suppressive properties and their metabolism. We showed that ATDCs create a lactate-rich microenvironment conducive to tolerance induction. This microenvironment dysregulates the aerobic glycolysis of T cells and induces a suppression of T cell proliferation,

as well as an expansion of regulatory T cells. In order to complete the understanding of their fate and their mechanisms of action observed *in vitro*, human ATDCs were also analyzed following their infusion in a model of human-into-mice xenogenic Graft versus Host Disease (GVHD). This humanized mouse model provides a useful tool to investigate the outcome of therapeutic cells post-injection (survival, viability, migration and stability), their safety and their *in vivo* mechanisms of action. Concomitantly to the Phase I/II trial evaluating the safety of ATDCs, this study allowed us to decipher themechanisms responsible for the tolerogenic activity of ATDCs and paves the way to the extension of their clinical application to various diseases.

RESULTS

Human ATDCs display strong tolerogenic features

Protocols of human tolerogenic DC generation have been described following treatment of blood monocytes with GM-CSF and IL-4, in association with other cytokines such as IL-10, immunomodulatory reagents such as rapamycin, dexamethasone or Vitamin D3 and DNA/RNA modifications, including anti-sense oligonucleotides (for review, (19)). Our previous works performed in mice and non-human primates demonstrated the robustness of a low-dose GM-CSF protocol (in the absence of other cytokines or agents) to derive tolerogenic DCs with regulatory properties *in vitro* and *in vivo* (10, 17). We thus developed a GMP-compatible manufacturing process to derive human ATDCs in thepresence of low-dose GM-CSF as the only cytokine used. ATDCs were obtained after a 6 day-culture of monocytes in AIMV medium supplemented with 100 IU/ml of GM-CSF. The main specificities of this ATDC protocol is to use a medium without serum and without IL-4. To characterize ATDCs, monocyte-derived DCs (MoDCs) were used as control cells. MoDCs were obtained following a classical protocol (20), in which monocytes were cultured in RPMI/FCS medium supplemented with GM-CSF and IL-4.

Consistent with a tolerogenic profile, ATDCs display an immature phenotype (HLA-$DR^{low}CD80^{-/low}CD83^{-}CD40^{low}$) whereas MoDCs express higher levels of these markers **(Figure 1A)**. Low to intermediate levels of CD86 marker were observed in ATDCs and MoDCs relating to the donors, without significant differences between these two DC types. We then determined that ATDCs maintain their immature state (with no overexpression of CD83/CD86 and a weak increase of CD80 expression) following exposure to LPS (**Figure 1B**) or other TLR ligands (**Figure S1**), in contrast to MoDCs which up-regulated co-stimulatory molecules under the same experimental conditions (**Figure 1B**). Furthermore, TLR ligand-stimuli favored IL-10 secretion by ATDCs in the absence of IL-12 production, reinforcing the tolerogenic features of ATDCs **(Figure S1).** Strikingly, even following a strong stimulation with LPS and IFNγ, ATDCs

preserved IL-10 secretion and still produced only a quarter of the IL-12 secreted by MoDCs under the same experimental conditions (**Figure 1C**).

Tolerogenic DCs are also defined by their ability to control T cell proliferation. Inhibition of T cell proliferation by ATDCs was assessedby MLR and suppression assays. The results highlighted that ATDCswere relatively poor stimulators of allogeneic T cells (**Figure 1D)** and were able to significantly suppress $CD4^+$ T cell proliferation (**Figure 1E**). This suppression was associated to an inhibition of IFNγ- and IL-17-producing T cells **(Figure S2)**. In comparison, immature MoDCs stimulatedallogeneic T cell proliferation much more potently and were significantly less efficient than ATDCs insuppressing $CD4^+$ T cell proliferation **(Figures 1D/1E)**.Taken together, these results demonstrate the strong tolerogenic profile of ATDCs.

ATDCs exhibit a unique phenotype

To better characterize ATDCs, these cells were phenotyped for the expression of an array of cell surface markers typically expressed on DCs, monocytes or macrophages. As expected, these *in vitro*-derived cells displayed a distinct phenotype compared to *in vivo* myeloid cells (**Figure 2A and Table S1)**. ATDCs appeared as a homogeneous population based on the analyzed markers. In comparison to MoDCs that represent the conventional phenotype of DCs derived from monocytes*in vitro* (depicted on **Figure S3A)**, both populations expressed CD11b, CD11c or CD206 markers. However, ATDCs did not express CD209 and FcεR1 markers but expressed CD64 and CX3CR1 markers that are more classically associated to a macrophage profile. ATDCs also expressed CCR7 suggesting an ability to migrate into secondary lymphoid organs. In relation to the stimulation assays described in the previous section, we showed that TLR ligation increases CCR7 expression on ATDCs despite a rather immature phenotype (**Figure S1)**. These data suggest that *in vivo*, the tolerogenic potential of ATDCs in secondary lymphoid organs could be reinforced after encountering TLR ligands. ATDCs expressed ILT-2 and ILT-3 inhibitory molecules but didnot express PD-L1, HLA-G, ILT-4 and ILT-7. This analysis

demonstrated that the phenotype of human ATDCs is unique given their concomitant expression of DC and macrophage markers.

To gain further insights pertaining tothis characterization, transcriptomic analysis was performed on ATDCs, MoDCs and resting monocyte-derived macrophages (rMoMacro), all differentiated from monocytes from 6 common donors. Expression data from our *in vitro*-generated ATDCs, MoDCs and rMoMacro were then compared with previously published expression data sets from i) monocytes, ii) three *in vitro*-differentiated monocyte-derived cells : GM-CSF/IL-4 mo-DCs, M-CSF mo-DCs and M-CSF mo-Mac, and iii) two *in vivo*-differentiated monocyte-derived cells : ascites mo-DCs and ascites mo-Mac (21, 22). To validate the combined analysis of all these data sets (**Figures 2B and S3B**), we observed that the cell types differentiated within the same protocol between the public data (GM-CSF/IL-4 mo-DCs) and our study (MoDCs) clustered together. Furthermore, our rMoMacro clustered close to the *in vitro*-differentiated M-CSF mo-Mac. On this principal component analysis, Principal component (PC) 1 allows the clear separation between DCs and Macrophages whereas PC2 dissociates monocytes, *in vitro*-differentiated cells and*in vivo*-differentiated cells. This analysis highlighted that ATDCs represent an individual subtype of cells that was distinct from monocytes but differentiated neither in DCs nor in macrophages (**Figure 2B**). Due to their closeness to monocytes, it is possible to speculate that ATDCs are less differentiated towards DCs or macrophages than other monocyte-derived myeloid cells (either DCs or macrophages). Nevertheless, we also validated that ATDCs are stable cells as shown on Figure 1B and no DC/Macrophage markers appear or disappear after LPS stimulation (data not shown). The singularity of ATDCs was also illustrated by a heatmap depicting the 50 most expressed genes in ATDCs versus MoDCs and rMoMacro (**Figure S3C)** and the full list of genes with expression fold change over 2 in ATDCs (**Table S2)**.

Therefore, ATDCs appear as a unique population by their phenotypic and transcriptomic profile. We classified these cells as dendritic cells in accordance to their close properties to the rodent

counterparts that we previously described (10, 11) but the term mononuclear phagocytes is also suitable.

ATDCs migrate preferentially to the spleen after injection in humanized mice and preserve an immature phenotype *in vivo*

Clinical evaluation of the safety of ATDC-based immunotherapy is currently being performed on recipients of a kidney transplant from a living-donor. Prior to ATDC administration to patients, we sought to determine the fate of ATDCs after injection. To follow ATDC migration over time, ATDCs were labeled with gold nanoparticles before intravenous (IV) injection into NOD/SCID/IL2rγ$^{-/-}$ (NSG) mice. Gold-labeled ATDCs were then tracked using inductively coupled plasma mass spectrometry (ICP-MS) as previously described (23). This highly sensitive technique allows the detectionof rare cells in tissues. Preliminary experiments were carried out to define the appropriate labeling protocol (incubation for one hour with $6.32x10^8$particles/ml) and to validate that this labeling did not modify the immature state or the hypostimulative effect of ATDCs (**Figure S4A-D**). Irradiated NSG mice received gold-labeled ATDCs and human autologous PBMCs simultaneously in order to approaching the clinical settings. ATDCs were then tracked from day 3 to day 14 post-cell injection. At all these time points, gold-labeled ATDCs were mainly detected in liver, lung and spleen with a peak at day 6 in the spleen (**Figure 3A**). In contrast, no gold staining was observed in heart and intestine, and low staining was present in kidney (data not shown). As gold was detected in homogenized tissue, it could not be excluded that the detected signal was expressed by dead ATDCs or mouse phagocytic cells. To formally prove that gold staining was coincident with the presence of injected and viable ATDCs, a second technique was used. In this new experiment, irradiated NSG mice received ATDCs, stained with Qtracker® marker (24), and PBMCs. This staining did not modify the expression of costimulatory and maturation markers by ATDCs (**Figure S4E)** and allowed the tracking of cells after *in vivo* injection by flow cytometry. Taking into account the

results of the gold-labeled ATDCs tracking experiment, mice were sacrificed at day 6 and splenocytes were analyzed. ATDCs were identified by their expression of human CD45, human CD11c, human HLA-DR and Qtrackermarkers (**Figure 3B**). These cells represent less than 2% of total human $CD45^+$ cells detected in the spleen of NSG mice (**Figure 3C**). The viability of ATDCs was quantified using a viability dye marker and was measured as 72.5% (±5.9%) viable cells indicating that ATDCs are still alive *in vivo* 6 days post-injection (**Figure 3B**). Furthermore, ATDCs maintained their immature phenotype post-injection (**Figures 3B and3D**) suggesting that ATDCs did not become immunogenic post injection. Indeed, one of the major concerns associated with injection of tolerogenicDCs into humans is the risk that these cells become immunogenic DCs in response to inflammatory signals encountered *in vivo*. Taken together, our results highlight that alive ATDCs migrated in second lymphoid organs and preserved their tolerogenic profile in order to favor tolerance induction *in vivo*.

ATDC suppressive ability is associated to an increase of lactate secretion in vitro

To clarify how ATDCs control $CD4^+$ T cell proliferation (observed in Figure 1E), a range of experiments was performed. In these assays, total $CD4^+$ T cells were stimulated with allogeneic mature DCs to induce their proliferation (mixed lymphocyte reaction, MLR condition). The addition of ATDCs was able to highly suppress T cell proliferation (suppression assay condition) compared to MoDCs. We first determined that this suppression was not associated to a competition between allogeneic stimulatory mature DCs and ATDCs as the addition of a higher number of stimulatory DCs does not control the suppression **(Figure 4A)**. Secondly, our results demonstrated that IL-10, TGF-β or inhibitory molecules known to be expressed by tolerogenic DCs as iNOS or IDO, were not involved in this suppression **(Figures4B and 4C)**. Furthermore, natural Tregs were not required for the suppressive activity of ATDC *in vitro* as the suppression of T cell proliferation was still observed in the absence of $CD4^+CD25^+$ natural Tregs **(Figure S5A).** Neither induction of anergy nor apoptosis were able to explain the cellular

mechanisms of ATDC suppression **(Figure S5B-C)**. However, the study of the cell cycle of T cells following stimulation with or without ATDCs highlighted that ATDCs block T cells in G1 phase preventing their proliferation **(Figure S5D)**.

In order to investigate whether ATDC/T cell interactions were required for the ATDC suppressive effect, the suppression experiment was performed with transwell inserts. Interestingly, this assay indicated that ATDC suppression is mediated by contact independent mechanism **(Figure 4D)**. We then validated these results by showing that the transfer of suppression assay medium (MLR + ATDCs supernatant) was able to suppress a new MLR, suggesting that soluble factors produced, or induced, by ATDCs were involved in T cell suppression **(Figure 4E)**. As a modification of medium color (indicating acidity) was observed when T cells were cultured with ATDCs, we hypothesized that ATDCs could suppress T cell proliferation by nutriment deprivation. In this line, the measure of glucose at the end of the culture suggested that ATDCs uptake glucose from the medium **(Figure 4F)**. However, the addition of glucose, glutamine or pyruvate to the suppression assay did not abrogate ATDC suppressive ability demonstrating that nutriment deprivation was not responsible of ATDC suppressive effect **(Figures4G and S6A/B)**. Strikingly, we found thata high amount of lactate (around 10mM) was detected in the suppression assay condition **(Figure 4H).** Among all the assays performed, our results suggest that this strong lactate secretion could be responsible for ATDC-mediated suppression.

High amount of lactate secreted by ATDCs is involved in their suppressive activity

We then investigated the metabolism of ATDCs to understand this increase of lactate detected in presence of ATDCs in suppression assay condition. Our results highlight that ATDCs display a higher glycolytic ability (ExtraCellular Acidification Rate (ECAR) -**Figure 5A)**, as well as a higher respiratory capacity **(Figure 5B)** than MoDCs using Seahorse analyses. Together, it demonstrated the dynamic metabolism of ATDCs **(Figure 5C),** associated with a high

intracellular ATP production **(Figure S6C)**. Concomitantly we tested whether the transcriptome of ATDCs also revealed their strong biological process of glycolysis. For that, we computed the rank of differential expression of genes associated to glycolysis in ATDCs versus MoDCs samples using Gene Set Enrichment Analysis (GSEA) and we found a significant enrichment in ATDCs (Normalized Enrichment Score: 1.29; nominal p-value:0.046; n=178 probes) **(Figure 5D)**. In accordance with ECAR and transcriptomic results, a significant increase of lactate deshydrogenase (LDH) activity was observed in ATDCs **(Figure 5E)**.

To formally prove that lactate was produced by ATDCs themselves and not by the other cell types present in the suppression assay (T cells and mature DCs), ATDCs were cultured alone for 6 days and the culture supernatant was analyzed (ATDC SN). Supernatants from MoDC culture (MoDC SN) were used as controls. Our results showed that ATDCs produce high amount of lactate **(Figure 5F)** indicating that ATDCs are a source of lactate and did not require T cell stimulation for this production. Accordingly, a decrease of pH was observed in ATDC SN compared to MoDC SN **(Figure S6D)**.

To evaluate the suppressive ability of the molecules secreted by ATDCs, these two supernatants were transferred to a new MLR. As shown in **Figure 5G**, we validated that the secretome of ATDCs was responsible for T cell suppression whereas MoDC SN did not modify T cell proliferation. Furthermore, only the fraction of ATDC SN containing molecules under 3KDa efficiently inhibited T cell proliferation. Molecules over 3KDa, as most of the proteins, are thus not involved in $CD4^+$ T cell suppression (**Figure 5G)**. In this experiment, the transfers of the fractions under 3KDa of MoDC SN or filtrated medium induced a non-significant decrease of T cell proliferation that could be explained by the reduction of FCS proteins following filtration.

Our goal was then to investigate the role of lactic acid secreted by ATDCs using a similar assay. Based on previous reports (25), we showed that lactic acid induces an inhibition of T cell proliferation in a MLR context **(Figure S6E)**. Furthermore, the addition of NaOH to this assay allowed to neutralize the acidification induced by lactic acid, and thus abrogated its effect by

suspending the proton and lactic acid symport by T cells **(Figure S6E)**. In this MLR, we determined that the suppression is not mediated exclusively by the acidification as both lactate and acidification were necessary to reduce T cell proliferation **(Figure S6F).** Back to our experiments, ATDC SN and MoDC SN were transferred to a new MLR in absence or presence of NaOH in order to investigate whether lactate production by ATDCs was involved in T cell suppression. Our results highlighted that the neutralization of acidification by NaOH partially reverted the inhibitory effect of ATDC SN suggesting that ATDCs inhibit T cell proliferation by lactate production (**Figures 5H and S6G**).

The secretome of ATDCs acts directly on T cells and enhances Treg expansion

In order to mimic the immune response that occurs following transplantation, T cell proliferation has been evaluated following their stimulation with allogeneic mature DCs in our assays. To now determine whether ATDCs act directly on T cells, $CD4^+$ T cells have been stimulated by polyclonal activators. Our experiments showed that ATDC SN reduced the proliferation of polyclonally stimulated $CD4^+$ T cells (**Figure 6A**), in association with an inhibition of IFNγ expression and Reactive Oxygen Species (ROS) productions (**Figures 6B-C**). ROS have been previously defined as molecules involved in distinct molecular pathways that promote pro-inflammatory cytokines production (27). Furthermore, ATDC SN induced a higher percentage of $CD4^+CD25^+Foxp3^+$Tregs amongst theseT cells **(Figure 6D)**, as well as an increase of TGFβ and Foxp3 expressions **(Figures 6E-F)**.As our hypothesis was that lactate secreted by ATDCs was uptaken by T cells to control their proliferation and differentiation, we performed mass spectrometry on T cells to measure their intracellular amount of lactate. As shown on **Figure 6G**, a significant increase of lactate was quantified on T cells cultured with ATDC SN. To confirm that this increase of lactate was a consequence of an uptake of lactate from the extracellular medium and not from a higher glycolytic activity of T cells, glucose uptake was monitored on T cells using the fluorescent glucose tracer 2-NBDG. We also

quantified the expression of Myc, a transcription factor responsible of glycolysis induction (28). A decrease of 2-NBDG intake and Myc expression were observed in T cells cultured with ATDC SN **(Figures 6H-I)**, showing that these T cells preferentially uptook lactate from the extracellular medium and consequently decreased their glycolytic activity.

As increases of the percentage of Tregs and Foxp3 expression were observed in T cells cultured with ATDC SN, we then investigated the ability of ATDC SN to expand purified $CD4^+CD25^+CD127^{low}$ Tregs. Our results highlighted that ATDC SN allowed a higher expansion of Tregs **(Figure 6J)**. Furthermore, ATDC SN-expanded Tregs preserved their Foxp3 expression (**Figure S6H**) and their suppressive ability **(Figures 6K)**. Taken together, these result show that the secretome of ATDCs favors Treg expansion and inhibits total $CD4^+$ T cell proliferation and Th1 differentiation.

ATDCs delay GVHD development in humanized mice and promote Treg expansion in vivo

Injection of human PBMCs into NSG immunodeficient mice provides a reliable model of human-into-mice xenogenic GVHD (29). This model was already used to evaluate the potential of human Tregs to regulate human cell engraftment (30). To investigate whether ATDCs achieve regulatory activity *in vivo*, irradiated NSG mice received human PBMCs in the presence or absence of autologous ATDCs and GVHD development was monitored. As shown in **Figure 7A**, ATDC injection leads to a significant delay in GVHD development as observed by an increase in survival time. The protective activity of ATDCs was not explained by a reduction of engraftment at early time points as no difference in the percentage of human $CD45^+$ cell engraftment was detected in the blood between these two groups of mice, either at day 7 or at day 21 post injection (**Figure 7B**). More precisely, the percentage of human $CD3^+$ cells were similar in the two groups (higher to 99% of $hCD45^+$ cells at 20 days post-cell injection - **Figure S7**), thereby excluding a possible difference in total T cell engraftment in these mice. To

understand how ATDCs control GVHD progression, mice were sacrificed three weeks post injection and spleen cells were analyzed by flow cytometry. At this time point, no difference in the percentage of $BrdU^+$ cells amongst $CD4^+$ T cells was found in these two groups of mice (**Figure 7C**). However,mice receiving ATDCs and PBMCs exhibited a higher number of $CD4^+CD25^+Foxp3^+$ human Treg cells suggesting that ATDCs promoted $Foxp3^+$Treg expansion or induction *in vivo* (**Figure 7D**). Interestingly, a higher concentration of lactate was also detected in the sera of mice receiving ATDCs one week post cell injection (**Figure 7E**). In correlation with our *in vitro* datas, these results suggest that ATDCs controlled GVHD development in humanized mice by their high lactate production that could favor Treg expansion.

DISCUSSION

Due to the complexity required to treat some autoimmune diseases and the toxicity of immunosuppressive drugs in transplantation, cell therapy with immunoregulatory cells is emerging as a promising strategy. In this study, we defined a new population of human tolerogenic monocyte-derived DCs. These ATDCs display a highly dynamic metabolism, are strongly suppressive and promote Treg expansion. To favor this tolerance, ATDCs secrete various molecules that create an original microenvironment. We showed in this paper that lactate is one compound of this microenvironment whereas classical immunomodulatory molecules such as TGF-β, IL-10, iNOS or IDO do not seem involved. To our knowledge, it is the first time that lactate production has been described as a T cell suppressive mechanism used by tolerogenic DCs. Our *in vivo* studies indicated that ATDCs preserve their tolerogenic phenotype after injection and effectively delayed GVHD development in humanized mice, in association with a Treg expansion and a higher lactate secretion.

Study of immunometabolism has become the focus of intense investigation and it allows to understand functional mechanisms of DC-target cells, T cells. It is then well described that effector T cells rely on aerobic glycolysis whereas Tregs preferentially used oxidative phosphorylation due to alterations in PI3K/Akt/mammalian target of rapamycin (mTOR) signaling axis (31). More precisely, aerobic glycolysis allows T cells to proliferate, produce cytokines and differentiate into effector T cells (31). As glycolysis is a good indicator of T cell proliferation, it was recently proposed to dose extracellular lactate to measure T cell proliferation (32). Concomitantly, the role of lactate as an immunosuppressive metabolite has been well described in the tumor environment. Indeed, high lactate concentrations were detected in tumor biopsies in correlation with metastatic spread and poor survival (33). As early as 1927, Otto Warburg described that tumor cells rely on glycolysis even in the presence of oxygen (34). This aerobic glycolysis is followed by increased lactate production by cancer cells leading to an accumulation of lactic acid in the tumor environment. Lactic acid inhibits T cell proliferation

and pro-inflammatory cytokine secretion (25), modifies pH and is involved in cytosolic NAD^+ regeneration; its strong contribution to immunosuppression is thus today studied in cancer (35).

Similarly, we highlight that ATDCs create a microenvironment conductive to T cell tolerance. This low pH microenvironment is rich in lactate and inhibit T cell proliferation. These results are in accordance with previous work from Fischer *et al.* showing that lactic acid was able to suppress T cell proliferation and strongly diminished IFN-γ production in T cells (25). In our in vitro assays, $CD4^+$ T cells cultured in this microenvironment display a low glycolysis as demonstrated by their low glucose uptake and their low Myc expression. Furthermore, we showed that these $CD4^+$T cells were blocked in G1 phase which could be explained by the decrease of Myc since the molecule has been shown to be involved in the control of cell cycle (36). The $CD4^+$ T cells cultured with ATDC SN also produced low levels of IFN-γ cytokine. This control of IFN-γ production could be correlated to the decrease of ROS observed in these T cells as previously described (27) or to a reduction of glycolysis. Indeed, it was previously shown that glycolytic enzymes, such as GAPDH, favor the production of inflammatory cytokines, when it is engaged in glycolysis and then promote effector T cell functions (37). Interestingly, our results also highlighted that T cells cultured with ATDC SN contained a high concentration of intracellular lactate. As previously demonstrated, T cells can uptake extracellular lactate by their monocarboxylate transporters following a concentration gradient between intracellular and extracellular lactate (25).We believe that this increase of intracellular lactate down-regulated the glycolysis pathway. Inversely, this lactate-rich environment induced by ATDCs favored Treg expansion as shown by the increase of $CD4^+CD25^+$ Tregs associated to increased expressions of TGF-β and Foxp3. We further showed that the secretome of ATDCs promoted Treg expansion without loss of their suppressive ability. These results coincide with a recent study reporting that Tregs are not impaired by lactate and even preserve their immunosuppressive function through Foxp3 expression (38). The authors showed that this transcription factor suppress *Myc* gene and consequently downregulates glycolysis. Foxp3 also controls metabolic changes by the upregulation of oxidative phosphorylation and an increased

NAD:NADH ratio that allow Treg functions in lactate-rich conditions (38). Our results are then in accordance with previous publications indicating that a low glucose, high lactate environment favors the induction of Tregs at the expanse of effector T cells. Although ATDC-secreted lactic acid is involved in T cell suppression, the inhibition of its symport (by NaOH treatment) only partially reverts ATDC suppressive ability. Further proteomic, metabolomic or lipidomic analyses are thus required to investigate the others soluble factors, secreted or converted by ATDCs, which allow this immunosuppression. For instance, it has been showed that fatty acid metabolism was highly involved in Treg proliferation (39) and in the maintenance of Treg stability (40).

Regarding the study of DC metabolism, Malinarich et al. reported that human tolerogenic DCs generated with GM-CSF, IL-4, dexamethasone and vitaminD3 display a higher glycolytic capacity, a higher mitochondrial respiration and a higher fatty acid oxydation activity than mature DCs (41). Here, using another protocol to derive human tolerogenic DCs, we confirmed their high metabolic plasticity in comparison to immature MoDCs. Furthermore, we associated their strong LDH activity and their high lactate secretion to their suppressive function on T cells. Contrastingly, in mouse, increases of glycolysis and lactate production were associated to immunostimulatory DCs, following DC stimulation with TLR agonists within minutes of stimulation (42). Interestingly, most of these studies were performed using murine bone–marrow derived DCs. Following stimulation, these murine GM-CSF-derived DCs express iNOS and produce NO that inhibits oxidative phosphorylation and thus favor glycolysis (43). This NO-dependent high glycolytic rate was shown to be inhibited by IL-10 (44). Thus in human DCs, high glycolytic capacity and lactate production are restricted to tolerogenic DCs, whereas in mouse DCs, this appears to be more pronounced in immunostimulatory DCs. This involvement of iNOS is clearly different between murine and human DCs. Indeed, our transcriptomic analysis revealed no overexpression of iNOS in our ATDCs (and also in human tolerogenic DCs reported above) compared to control DCs. Furthermore, iNOS inhibitor did not counter the suppressive capacity of ATDCs in our experiments. As these two human tolerogenic

DCs secrete IL-10, it could be speculated that Il-10 inhibits the effect of NO on oxidative phosphorylation. To explain the differences between murine and human DCs, it is thus worth considering the glycolysis-respiration balance of the cells instead of their glycolytic capacity only. Regarding lactate and DCs, it was also reported that human MoDCs stimulated with LPS and lactic acid produce less IL-12 compared to MoDCs stimulated with LPS only (26, 45). We can then postulate that lactate produced by ATDCs favor their own resistance to TLR ligand stimulation. Further experiments will be necessary to investigate whether lactic acid contains in ATDC SN could influence the maturation of surrounding DCs.

Analysis of ATDC phenotype and transcriptome highlighted that ATDCs shared also some features with macrophages. Two main *in vitro*-derived populations of macrophages are studied: inflammatory macrophages induced by LPS±IFNγ (M1) and anti-inflammatory macrophages, induced by IL-4 (M2). Due to their anti-inflammatory properties (IL-10 secretion), we are mainly interested in M2 macrophages which are known to be involved in the resolution of inflammation, the resistance to helminth parasites and in tumors. Indeed, it has been reported that Tumor-Associated Macrophages (TAM) displayed a M2-like profile, based on phenotype and cytokine secretion. Furthermore, works from the team of Medzhitov showed that lactic acid from the tumor microenvironment favors the M2-like polarization of TAM (46). However, initial studies reported that inflammatory macrophages displayed a glycolytic metabolism whereas the metabolism of anti-inflammatory macrophages was mainly based on oxidative phosphorylation fueled by fatty acid oxidation. Consequently, TAM appear to share the tolerogenic profile (phenotype and cytokine secretion) of M2 macrophages and the metabolic profile of M1 macrophages. Nevertheless it appears today that fatty acid oxidation could also driveinflammasome activation and that glycolysis is involved in the metabolism of M2-like macrophages as the deletion of mTORC2 prevents their anti-inflammatory activation and consequently suppressestumoral growth (47, 48). In this manuscript, we focused on the high glycolysis of ATDCs that explained their control of $CD4^+$T cell proliferation but we also showed that these cells displayed a high oxidative phosphorylation.In addition to the

understanding ofthe effects of ATDCs on T cells and DCs, further analyses of their own metabolism would help us to clarify their place in the myeloid field. Previous reports highlighted for instance that lipid metabolism and extracellular metabolites, such as succinate, regulate the function of DCs (49) whereas glutamine metabolism is critical for the M2 polarization (50).

We studied here human ATDCs whose the safety is currently evaluated in patients receiving a kidney transplantation. Prior to our trial, a study performed in non-human primates reinforced the therapeutic promise of tolerogenic DCs by providing important evidence of safety and efficacy of donor regulatory DCs in organ transplantation (51). Furthermore, the administration of donor regulatory macrophages in two renal transplant recipients gave encouraging results as no toxicity was reported and a stable renal function was observed in these two patients (52). While these two teams and others have studied the potential of donor derived tolerogenic antigen presenting cells (DCs or macrophages) or donor antigen-pulsed recipient tolerogenic DCs in transplantation, the originality of our team is the use of unloaded autologous cells. Indeed, our group reported the potential of non-pulsed recipient tolerogenic DCs to significantly prolong cardiac, skin and islet allograft survivals in rodents (6, 9,10). In addition to the safety reasons (no risks of donor sensitization andno non-self-recognition by the recipient), it means that ATDC therapy is not restricted to the transplantation field and its application could be then extended to the treatment of others pathologies.

METHODS

Cell generation

Dendritic cells were generated from monocytes isolated from healthy volunteer donors (French Blood Service, Nantes, France).ATDCs were differentiated following a 6 day-culture of monocytes in AIMV medium CTS (Life Technologies) supplemented with recombinant human GM-CSF (CellGenix) at 100U/ml. MoDC were derived from monocytes cultured in complete RPMI 1640 medium (Life Technologies, France) supplemented with 10% decomplemented Fetal Calf serum (Eurobio), recombinant human IL-4 (200U/ml, CellGenix) and recombinant human GM-CSF (1000U/ml, CellGenix) for 6 days. ATDC and MoDC SN were obtained following cultures of DCs in complete RPMI medium for 6 days.Mature DCs were obtained after a 48h stimulation of MoDC with LPS (Sigma Aldrich) at 1µg/ml. In some experiments, ATDC SN were added during the 48H-stimulation.See suppl. Methods for details.

T cell assays

For MLR assay, allogeneic $CD3^+$ T cells were isolated using pan T cell isolation kit (Miltenyi) and cultured in 96-well plates with DCsat different ratios. Cells were cultured for 5 days and proliferation was assessed by the addition of 1µCi ^{3}H-thymidine/well (Perkin Elmer) for the last 8 hours. In some experiments, T cells were labeled with Cell Proliferation Dye (CPD) efluor 450 (ebioscience) and T cell proliferation was measured by CPD dilution by flow cytometry.

To investigate the suppressive function of ATDCs, autologous $CD4^+$ T cells were selected using a $CD4^+$ T Cell isolation kit II (Miltenyi). In some experiments, T cells were labeled with CPD and cultured in 96-well plates with DC (ATDCs or MoDCs) at a 1:1 ratio and with mature DCs at a 1:0.1ratio for 6 days.In some experiments, supernatants from MLR in the presence or not of ATDCs were collected. These supernatant were then frozen at -80^{o}C until use. A volume of 100µL of supernatants were added to a new assay to test the suppressive activity. The

neutralization of supernatants was performed by the addition of NaOH(Sigma) to a final concentration of 5mM. See suppl. Methods for details.

Metabolic assay.

ATDC or MoDC were plated on Seahorse Bioanalyzer XFe24 culture plates (120.000 cells/well) in Seahorse XF-base medium (Sigma) supplemented with 9mM of glucose, 0,86mM of NaOH, 1mM of pyruvate and 2mM of glutamine. Before the experiment, cells were incubated 20 minutes at 37°C and 0% CO_2. OCAR and ECAR readout were obtained using Seahorse XF24 analyzer. Seahorse data analysis was performed using Seahorse Wave 2.4 software.

Agilent Microarray and expression data analysis

Transcriptomic analysis was performed on ATDCs, MoDCs and rMoMacro. The Agilent data have been deposited in GEO (Accession number GSE104438). Background corrected intensity values were quantile normalized and log2 transformed.Based on the common genes, these expression datas were then combined with public Affymetrix microarray data sets obtained from human monocytes, and in vitro- and in vivo- differentiated DCs and Macrophages (GSE40484, GSE102046) (21, 22). Principal component analysis illustrates the comparison of all these myeloid subsets. See suppl. Methods for details.

Xenogeneic GVHD model

A humanized mouse model was used to evaluate the function of ATDCs *in vivo*. NOD/SCID/*Il2rg*$^{-/-}$ (Charles River) bred in the Labex IGO humanized rodent facility were exposed to a conditioning dose of 1.5 Gray of whole-body gamma irradiation. Mice received $5x10^6$ thawed PBMCs and $5x10^6$autologous ATDCs simultaneously by IV route. Animals that

displayed a reduction greater than 20% total of body weight were sacrificed according to the local ethical committee guidelines.

In vivo tracking and phenotype of ATDCs were assessed following the injection of ATDCs labeled with gold nanoparticles (Sigma-Aldrich) or Qtracker® 605 (Life Technologies). The presence of gold nanoparticles in different tissues was analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as previously described(23). Qtracker® 605 staining was performed on splenic cells and analyzed by flow cytometry. See suppl. Methods for details.

Statistical analysis

Results were expressed as the mean ± SEM. As detailed in Figure legends, group comparisons were made using 1way ANOVA, 2way ANOVAor Student's *t* tests (paired or unpaired). Survival curve was analyzed by log-rank (Mantel-Cox) test. *p<0.05, **p<0.01, *** p<0.001.

Study approval

Human blood cells from healthy volunteer donors were obtained from the French Blood Service (Nantes, France).Experiments performed in mice were carried out in strict accordance with the protocol approved by the Committee on the Ethics of Animal Experiments of Pays de la Loire.

AUTHOR CONTRIBUTIONS:

E.M, LBD, MCC and AMo conceived the project and designed the experiments. EM, LBD, AE, AMa, VD, GBe, JAH, NO, CP, AA and AMo performed the experiments. EM, LBD, CL, MG, TPVM, BS, EC, EG, BV, GBl, MD, RJ, MCC and AMo analyzed the data. EM, LBD, BAL, MCC and AMo wrote the manuscript.

ACKNOWLEDGEMENTS

We are grateful to Marc Grégoire and Delphine Coulais from the Clinical Development and Transfer facility (SFR Santé Bonamy, Nantes, France) for providing human monocytes. We thank Emilie Varey, PierrickGuerif and Nephrology and Transplantation Department from Nantes Hospital for providing patient blood samples. We also thank Dorian McIlroy and Ignacio Anegon for critically reading the manuscript.

Funding: This work was funded by IMBIO-DC, The ONE Study (FP7-260687) and BIODRIM (FP7-305147) European Union 7th Framework Programs. This work was also supported by funds from IHU-CESTI (Investissementd'Avenir ANR-10-IBHU-005, Région Pays de la Loire and Nantes Métropole), ANR JeunesChercheurs (ANR-16-CE18-0001-01), DHU Oncogreffe and the Labex IGO project (ANR-11-LABX-0016-01).

REFERENCES

1. Morelli, A.E., and Thomson, A.W. 2007. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol* 7:610-621.
2. Moreau, A., Varey, E., Beriou, G., Hill, M., Bouchet-Delbos, L., Segovia, M., and Cuturi, M.C. 2012. Tolerogenic dendritic cells and negative vaccination in transplantation: from rodents to clinical trials. *Front Immunol.* 3:218.:10.3389/fimmu.2012.00218. eCollection 02012.
3. Machen, J., Harnaha, J., Lakomy, R., Styche, A., Trucco, M., and Giannoukakis, N. 2004. Antisense oligonucleotides down-regulating costimulation confer diabetes-preventive properties to nonobese diabetic mouse dendritic cells. *J Immunol* 173:4331-4341.
4. Stoop, J.N., Robinson, J.H., and Hilkens, C.M. 2011. Developing tolerogenic dendritic cell therapy for rheumatoid arthritis: what can we learn from mouse models? *Ann Rheum Dis.* 70:1526-1533. doi: 1510.1136/ard.2011.151654.
5. Mansilla, M.J., Selles-Moreno, C., Fabregas-Puig, S., Amoedo, J., Navarro-Barriuso, J., Teniente-Serra, A., Grau-Lopez, L., Ramo-Tello, C., and Martinez-Caceres, E.M. 2015. Beneficial effect of tolerogenic dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. *CNS Neurosci Ther.* 21:222-230. doi: 210.1111/cns.12342. Epub 12014 Nov 12318.
6. Peche, H., Trinite, B., Martinet, B., and Cuturi, M.C. 2005. Prolongation of heart allograft survival by immature dendritic cells generated from recipient type bone marrow progenitors. *Am J Transplant* 5:255-267.
7. Lutz, M.B., Suri, R.M., Niimi, M., Ogilvie, A.L., Kukutsch, N.A., Rossner, S., Schuler, G., and Austyn, J.M. 2000. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *Eur J Immunol* 30:1813-1822.
8. Turnquist, H.R., Raimondi, G., Zahorchak, A.F., Fischer, R.T., Wang, Z., and Thomson, A.W. 2007. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol* 178:7018-7031.
9. Baas, M.C., Kuhn, C., Valette, F., Mangez, C., Duarte, M.S., Hill, M., Besancon, A., Chatenoud, L., Cuturi, M.C., and You, S. 2014. Combining autologous dendritic cell therapy with CD3 antibodies promotes regulatory T cells and permanent islet allograft acceptance. *J Immunol.* 193:4696-4703. doi: 4610.4049/jimmunol.1401423. Epub 1402014 Sep 1401424.
10. Segovia, M., Louvet, C., Charnet, P., Savina, A., Tilly, G., Gautreau, L., Carretero-Iglesia, L., Beriou, G., Cebrian, I., Cens, T., et al. 2014. Autologous dendritic cells prolong allograft survival through Tmem176b-dependent antigen cross-presentation. *Am J Transplant* 14:1021-1031.
11. Hill, M., Thebault, P., Segovia, M., Louvet, C., Beriou, G., Tilly, G., Merieau, E., Anegon, I., Chiffoleau, E., and Cuturi, M.C. 2011. Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and epstein-barr virus-induced gene 3. *Am J Transplant* 11:2036-2045.
12. Lan, Y.Y., Wang, Z., Raimondi, G., Wu, W., Colvin, B.L., de Creus, A., and Thomson, A.W. 2006. "Alternatively activated" dendritic cells preferentially secrete IL-10, expand Foxp3+CD4+ T cells, and induce long-term organ allograft survival in combination with CTLA4-Ig. *J Immunol.* 177:5868-5877.
13. Giannoukakis, N., Phillips, B., Finegold, D., Harnaha, J., and Trucco, M. 2011. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* 34:2026-2032.

14. Bell, G.M., Anderson, A.E., Diboll, J., Reece, R., Eltherington, O., Harry, R.A., Fouweather, T., MacDonald, C., Chadwick, T., McColl, E., et al. 2016. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann Rheum Dis*.
15. Benham, H., Nel, H.J., Law, S.C., Mehdi, A.M., Street, S., Ramnoruth, N., Pahau, H., Lee, B.T., Ng, J., ME, G.B., et al. 2015. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci Transl Med.* 7:290ra287. doi: 210.1126/scitranslmed.aaa9301.
16. Jauregui-Amezaga, A., Cabezon, R., Ramirez-Morros, A., Espana, C., Rimola, J., Bru, C., Pino-Donnay, S., Gallego, M., Masamunt, M.C., Ordas, I., et al. 2015. Intraperitoneal Administration of Autologous Tolerogenic Dendritic Cells for Refractory Crohn's Disease: A Phase I Study. *J Crohns Colitis.* 9:1071-1078. doi: 1010.1093/ecco-jcc/jjv1144. Epub 2015 Aug 1024.
17. Moreau, A., Hill, M., Thebault, P., Deschamps, J.Y., Chiffoleau, E., Chauveau, C., Moullier, P., Anegon, I., Alliot-Licht, B., and Cuturi, M.C. 2009. Tolerogenic dendritic cells actively inhibit T cells through heme oxygenase-1 in rodents and in nonhuman primates. *FASEB J* 23:3070-3077.
18. Moreau, A., Vandamme, C., Segovia, M., Devaux, M., Guilbaud, M., Tilly, G., Jaulin, N., Le Duff, J., Cherel, Y., Deschamps, J.Y., et al. 2014. Generation and in vivo evaluation of IL10-treated dendritic cells in a nonhuman primate model of AAV-based gene transfer. *Mol Ther Methods Clin Dev* 1:14028.
19. Moreau, A., Varey, E., Beriou, G., Hill, M., Bouchet-Delbos, L., Segovia, M., and Cuturi, M.C. 2012. Tolerogenic dendritic cells and negative vaccination in transplantation: from rodents to clinical trials. *Front Immunol* 3:218.
20. Sallusto, F., and Lanzavecchia, A. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 179:1109-1118.
21. Goudot, C., Coillard, A., Villani, A.C., Gueguen, P., Cros, A., Sarkizova, S., Tang-Huau, T.L., Bohec, M., Baulande, S., Hacohen, N., et al. 2017. Aryl Hydrocarbon Receptor Controls Monocyte Differentiation into Dendritic Cells versus Macrophages. *Immunity* 47:582-596 e586.
22. Segura, E., Touzot, M., Bohineust, A., Cappuccio, A., Chiocchia, G., Hosmalin, A., Dalod, M., Soumelis, V., and Amigorena, S. 2013. Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* 38:336-348.
23. Managh, A.J., Hutchinson, R.W., Riquelme, P., Broichhausen, C., Wege, A.K., Ritter, U., Ahrens, N., Koehl, G.E., Walter, L., Florian, C., et al. 2014. Laser ablation-inductively coupled plasma mass spectrometry: an emerging technology for detecting rare cells in tissue sections. *J Immunol.* 193:2600-2608. doi: 2610.4049/jimmunol.1400869. Epub 1402014 Jul 1400823.
24. Rosen, A.B., Kelly, D.J., Schuldt, A.J., Lu, J., Potapova, I.A., Doronin, S.V., Robichaud, K.J., Robinson, R.B., Rosen, M.R., Brink, P.R., et al. 2007. Finding fluorescent needles in the cardiac haystack: tracking human mesenchymal stem cells labeled with quantum dots for quantitative in vivo three-dimensional fluorescence analysis. *Stem Cells.* 25:2128-2138. Epub 2007 May 2110.
25. Fischer, K., Hoffmann, P., Voelkl, S., Meidenbauer, N., Ammer, J., Edinger, M., Gottfried, E., Schwarz, S., Rothe, G., Hoves, S., et al. 2007. Inhibitory effect of tumor cell-derived lactic acid on human T cells. *Blood* 109:3812-3819.
26. Gottfried, E., Kunz-Schughart, L.A., Ebner, S., Mueller-Klieser, W., Hoves, S., Andreesen, R., Mackensen, A., and Kreutz, M. 2006. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood* 107:2013-2021.
27. Naik, E., and Dixit, V.M. 2011. Mitochondrial reactive oxygen species drive proinflammatory cytokine production. *J Exp Med* 208:417-420.

28. Wang, R., Dillon, C.P., Shi, L.Z., Milasta, S., Carter, R., Finkelstein, D., McCormick, L.L., Fitzgerald, P., Chi, H., Munger, J., et al. 2011. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* 35:871-882.
29. Shultz, L.D., Ishikawa, F., and Greiner, D.L. 2007. Humanized mice in translational biomedical research. *Nat Rev Immunol.* 7:118-130.
30. Mutis, T., van Rijn, R.S., Simonetti, E.R., Aarts-Riemens, T., Emmelot, M.E., van Bloois, L., Martens, A., Verdonck, L.F., and Ebeling, S.B. 2006. Human regulatory T cells control xenogeneic graft-versus-host disease induced by autologous T cells in RAG2-/-gammac-/- immunodeficient mice. *Clin Cancer Res.* 12:5520-5525.
31. Scharping, N.E., and Delgoffe, G.M. 2016. Tumor Microenvironment Metabolism: A New Checkpoint for Anti-Tumor Immunity. *Vaccines (Basel)* 4.
32. Grist, J.T., Jarvis, L.B., Georgieva, Z., Thompson, S., Kaur Sandhu, H., Burling, K., Clarke, A., Jackson, S., Wills, M., Gallagher, F.A., et al. 2018. Extracellular Lactate: A Novel Measure of T Cell Proliferation. *J Immunol* 200:1220-1226.
33. Walenta, S., Wetterling, M., Lehrke, M., Schwickert, G., Sundfor, K., Rofstad, E.K., and Mueller-Klieser, W. 2000. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. *Cancer Res* 60:916-921.
34. Warburg, O., Wind, F., and Negelein, E. 1927. THE METABOLISM OF TUMORS IN THE BODY. *J Gen Physiol* 8:519-530.
35. Choi, S.Y., Collins, C.C., Gout, P.W., and Wang, Y. 2013. Cancer-generated lactic acid: a regulatory, immunosuppressive metabolite? *J Pathol* 230:350-355.
36. Eilers, M., and Eisenman, R.N. 2008. Myc's broad reach. *Genes Dev* 22:2755-2766.
37. Chang, C.H., Curtis, J.D., Maggi, L.B., Jr., Faubert, B., Villarino, A.V., O'Sullivan, D., Huang, S.C., van der Windt, G.J., Blagih, J., Qiu, J., et al. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153:1239-1251.
38. Angelin, A., Gil-de-Gomez, L., Dahiya, S., Jiao, J., Guo, L., Levine, M.H., Wang, Z., Quinn, W.J., 3rd, Kopinski, P.K., Wang, L., et al. 2017. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. *Cell Metab* 25:1282-1293 e1287.
39. Pacella, I., Procaccini, C., Focaccetti, C., Miacci, S., Timperi, E., Faicchia, D., Severa, M., Rizzo, F., Coccia, E.M., Bonacina, F., et al. 2018. Fatty acid metabolism complements glycolysis in the selective regulatory T cell expansion during tumor growth. *Proc Natl Acad Sci U S A*.
40. Fan, M.Y., and Turka, L.A. 2018. Immunometabolism and PI(3)K Signaling As a Link between IL-2, Foxp3 Expression, and Suppressor Function in Regulatory T Cells. *Front Immunol* 9:69.
41. Malinarich, F., Duan, K., Hamid, R.A., Bijin, A., Lin, W.X., Poidinger, M., Fairhurst, A.M., and Connolly, J.E. 2015. High mitochondrial respiration and glycolytic capacity represent a metabolic phenotype of human tolerogenic dendritic cells. *J Immunol* 194:5174-5186.
42. Everts, B., Amiel, E., Huang, S.C., Smith, A.M., Chang, C.H., Lam, W.Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J., et al. 2014. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. *Nat Immunol* 15:323-332.
43. Everts, B., Amiel, E., van der Windt, G.J., Freitas, T.C., Chott, R., Yarasheski, K.E., Pearce, E.L., and Pearce, E.J. 2012. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* 120:1422-1431.
44. Krawczyk, C.M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R.J., Cross, J.R., Jung, E., Thompson, C.B., Jones, R.G., et al. 2010. Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood* 115:4742-4749.

45. Nasi, A., Fekete, T., Krishnamurthy, A., Snowden, S., Rajnavolgyi, E., Catrina, A.I., Wheelock, C.E., Vivar, N., and Rethi, B. 2013. Dendritic cell reprogramming by endogenously produced lactic acid. *J Immunol* 191:3090-3099.
46. Colegio, O.R., Chu, N.Q., Szabo, A.L., Chu, T., Rhebergen, A.M., Jairam, V., Cyrus, N., Brokowski, C.E., Eisenbarth, S.C., Phillips, G.M., et al. 2014. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* 513:559-563.
47. Huang, S.C., Smith, A.M., Everts, B., Colonna, M., Pearce, E.L., Schilling, J.D., and Pearce, E.J. 2016. Metabolic Reprogramming Mediated by the mTORC2-IRF4 Signaling Axis Is Essential for Macrophage Alternative Activation. *Immunity* 45:817-830.
48. Van den Bossche, J., O'Neill, L.A., and Menon, D. 2017. Macrophage Immunometabolism: Where Are We (Going)? *Trends Immunol* 38:395-406.
49. O'Neill, L.A., and Pearce, E.J. 2016. Immunometabolism governs dendritic cell and macrophage function. *J Exp Med* 213:15-23.
50. Liu, P.S., Wang, H., Li, X., Chao, T., Teav, T., Christen, S., Di Conza, G., Cheng, W.C., Chou, C.H., Vavakova, M., et al. 2017. alpha-ketoglutarate orchestrates macrophage activation through metabolic and epigenetic reprogramming. *Nat Immunol* 18:985-994.
51. Ezzelarab, M.B., Zahorchak, A.F., Lu, L., Morelli, A.E., Chalasani, G., Demetris, A.J., Lakkis, F.G., Wijkstrom, M., Murase, N., Humar, A., et al. 2013. Regulatory dendritic cell infusion prolongs kidney allograft survival in nonhuman primates. *Am J Transplant.* 13:1989-2005. doi: 1910.1111/ajt.12310. Epub 12013 Jun 12311.
52. Hutchinson, J.A., Riquelme, P., Sawitzki, B., Tomiuk, S., Miqueu, P., Zuhayra, M., Oberg, H.H., Pascher, A., Lutzen, U., Janssen, U., et al. 2011. Cutting Edge: Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. *J Immunol.* 187:2072-2078. doi: 2010.4049/jimmunol.1100762. Epub 1102011 Jul 1100729.

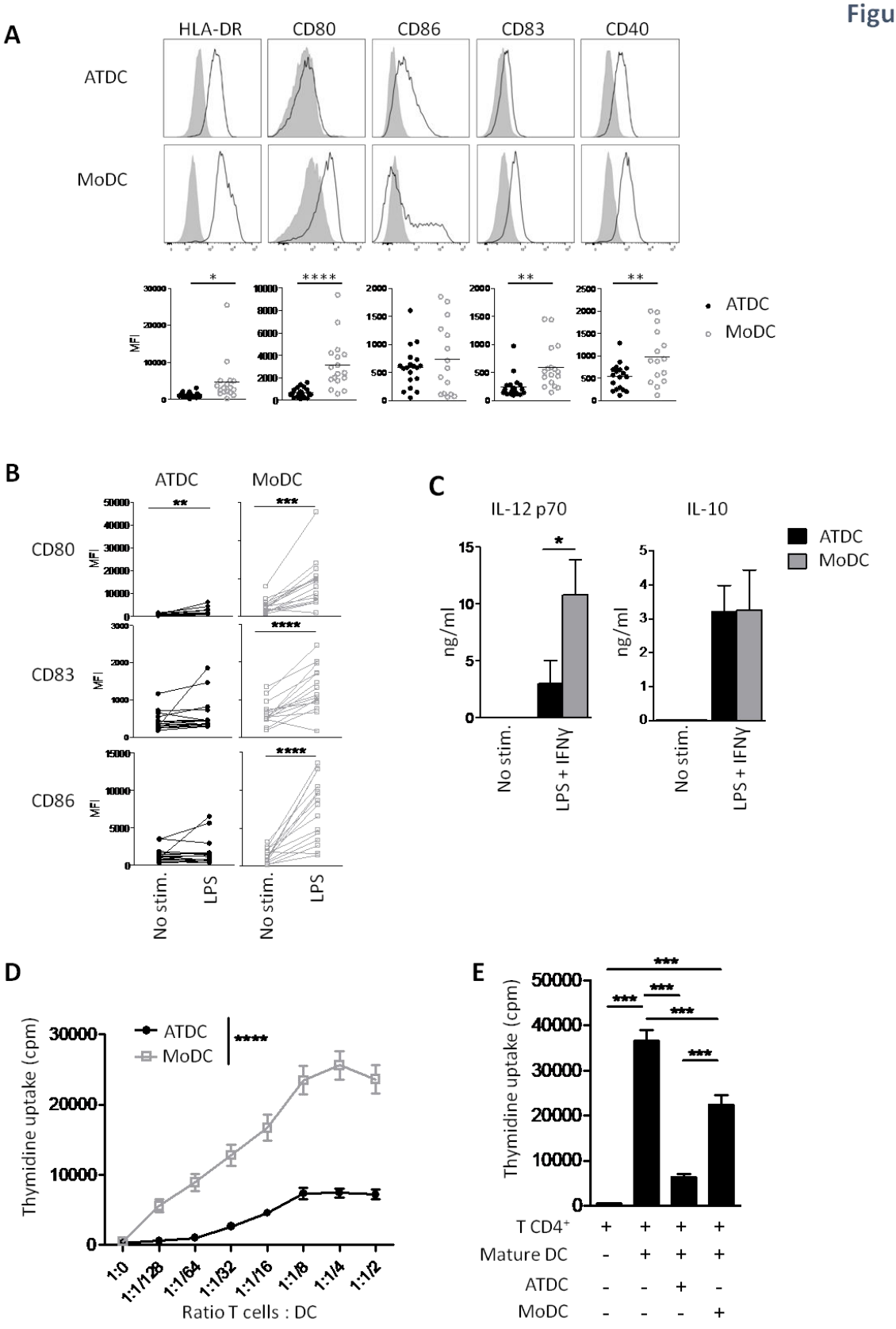

A
HLA-DR
CD80
CD86
CD83
CD40
ATDC
MoDC
MFI
ATDC
MoDC
B
ATDC
MoDC
CD80
CD83
CD86
MFI
No stim.
LPS
C
IL-12 p70
IL-10
ng/ml
No stim.
LPS + IFNγ
ATDC
MoDC
D
Thymidine uptake (cpm)
ATDC
MoDC
Ratio T cells : DC
1:0
1:1/128
1:1/64
1:1/32
1:1/16
1:1/8
1:1/4
1:1/2
E
Thymidine uptake (cpm)
T CD4+
Mature DC
ATDC
MoDC

Figure 1: Human ATDCs display tolerogenic features

A. Representative histograms of the expression of the surface markers by ATDCs and MoDCs (black line) and their respective isotype controls (grey shaded) are shown. Scatter plots of the MFI of the different markers are shown (n≥15 donors). **B.** ATDCs and MoDCs were left untreated or treated with LPS for 48 hours. Graphs show the expression (MFI) of CD80, CD86, CD83 markers (n≥14). **C.** IL-10 and IL-12 concentrations in the supernatants of DCs stimulated for 48 hours with LPS and IFNγ or left untreated were assessed by ELISA (n≥4). **D.** ATDCs and MoDCs were cultured with allogeneic $CD3^+$ T cells at different T: DC ratio for 6 days. T cell proliferation was measured by ^3H thymidine uptake during the last 8 hours of culture. (n=16). **E.** $CD4^+$ T cells were cultured with allogeneic mature DCs in the presence of absence of ATDCs or MoDCs (autologous to $CD4^+$ T cells) (10 T cells: 1 mature DC: 10 ATDC/MoDC ratio) for 6 days. T cell proliferation was measured as described in D (n=26). p values were calculated by paired t tests on graphs A-C, by 2way ANOVA on graph D and by 1way ANOVA on graph E; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

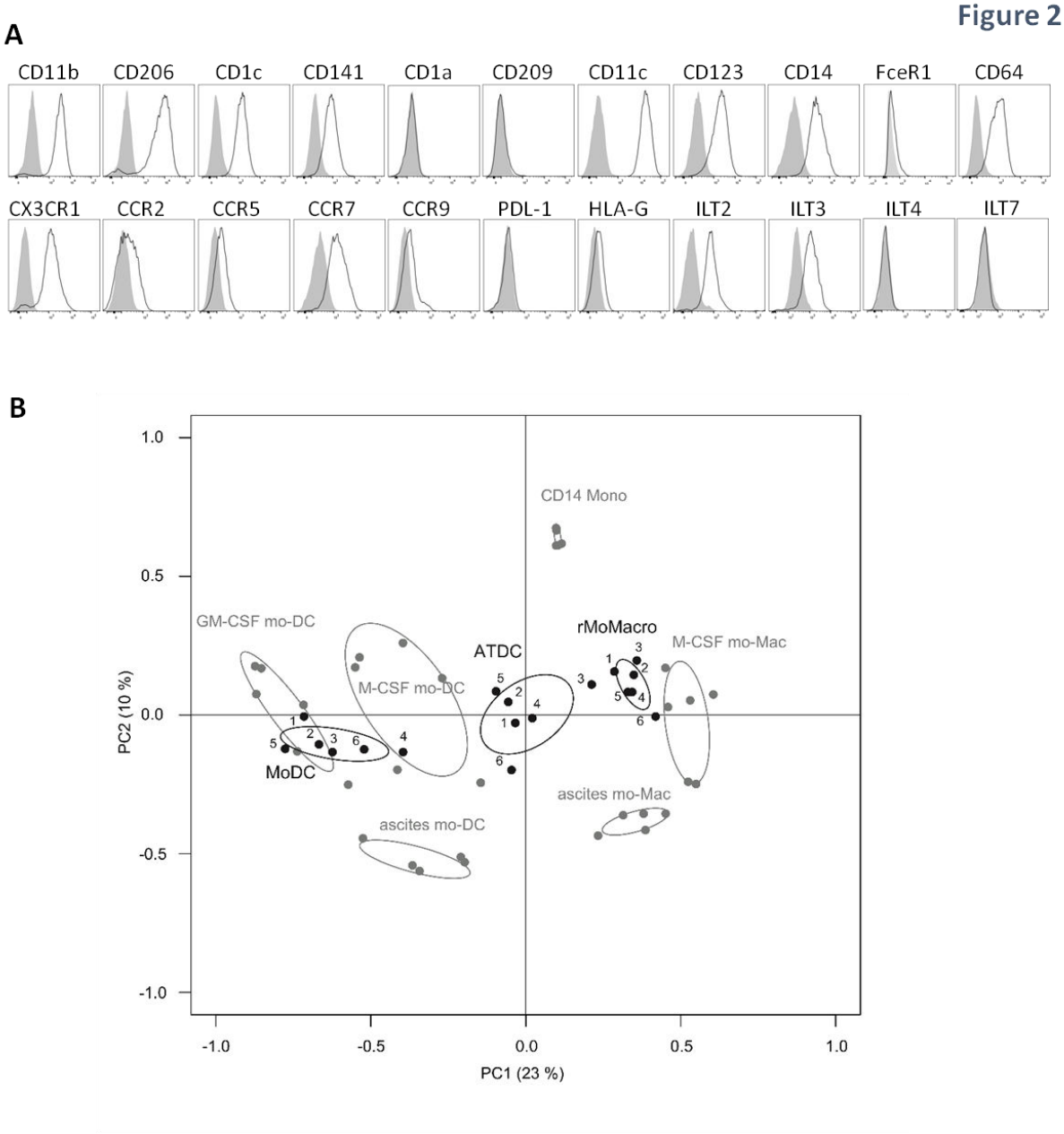


Figure 2: In depth phenotyping and comparison with monocytes and in vitro-and in vivo-derived cells

A. ATDCs were stained with myeloid cells, monocytes, DCs, chemokines and inhibitory molecules markers and analyzed by flow cytometry. Representative histograms of the expression of the markers by ATDCs (black line; n≥4 donors) and their respective isotype controls (grey shaded) are shown. **B.** Microarrays were performed on ATDCs, MoDCs and rMoMacro generated from monocytes of 6 healthy donors. Transcriptomic datas of these three populations (in black) were compared with human populations obtained from public datasets (in grey). Public data sets include monocytes (n=4), GM-CSF/IL-4 mo-DCs (n=6), M-CSF mo-DCs (n=6), M-CSF mo-Mac (n=6), ascites mo-DCs (n=5) and ascites mo-Mac (n=5). This graph

depicts the principal component analysis of these different subsets. Numbers indicate the donor ID used to generate ATDCs, MoDCs and rMoMacro.

Figure 3

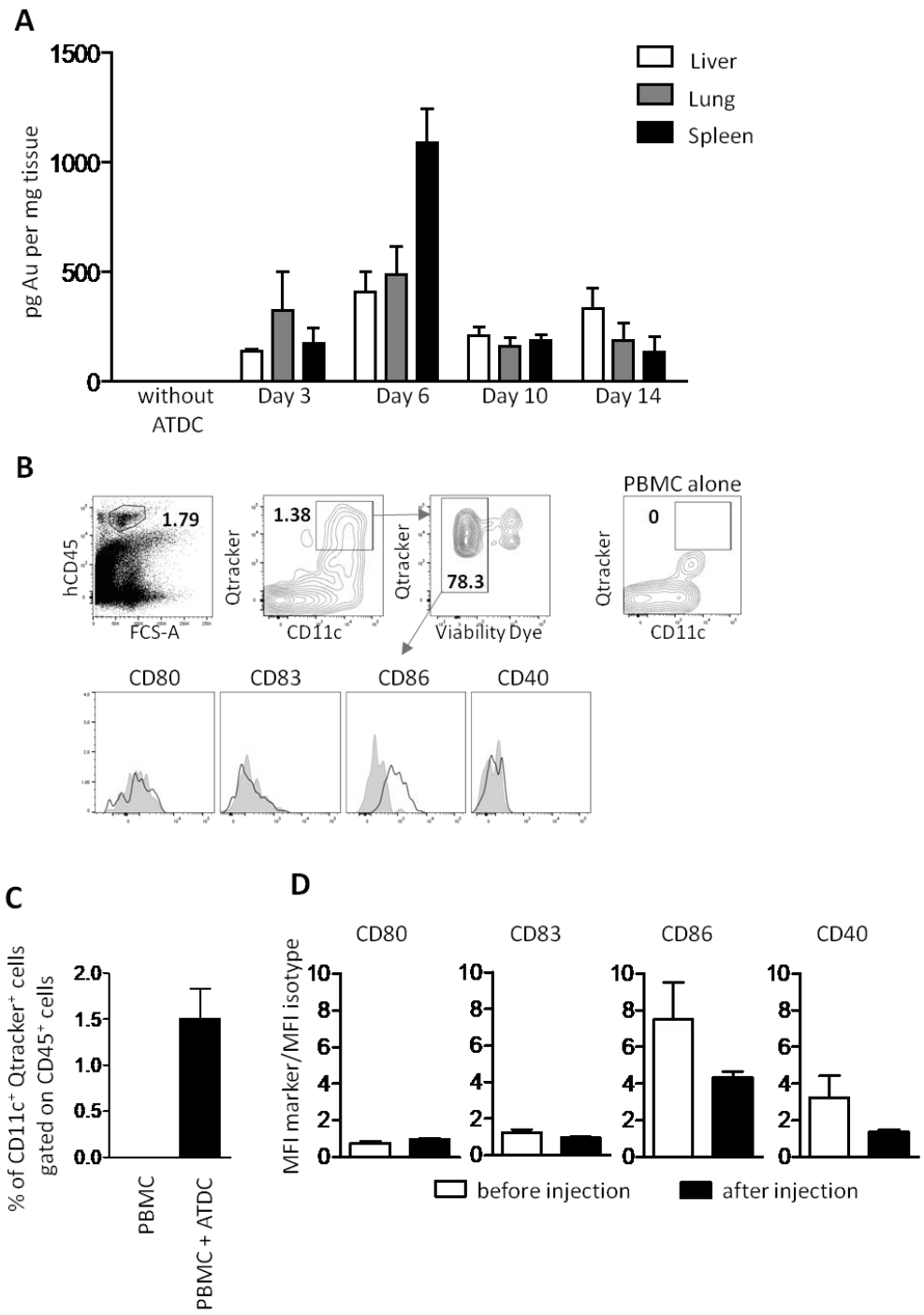


Figure 3: ATDCs migrate preferentially to the spleen and preserve an immature phenotype after injection in humanized mice.

A.Gold-labeled ATDCs were intravenously injected with autologous PBMCs into irradiated NSG mice. Mice injected with PBMCs alone were used as controls. The amount of Gold was

determined by ICP-MS (Au mean of 3 biopsies/organ) in liver, lung and spleen at marked days post-injection. Bar graph depicts Au labeling per mg of tissue (n=3 mice for day 3 and day 14; n=5/6 mice from two independent experiments for day 6, day 10 and without ATDC condition). **B-D.** ATDCs were labeled with Qtracker® and then intravenously injected with autologous PBMCs in irradiated NSG mice. Mice injected with PBMCs alone were used as controls. Spleens were recovered 6 days later and ATDCs were analyzed using human CD45, CD11c and HLA-DR antibodies and Qtracker® marker. The viability (viability dye marker) and the expression of surface markers were assessed by flow cytometry. (B) Representative dot plots showing gating for ATDCs, their viability, and the expression of different markers by ATDCs (black line) and their respective isotype controls (grey shaded). (C) Bar chart depicts mean percentage of ATDC in spleen of NSG mice (n=5 mice injected with PBMC+ATDC and n=2 mice injected with PBMC alone). (D) Bar charts depict the ratio of MFI marker/MFI isotype control of CD80, CD83, CD86 and CD40 in ATDCs before (n= 3 donors) or after (n=5 mice) injection. Ratio of marker expression were compared with unpaired t test analysis.

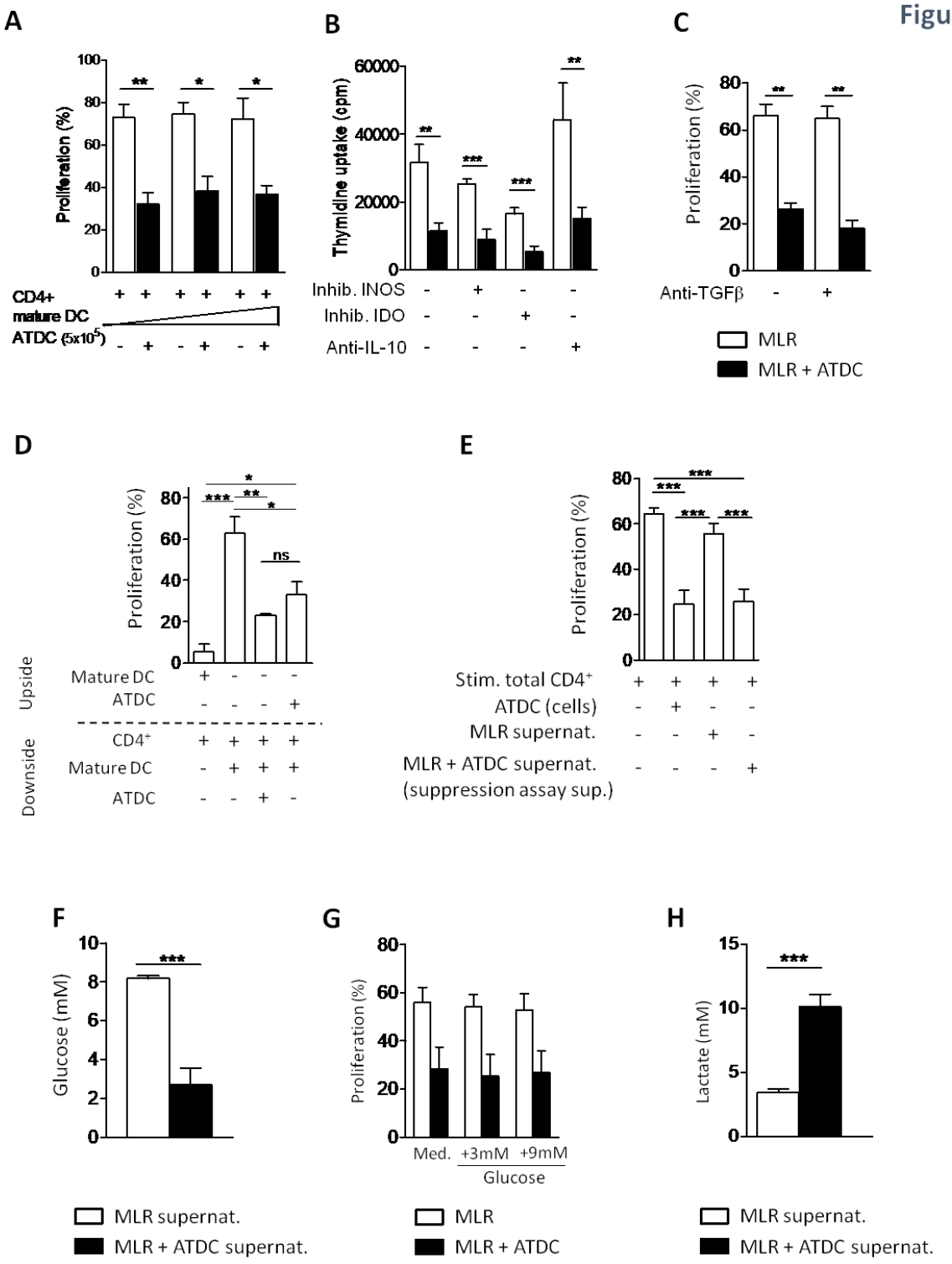


Figure 4: ATDCs inhibit T cellproliferation by soluble factors and favor lactate secretion.

A. CPD-labeled $CD4^+$ T cells were cultured with different amounts of allogeneic mature DCs ($5x10^4$, $1x10^5$ and $2x10^5$) with or without autologous ATDCs for 6 days. T cell proliferation was assessed by CPD dilution. (n=3 donors). **B-C.** Inhibitors of INOS (Aminoguanonidine, 100 µM) or IDO (1-Methyl-D-tryptophan, 20µM) or anti-IL10 antibody (10µg/ml) (B) and anti-TGFβ

(C) were added in a suppressive assay where $CD4^+$ T cells were incubated with allogenic mature DCs and autologous ATDCs (ratio 10 T cells : 1 mature DC :10 ATDC). After 6 days, $CD4^+$ T cell proliferation was evaluated by [^{3}H]-thymidine incorporation (B) or CPD dilution (C) ($n \geq 3$ donors). **D.** $CD4^+$ T cell proliferation was assessed following culture with allogeneic mature DCs with or without autologous ATDCs in a transwell assay; (n=3 donors).**E.**Supernatants from culture of $CD4^+$ T cells with allogeneic mature DCs in the absence (MLRcondition) or presence of ATDCs (MLR + ATDC, suppression assay condition) were harvested and transfered to a new MLR. $CD4^+$ T cell proliferation was assessed by CPD dilution($n \geq 9$donors). **F and H.**Glucose and lactate concentrations were measured in the supernatants from MLR and MLR + ATDC conditions (n=4-10 donors). **G.** CPD-labeled $CD4^+$ T cells were cultured with allogeneic mature DCs in presence or absence of ATDCs and with increasing concentrations of glucose. Glucose concentration of the RPMI medium is 9mM. T cell proliferation was assessed after 6 days (n=3 experiments). p values were calculated by 1way ANOVA on Graphs D-E while paired t tests were used in others panels; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

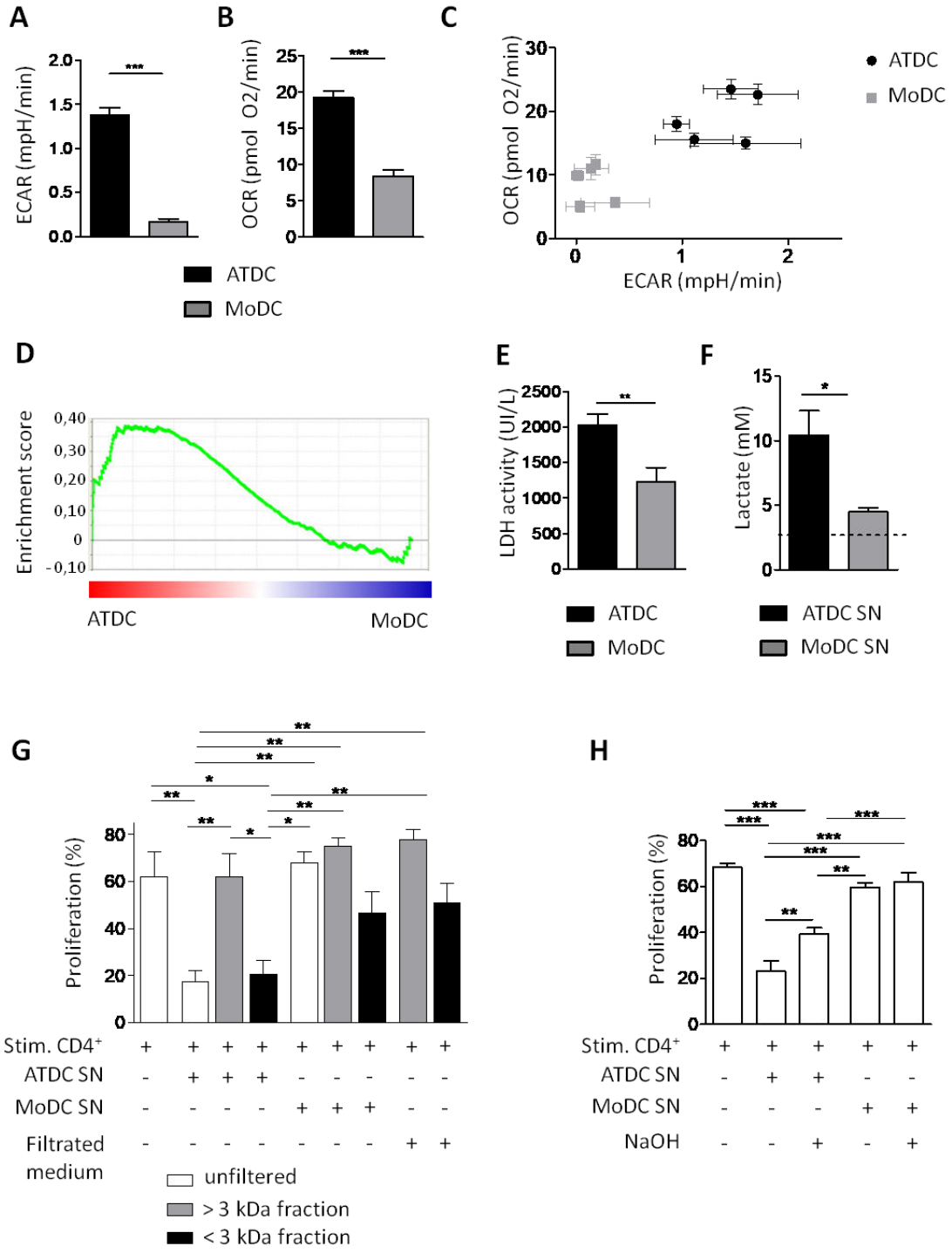


Figure 5: ATDCs display high glycolytic activity that is involved in their suppression ability

A-C. Glycolytic (Extra Cellular Acidification Rate, ECAR, panel A) and Respiratory (Oxygen Consumption Rate, OCR, panel B) capacities of ATDCs and MoDCs were measured by Seahorse XF analyzer (n=5 donors). **D.**Gene Set Enrichment Analysis (GSEA) of glycolysis pathway was performed on ATDCs versusMoDCs transcriptomic data (described on Figure 2,

n=6 donors) using the list of “hallmark glycolysis” gene sets from the Molecular Signature Database. The graph depicts the enrichment of gene sets associated to glycolysis. **E.** Lactate deshydrogenase (LDH) activity was quantified in the two types of cells (n=6 donors). **F.** ATDC or MoDCs were cultured alone in complete RPMI mediumfor 6 days. Lactate concentration was measured in cell supernatants at the end of culture. Dotted line represents the amount of lactate measured in medium without cells (n=5 donors). **G.** Supernatants from ATDCs (ATDC SN) or MoDCs (MoDC SN) cultured alone were collected and transferred to a new MLR. $CD4^+$ T cell proliferation was assessed by CPD dilution. On graph G, SN were filtrated on 3KDa filter and both fractions were tested in the MLR and compared with unfiltrated SN conditions ($n \geq 3$ independent experiments). On graph H, the acidification was neutralized (or not) by the addition of 5mM NaOH ($n \geq 8$ independent experiments). A representative experiment of Graph H is depicted on S6G. p values were calculated by unpaired t teston graphs A-B, paired t test on graphs E-F and 1way ANOVA on graphs G-H; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

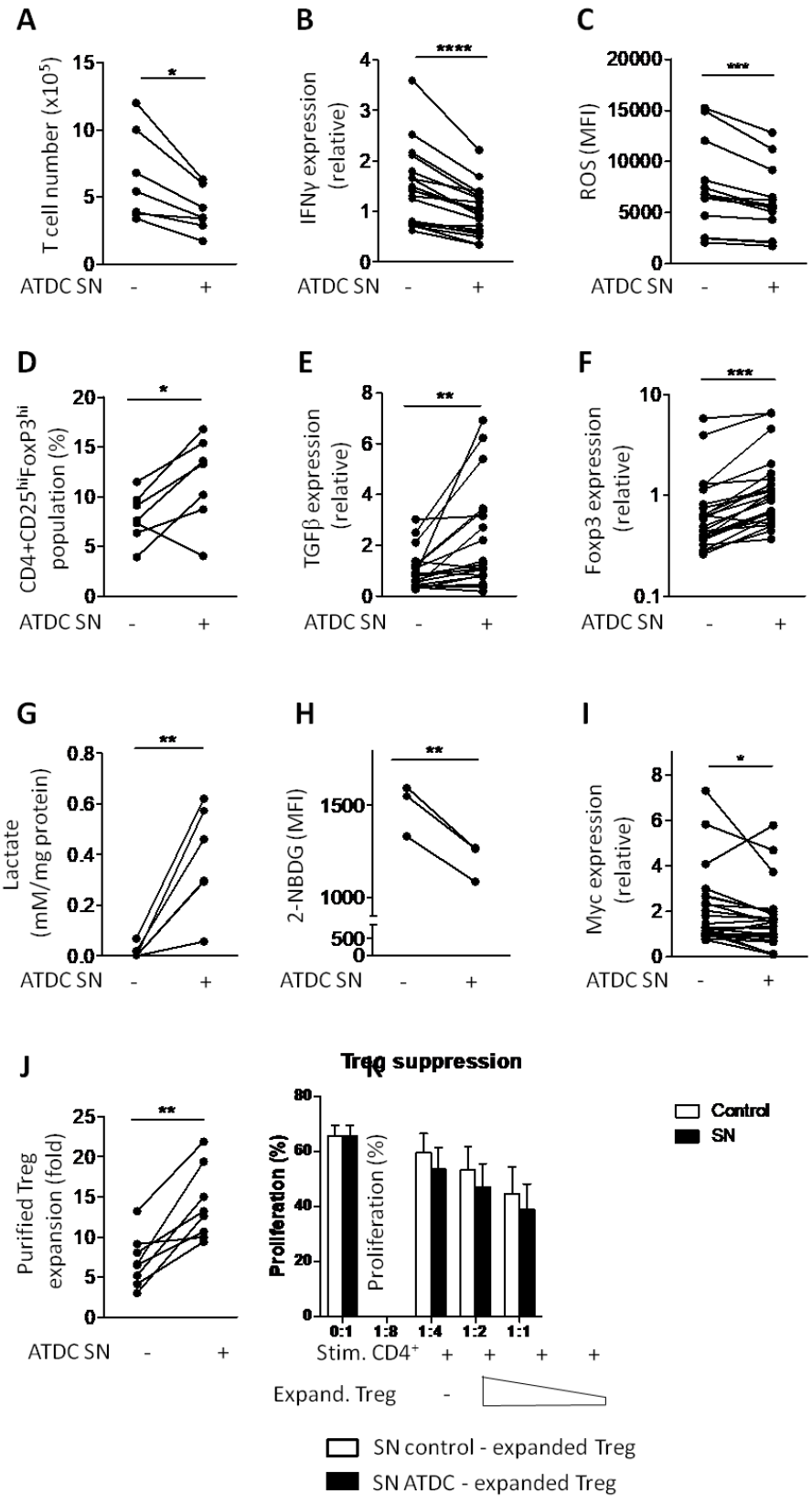


Figure 6: ATDCs induce lactate uptake by T cells and favors the induction of Tregs at the expanse of effector T cells

A-I. $CD4^+$T cells were polyclonally stimulated either with anti-CD3 and anti-CD28 antibodies for 3 days (A,D) or with PMA and Ionomycin for 5 hours (B-C, E-I) in presence of absence of ATDC SN. Graph A representsthe T cell number (n=7 donors) whereas their percentage of $CD4^+CD25^+Foxp3^+$ cells was shown on D (n=7 donors). Graphs B, C, E, F, H and I depict the expressions of IFNγ (n=19 donors), Myc (n=25 donors), TGFβ (n=19 donors), Foxp3 (n=25 donors), ROS production (n=13 donors) and 2-NBDG intake (n=3 donors). ROS and 2-NBDG were quantified by flow cytometry whereas Foxp3, Myc and the cytokines were measured by RT-PCR. On graph G, intracellular lactate was assessed by mass spectrometry (n=5 donors). **J-K.** Purified $CD4^+CD25^-CD127^{low}$ Tregs were expanded with anti-CD3 and anti-CD28 antibodies for 15 days in presence of absence of ATDC SN. Graph J represents the fold of Treg expansion (n=8 donors). These expanded Tregs were cultured with autologous $CD4^+CD25^-$cells and allogeneic mature DCs to evaluate their suppressive ability (K) (n=8 donors). In A-J, p values were calculated by paired t test whereas unpaired t test was used on graph K; *$p<0.05$, **$p<0.01$, ***$p<0.001$. No statistical difference was also observed on graph K between SN control and SN ATDC using 2way ANOVA.

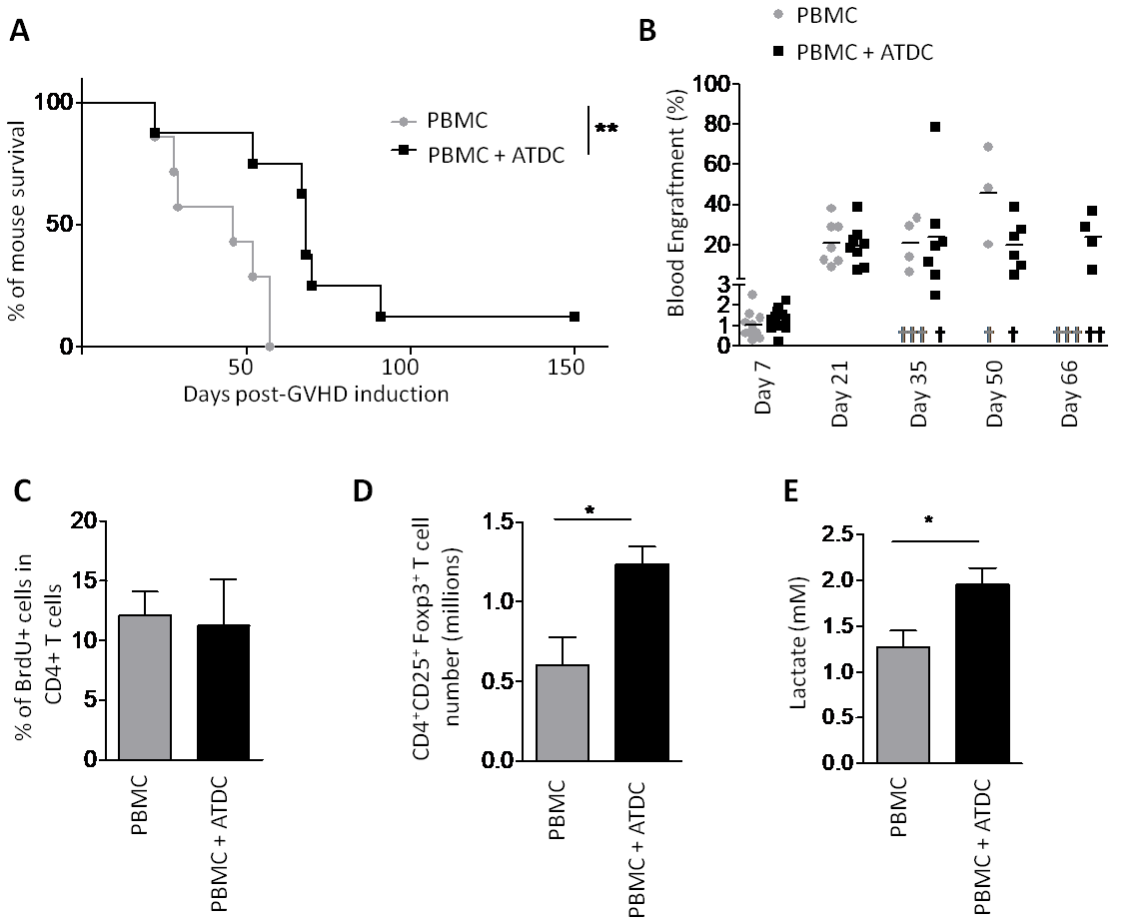


Figure 7: ATDCs delay GVHD development in humanized mice and promote Treg expansion, associated to an increase of lactate secretion.

Irradiated NSG mice received PBMCs in absence or presence of autologous ATDCs. **A.** Survival curve displays mice survival (n=7-8 mice per group pooled from two independent experiments). Mice were sacrificed when they lost 20% of their initial weight. Median survivals are 46 days in PBMC group and 68 days in PBMC + ATDC group. Survival curve was analyzed by log-rank (Mantel-Cox) test; ** $p \leq 0.01$. **B.** The engraftment of human cells was assessed in the blood of injected mice at different times post cell infusion (n≥7/8 mice per group pooled from 2-3 independent experiments). Each "†" symbol represents a mouse sacrificed as its weight was inferior to 80% of its initial weight. **C-D**. Irradiated NSG mice receiving PBMCs in absence or presence of autologous ATDCs were sacrificed at day 21. The percentage of $BrdU^+$ cells in splenic $CD4^+$ T cells (C), as well as the number of splenic human $CD4^+CD25^+Foxp3^+$Tregs (D) were assessed by flow cytometry(n=3 mice per group). **E.**Lactate concentration was measured one week post cell injection in the sera of mice (n=5 mice per group) by mass spectrometry. On graphsC-E, p values were calculated by unpaired t test; *$p<0.05$.

SUPPLEMENTARY MATERIALS

SUPPL. METHODS

Cells and SN generation

Monocytes were isolated either by elutriation of PBMCs (Clinical Development and Transfer Platform, Nantes, France) or by magnetic labeling (untouched cells, Human monocyte Isolation kit II, Miltenyi Biotec). ATDCs were differentiated following a 6 day-culture of 2.5 x 10^6 monocytes in 5ml of AIMV medium CTS (Life Technologies) supplemented with recombinant Human GM-CSF (CellGenix) at 100U/ml in a 6-well plate. To obtain MoDC, 2.5x10^6 monocytes were incubated in a 6-well plate in 5 ml of complete medium (RPMI 1640 medium (Life Technologies, France) containing 10% Fetal Calf serum (FCS), 1% L-glutamin, 1% antibiotics, 1mM Sodium Pyruvate, 1mM Hepes, 1% non-essential amino acids) supplemented with recombinant Human IL-4 (200U/ml, CellGenix) and recombinant Human GM-CSF (1000U/ml, CellGenix) for 6 days. LDH activity were measured in DCs using the Roche diagnostic kit on a Cobas 8000 device (Roche Diagnostics, Mannheim DE) as described previously(1).

ATDC and MoDC SN were obtained following cultures of DCs (0.25 x10^6 cells/ml) in complete RPMI medium. After 6 days, supernatants were collected and frozen at -80°C until use.In some experiments, supernatants were filtrated to 3KDa filters (VWR) and both fractions were tested in MLR assay.

Mature DCs were obtained after a 48h stimulation of MoDC with LPS (Sigma Aldrich) at 1µg/ml. To evaluate the maturation resistance, DCs (either ATDCs or MoDCs) were cultured 48h in a 96-well plate at 0.5x10^5 cells/well or in a 24-well plate at 1x10^6 cells/well in complete medium in presence of LPS (200ng/ml, Sigma) alone or LPS and IFN-γ (50ng/ml, R&D systems).

Antibodies and flow cytometry

Antibodies used for surface staining were provided from BD Biosciences and eBioscience (Table S3). For intracellular staining, cells were stained then fixed/permeabilized according to the eBioscience protocol. Permeabilized cells were incubated in Perm. Buffer with anti-IFN-γ, IL-17A or appropriate isotype control (with human sera). Foxp3 staining was performed according to the manufacturer's protocol (ebiosciences). For reactive oxygen species quantification, cells were incubated with DHE (Thermofisher Scientific) according to manufacturer protocol. For glucose intake assay, cells were incubated with 200 µM 2-NBDG (Life technologies) diluted in free glucose medium RPMI 1640 medium (+) L-Glutamine, (-) D-Glucose, (Gibco) for 30 minutes at 37°C and 5% of CO_2.For the detection of intracellular cytokines, PMA (50ng/ml), ionomycin (1µM) and brefeldinA were added for 5 hours before staining. Dead cells were excluded using DAPI or Fixable Viability Dye eFluor® 450 or eFluor® 506 (eBioscience). Flow cytometry was performed on a FACS Canto II or a FACS LSR (BD Biosciences) and analyzed with FlowJo software (Tree Star).

T cell assays

Suppressive assays with mature DCs: To investigate the suppressive function of ATDCs, autologous $CD4^+$ T cells were selected using a $CD4^+$ T Cell isolation kit II (Miltenyi) and $CD4^+CD25^-$ cells were selected following an additional depletion of $CD25^+$ cells using a $CD25^+$ isolation kit (Miltenyi). In most of the experiments, T cells were labeled with Cell Proliferation Dye (CPD) efluor 450 (ebioscience) and cultured in 96-well plates with DC (ATDCs or MoDCs) at a 1:1 ratio and with mature DCs at a 1:0.1 ratio for 6 days. In some experiments, transwells (3 µm pore size, Corning) were added to the well to define whether contact dependent mechanisms occurred. In some experiments, Glutamine (Sigma), Pyruvate (Gibco), HCl (VWR), lactic acid (Sigma) or NaLactate (Sigma) were added to the medium. In other assays, inhibitors of INOS (Aminoguanonidine, 100 µM, Sigma) and IDO (1-Methyl-D-tryptophan, 20µM, Sigma) or anti-IL10 antibody (clone JES3-9D7, 10µg/ml, BD Biosciences) or anti-TGF-β (clone 2GT, 2µg/ml) were added in the suppressive assay.

Anti-CD3/anti-CD28 stimulation. $CD4^+$ purified T cells ($5x10^5$ cells) were stimulated with 1µg/mL of anti-CD3 (OKT3 clone), 1µg/mL of anti-CD28 (28.6 clone) in 48 well-plate for 5 days at 37°C and 5% CO_2. ATDC SN was added at day 0 and represented 50% of the final medium volume.

PMA-Ionomycin stimulation. $CD4^+$ purified T cells ($5x10^5$ cells) were cultured in 96 well-plate overnight before the stimulation with Phorbol 12-Myristate 13-acetate (PMA) and ionomycin(both from Sigma) in presence or absence of ATDC SN for 5h.

Regulatory T-cell expansion and suppressive ability.

$CD4^+CD25^+CD127^-$ T cells were purified by cell sorter (ARIAII, BD Biosciences) and $3x10^5$ cells were stimulated with 1mg/mL of anti-CD3 (OKT3 clone), 1mg/mL of anti-CD28 (28.6 clone) and 1000U/mL of IL-2 (Proleukin®, Novartis) for 14 days in 48 well plates. Medium was replaced at day 7. ATDC SN was added at day 0 and day 7 and represented 50% of the final medium volume. To evaluate their suppressive ability, expanded Tregs (1.25; 2.5 or $5x10^5$ cells) were cultured with autologous purified CPD-labeled $CD4^+CD25^-$ T cells ($5x10^5$ cells) and mature DCs ($5x10^4$ cells). T cell proliferation was evaluated 6 days later by CPD dilution.

IL-10 and IL-12 ELISAs

To measure IL-10 and IL-12p70 production after LPS/IFNγ stimulation, culture supernatant of unstimulated and stimulated ATDCs and MoDCs were harvested after 48 hours. Level of these cytokines was determined by capture ELISA according to the manufacturer's instructions (BD OptEIA, BD Biosciences).

Lactacte/glucose/ pH measurements

Glucose and lactate were quantified from supernatants of DC cultures and from MLR in presence or not of ATDC with Glu – Test strips and Lac – Test strips (NOVA biomedical) respectively. pH was measured in supernatants of DC cultures with HANNA pH210 microprocessor pH meter at room temperature.

ATP measurement. ATDC or MoDC were plated on flat bottom 96 wells plate (50.000 cells/well) in complete media. Intracelullar ATP was measured using ATPlite Luminescence ATP Detection Assay System (PerkinElmer) According to manufacturer protocol. Luminescence was measured with a SPARK 10M (TECAN) plate reader.

RNA extraction and real-time quantitative PCR.

Total RNA from cell cultures was extracted with RNeasy mini kit (Qiagen) according to manufacturer protocol. mRNA were reverse transcribed into first strand cDNA using poly dT oligonucleotide and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed in a ViiA7 Real-Time PCR system (Thermofisher) Detection System using Fast SYBR Green master mix (Applied Biosystems). The oligonucleotides used are listed on Table S4. Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

Agilent Microarray Hybridization

The Agilent data have been deposited in GEO (Accession number GSE104438). In the microarray experiments, $CD14^+$ sorted monocytes from 6 different donors were used to differentiate ATDCs, MoDCs and rMoMacro from the same samples. ATDCs were generated as described in cell generation section, whereas MoDCs were cultured for 6 days in IMDM medium (Invitrogen) supplemented with 10% FBS (Gemini), 1000 U/ml rhGM-CSF (Miltenyi), 1000 U/ml rhIL-4 (Miltenyi) at a concentration of $0{,}5x10^6$ cells/ml. rMoMacro were obtained following a 7 day-culture of 1 x 10^6 monocytes in 3 ml of RPMI 1640 medium (Lonza) supplemented with 10% FCS (Biochrom), 2 mM Glutamax (Invitrogen), 100 U/mL penicillin-100 ug/mL streptomycin (Lonza), and 100ng/ml rhM-CSF (R&D) in 6-well plate. RNA were extracted from cultured cells using the RNeasy Plus Mini kit (Qiagen) and then labeled and hybridized on Agilent whole genome oligo Microarrays (8x60K) according to the "One-Color Microarray-Based Gene Expression Analysis" protocol (Agilent)(2). Background corrected

intensity values were quantile normalized and log2 transformed. Heatmap of gene expression was normalized and centered across each gene (row), and displayed using the matrix2png program (https://matrix2png.msl.ubc.ca).Gene Set Enrichment Analysis of glycolysis pathway was performed on ATDCs and MoDCs using the list of "hallmark glycolysis" gene sets from the Molecular Signature Database (http://software.broadinstitute.org/gsea/msigdb/cards/HALLMARK_GLYCOLYSIS).

Affymetrix HuGene1.1 ST raw data files were normalized by the robust multiarray average (RMA) algorithm and summarized, after background correction, using the R package *aroma.affymetrix*(www.aroma-project.org). The Agilent and Affymetrix processed data sets were matched using gene symbol as primary identifier. The Agilent gene expression data was then adjusted to the Affymetrix one using the ComBat function of the *sva* package(3),as done in (4). Principal Component Analysis (PCA) was performed as described in(5) in order to compare all the above myeloid subsets within each other. Briefly, we used the dudi.pca function of the *ade4* package (http://pbil.univ-lyon1.fr/ADE-4) and ellipses were drawn using the dataEllipse function of the *car* package (https://cran.r-project.org/web/packages/car/index.html) with the level parameter set at 0.5.

Quantification of lactate by mass spectrometry

Lactate was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in serum or cell lysate samples. All solvents were LC-MS grade and purchased from Biosolve (Valkenswaard, Netherlands). Standard compounds were obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). A pool of reference standard solutions was prepared and serially diluted in water to obtain 7 standard solutions ranging 0.1-100 µmol/L. $CD4^+$ purified T cells ($2,5x10^6$ cells) were incubated overnight before their stimulation with PMA/ionomycin in presence or absence of ATDC SN for 5h. After stimulation cells were washed twice at 4^oC and frozen in liquid nitrogen. Cells were then lysed with distilled water and centrifugated. One part of supernatants was used for protein quantification with bicinchoninic acid assay kit according

to supplier's instructions (Sigma Aldrich) and the other part was used for metabolite quantification.

Briefly, exogenous ^{13}C-lactate labeled internal standard was added and supernatant were deproteinized by adding 4:1 methanol and then centrifuged. Supernatants were recovered and dried using nitrogen stream. Finally, dried samples were recovered in water containing 0,1% formic acid and then injected into the LC-MS/MS system. Analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses, was performed on a Xevo TQD mass spectrometer with an electrospray interface and an Acquity H-Class® UPLC™ device (Waters Corporation). Data acquisition and analyses were performed with MassLynx® and TargetLynx®1 version 4.1 software, respectively (Waters Corporation). Injection and data analysis was performed by mass spectrometry core facility of Biogenouest CORSAIRE platform.Lactate concentrations in cell lysates were normalized with total protein contents of samples.

In vivo ATDC tracking

ATDC tracking was evaluated by the injection of gold labeled ATDCs. At the end of the differentiation of the ATDCs, 50-nm gold nanoparticles (Sigma-Aldrich) were added to each well. After one hour of incubation at 37°C and 5% CO2, cells were harvested and washed 3 times in PBS. Cells were injected as described above. Mice were sacrificed at day 3, day 6, day 10 and day 14. The presence of gold nanoparticles in different tissues was analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as previously described(6, 7).

To analyze ATDC phenotype *in vivo*, cells were labeled with Qtracker® 605 (Life Technologies) before injection according to the manufacturer instructions. Briefly, a 10nM labeling solution containing Qtracker Component A and Component B was prepared, incubated 5min at room temperature and added to the cells ($1X10^6$ cells/ml) in complete medium. After 30min incubation at 37°C and 5% CO2, cells were washed 3 times in PBS before injection. Animals were sacrificed 6 days later and spleen was collected and digested with collagenase D

during 30min at 37°C (Sigma-Aldrich). ATDC staining was then performed on splenic cells and analyzed by flow cytometry.

To evaluate the engraftment of the human cells, blood cells were stained with anti-human CD45, anti-mouse CD45 and anti-human CD3 and were then analyzed by flow cytometry The engraftment of human cells was calculated as [% of human $CD45^+$ cells / (% of human $CD45^+$ cells + % of mouse $CD45^+$cells)]x100. Animals that displayed greater than 20% total body weight were sacrificed according to the local ethical committee guidelines.

To quantify lactate, blood was collected on day 7 and serum was obtained following centrifugation. Lactate concentration was measured by mass spectrometry.

To analyze in vivo mechanism of action of ATDCs, mice were sacrificed 21 days after injection of PBMC or PBMC/ATDCs. Splenocytes were collected as described above before T cell staining. On the same day, in vivo T cell proliferation was analyzed following the intraperitoneal injection of 2mg/ml 5-bromo-2'-deoxyuridine (BrdU, BD Biosciences) 18 hours before the sacrifice. Detection of BrdU was done according to the manufacturer's instruction (BD Biosciences).

SUPPL.REFERENCES

1. Gratas, C., Sery, Q., Rabe, M., Oliver, L., and Vallette, F.M. 2014. Bak and Mcl-1 are essential for Temozolomide induced cell death in human glioma. *Oncotarget* 5:2428-2435.
2. Riquelme, P., Tomiuk, S., Kammler, A., Fandrich, F., Schlitt, H.J., Geissler, E.K., and Hutchinson, J.A. 2013. IFN-gamma-induced iNOS expression in mouse regulatory macrophages prolongs allograft survival in fully immunocompetent recipients. *Mol Ther.* 21:409-422. doi: 410.1038/mt.2012.1168. Epub 2012 Aug 1028.
3. Johnson, W.E., Li, C., and Rabinovic, A. 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 8:118-127.
4. Carpentier, S., Vu Manh, T.P., Chelbi, R., Henri, S., Malissen, B., Haniffa, M., Ginhoux, F., and Dalod, M. 2016. Comparative genomics analysis of mononuclear phagocyte subsets confirms homology between lymphoid tissue-resident and dermal XCR1(+) DCs in mouse and human and distinguishes them from Langerhans cells. *J Immunol Methods* 432:35-49.
5. Goudot, C., Coillard, A., Villani, A.C., Gueguen, P., Cros, A., Sarkizova, S., Tang-Huau, T.L., Bohec, M., Baulande, S., Hacohen, N., et al. 2017. Aryl Hydrocarbon Receptor Controls Monocyte Differentiation into Dendritic Cells versus Macrophages. *Immunity* 47:582-596 e586.
6. Managh, A.J., Edwards, S.L., Bushell, A., Wood, K.J., Geissler, E.K., Hutchinson, J.A., Hutchinson, R.W., Reid, H.J., and Sharp, B.L. 2013. Single Cell Tracking of Gadolinium Labeled CD4(+) T Cells by Laser Ablation Inductively Coupled Plasma Mass Spectrometry. *Anal Chem* 85:10627-10634.
7. Managh, A.J., Hutchinson, R.W., Riquelme, P., Broichhausen, C., Wege, A.K., Ritter, U., Ahrens, N., Koehl, G.E., Walter, L., Florian, C., et al. 2014. Laser ablation-inductively coupled plasma mass spectrometry: an emerging technology for detecting rare cells in tissue sections. *J Immunol.* 193:2600-2608. doi: 2610.4049/jimmunol.1400869. Epub 1402014 Jul 1400823.
8. See, P., Dutertre, C.A., Chen, J., Gunther, P., McGovern, N., Irac, S.E., Gunawan, M., Beyer, M., Handler, K., Duan, K., et al. 2017. Mapping the human DC lineage through the integration of high-dimensional techniques. *Science* 356.
9. Segura, E., Touzot, M., Bohineust, A., Cappuccio, A., Chiocchia, G., Hosmalin, A., Dalod, M., Soumelis, V., and Amigorena, S. 2013. Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* 38:336-348.
10. Cao, W., Rosen, D.B., Ito, T., Bover, L., Bao, M., Watanabe, G., Yao, Z., Zhang, L., Lanier, L.L., and Liu, Y.J. 2006. Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production. *J Exp Med* 203:1399-1405.
11. Haniffa, M., Bigley, V., and Collin, M. 2015. Human mononuclear phagocyte system reunited. *Semin Cell Dev Biol* 41:59-69.
12. Merad, M., Sathe, P., Helft, J., Miller, J., and Mortha, A. 2013. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 31:563-604.

SUPPL. FIGURES

Figure S1

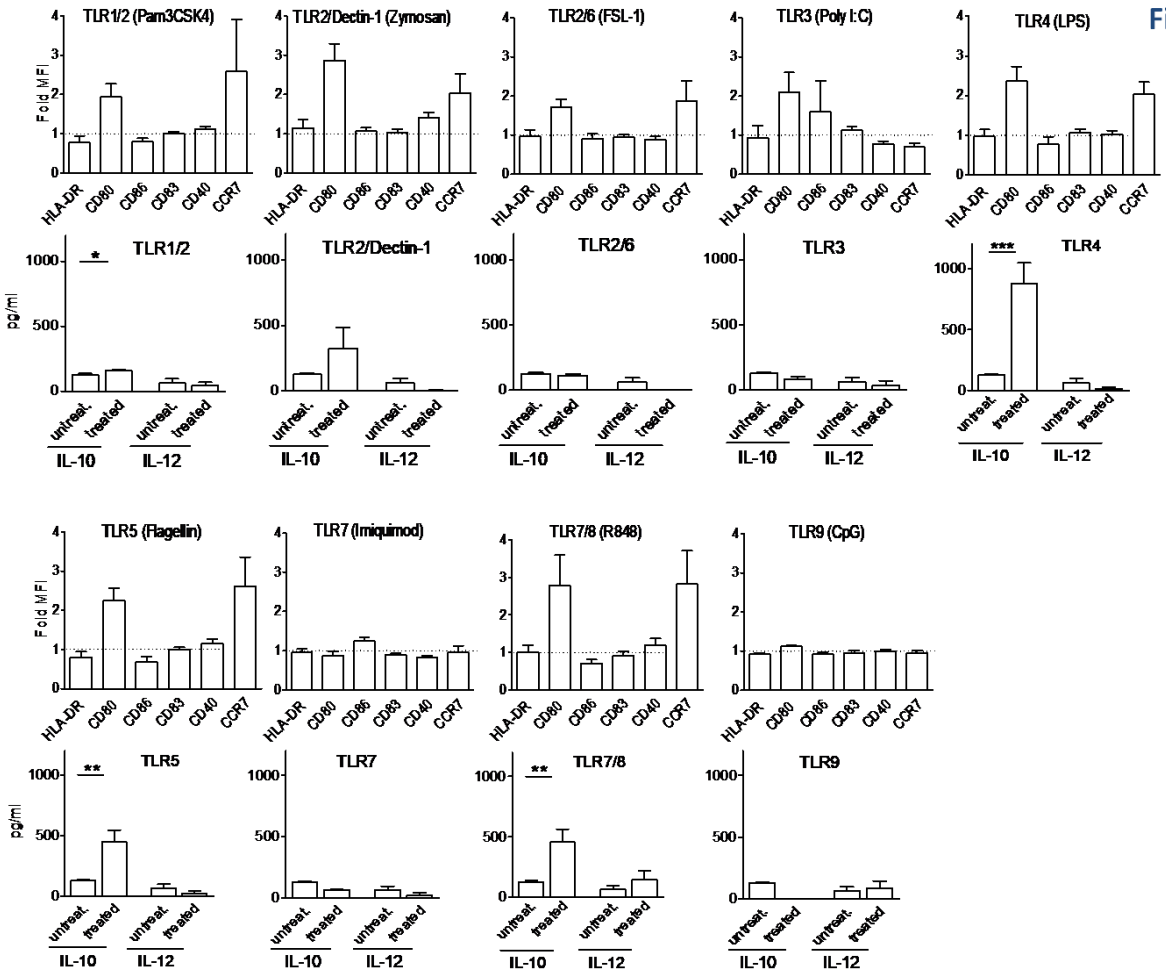


Figure S1: Maturation resistance assay of ATDCs

ATDCs were left untreated or treated with 1µg/ml Pam3CSK4,1µg/ml Zymosan,1µg/ml FSL-1,5µg/ml PolyI:C,1µg/ml LPS, 5µg/ml Flagellin, 2.5µg/ml Imiquimod,2.5µg/ml R848 or 5µg/ml CpG ODN2006 for 24 hours. Expression of HLA-DR, CD80, CD86, CD83, CD40 and CCR7 markers were assessed by flow cytometry after stimulation. Graphs show the overexpression (Fold MFI) of the different markers between untreated and TLR ligand-treated DCs from the same individual (n=5 donors). The baseline defines the expression of each marker in untreated ATDCs which was related to 1. IL10 and IL12 concentrations in the supernatants of TLR ligand-treated and left untreated ATDCs were assessed by ELISA. Bar graphs depict mean+SEM of IL10 and IL12 concentration produced by untreated (untreat.) or TLR ligand-treated (treated) ATDCs (n=4 donors). p values were calculated by paired t test; *p<0.05, **p<0.01; ***p<0.001.

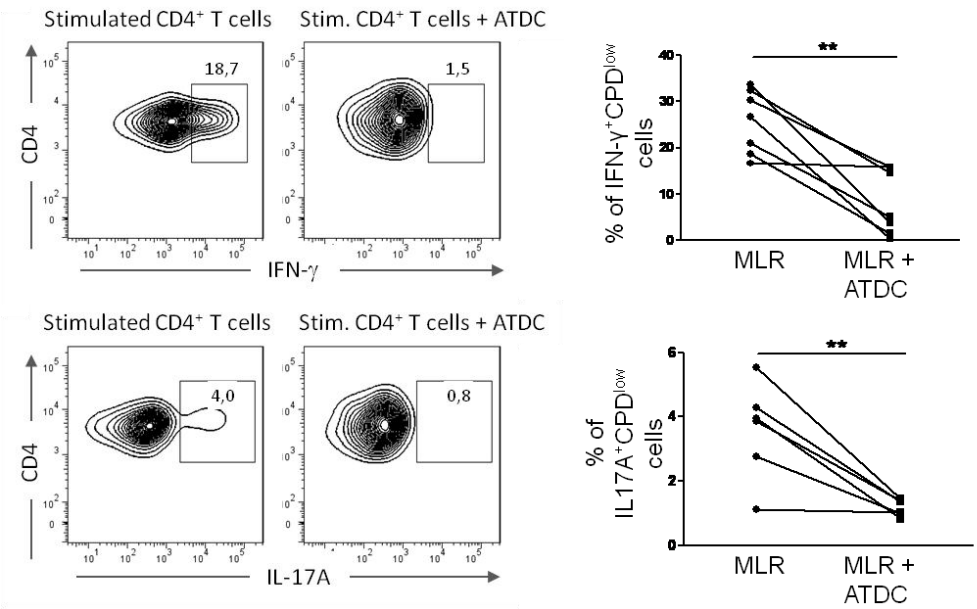


Figure S2: ATDCs inhibit T cell differentiation.

CPD-labeled $CD4^+$ T cells were cultured with allogeneic mature DCs with or without autologous ATDCs and then analyzed for their cytokine secretion. Plots depict the percentage of $CD4^+$IFN-γ^+ Th1 cells, and $CD4^+$IL-17^+ Th17 cells among CPD^{low} $CD4^+$ proliferating cells in absence or presence of ATDCs. Graphs on the right show cumulative data of ≥6donors. p values were calculated by paired t test; **p<0.01.

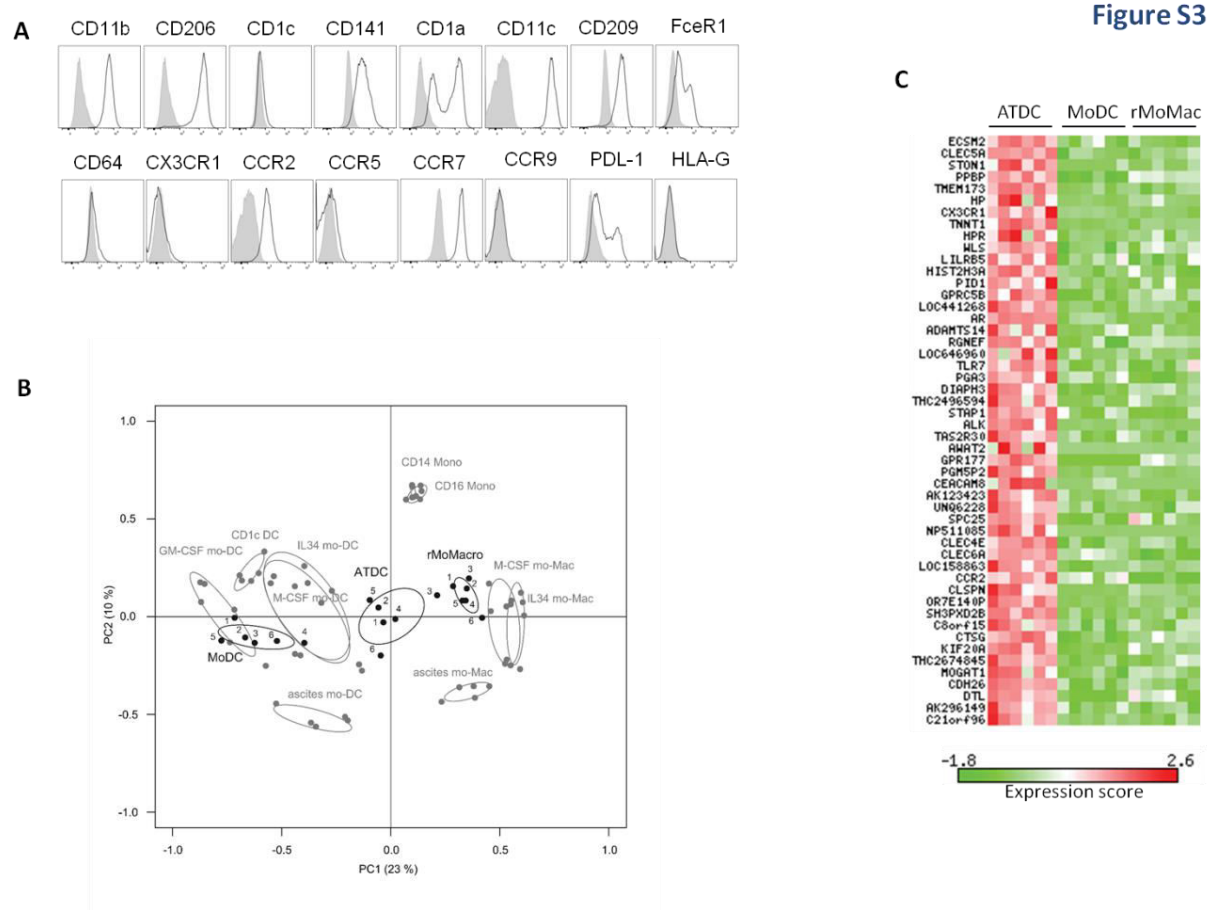


Figure S3:Transcriptomic analysis of ATDCs, MoDCs, rMoMacro and all public datasets from GSE40484 and GSE102046 studies.

A.MoDCs were stained with myeloid cells, monocytes, DCs, chemokines and inhibitory molecules markers and analyzed by flow cytometry. Representative histograms of the expression of the markers by MoDCs (black line; n≥4 donors) and their respective isotype controls (grey shaded) are shown. **B-C.**Microarrays were performed on ATDCs, MoDCs and rMoMacro generated from monocytes of 6 healthy donors. **B.**Transcriptomic datas of these three populations (in black) were compared with human populations obtained from public datasets GSE40484 and GSE102046(in grey). The principal component analysis presented in Figure 2B illustrated only 6 populations from these two public data sets. In this Figure S2, all the populations from GSE40484 and GSE102046 were shown. Public data sets include $CD14^{+}$monocytes (n=4), $CD16^{+}$ monocytes (n=4), blood $CD1c^{+}$ DCs (n=4), GM-CSF/IL-4 mo-DCs (n=6), M-CSF mo-DCs (n=6), IL34 mo-DCs (n=6), M-CSF mo-Mac (n=6), IL34 mo-Mac (n=6), ascites mo-DCs (n=5) and ascites mo-Mac (n=5). The graph depicts the principal component analysis of these different subsets. Numbers indicate the donor ID used to generate ATDCs, MoDCs and rMoMacro.**C.** Heatmap illustrates the expression patterns of the top 50 highly overexpressed genes in ATDCs compared to MoDCs and rMoMacro.

Figure S4

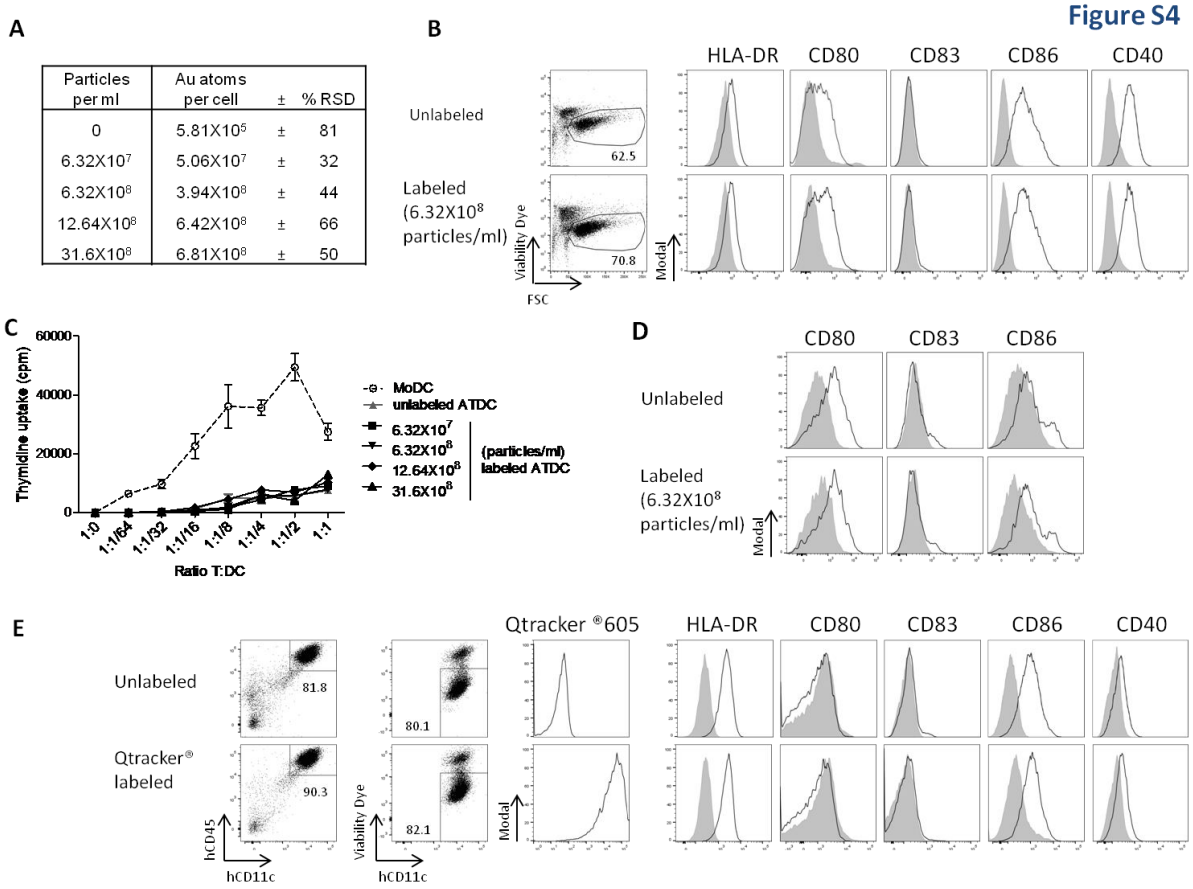


Figure S4: Incubation of ATDCs with Gold nanoparticles or Qtracker® does not modify their properties

Preliminary experiments were performed to define a protocol for labelling ATDCs with Gold nanoparticles. The staining of ATDCs by nanoparticles has to be strong enough for an in vivo detection of the signal but without leading to ATDC death or modification of their properties. **A.** ATDCs were incubated with various concentrations of Gold nanoparticles and their Gold uptake was determined by ICP-MS. Data are the average number of gold atoms internalized/cell for each labeling condition (n= 2-3 donors). **B.** Expression of HLA-DR, CD80, CD86, CD83 and CD40 markers were assessed in unlabeled or gold labeled ATDCs by flow cytometry. Representative histograms of the expression of the markers by ATDCs (black line) and their respective isotype controls (grey shaded) are shown (n=4 donors). **C.** MLR assay described in Figure 1D was performed using unlabeled or gold labeled ATDCs. **D.** Unlabeled or gold labeled ATDCs were left untreated (grey shaded) or treated with 200ng/ml LPS (black line) for 48 hours. Graphs show the expression (MFI) of CD80, CD86, CD83 markers assessed by flow cytometry (n=2 donors). **E.** Expression of Qtracker, HLA-DR, CD80, CD86, CD83 and CD40 markers were assessed in unlabeled or Qtracker-labeled ATDCs by flow cytometry. Representative histograms of the expression of the markers by ATDCs (black line) and their respective isotype controls (grey shaded) are shown (n=2 donors).

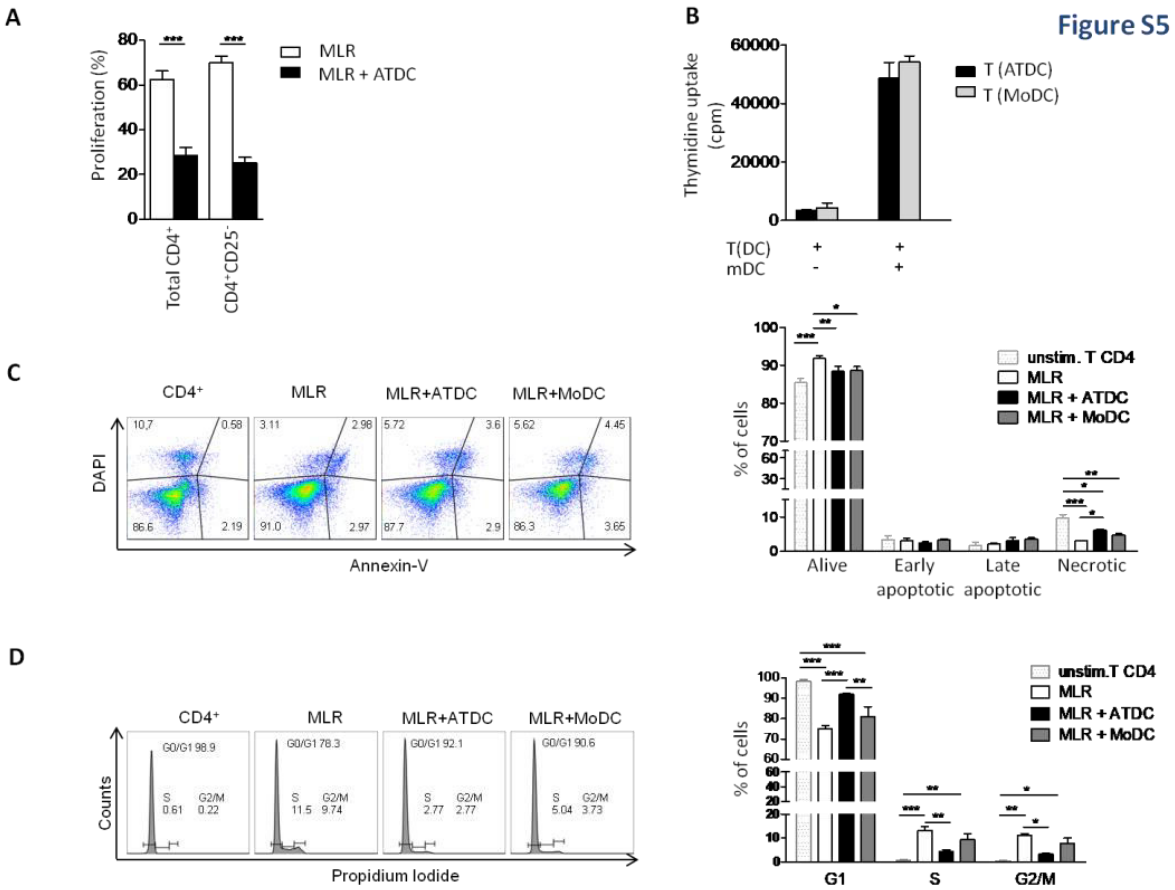


Figure S5: ATDCs do not induce T cell apoptosis or anergy but block CD4$^+$ T cells in G1 phase of cell cycle

A. CPD-labeled total CD4$^+$ T cells or CPD-labeled CD4$^+$CD25$^-$ T cells were cultured with allogeneic mature DCs in presence or absence of ATDCs. T cell proliferation was assessed after 6 days (n=12 experiments). **B.** Anergy assay. Naïve CD4$^+$ T cells were incubated with ATDCs or MoDCs (1 T cell : 1 DC). After 6 days of culture, CD4$^+$ T cells were sorted by cell sorter (ARIA II, BD Biosciences) and incubated 6 to 8 days with recombinant human IL-2 (50U/ml) (CellGenix) to obtain T cell lines (T(DC)). These cell lines were tested for their ability to proliferate in response to mature DC provided from the same donor as DCs (ratio 10 T(DC) : 1 mature DC). After 3 to 6 days, proliferation was evaluated by [^{3}H]-thymidine incorporation for an additional 8 hours. The graph depicts the proliferation of CD4$^+$ T cells from 2 experiments. **C.** Apoptosis assay. Cells were harvested after suppression assay, stained with CD3 antibody, washed and labeled with Annexin V (BD Biosciences) for 20 minutes at room temperature. After labeling, DAPI was added and samples were analyzed in the following hour (n=4 experiments).**D.** Cell cycle assay. Cells were harvested after suppression assay, stained with CD3-PE antibody, washed and fixed with PFA 1% during 1h on ice. After fixation, cells were washed, recovered in 70% cold ethanol and incubated at 4°C overnight. Following the incubation, cells were washed and labeled with Propidium Iodide (50µg/mL Sigma Aldrich) in

presence of RNAase (50µg/mL) diluted in PBS for 30 minutes at 37°C. After labeling, samples were analyzed without washing (n=3 experiments). In these assays, p values were calculated by 2way ANOVA; *p<0.05, **p<0.01,***p<0.001.

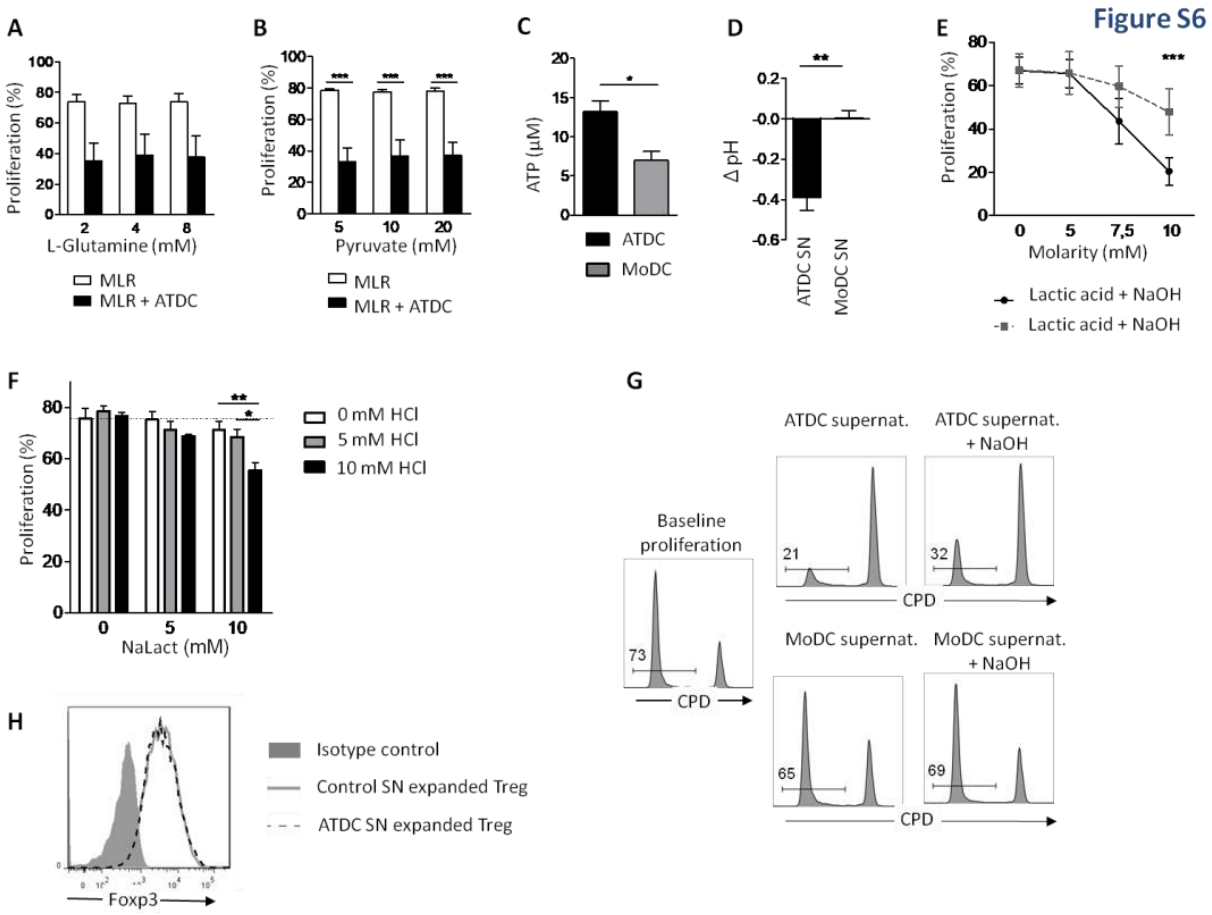


Figure S6: Involvement of lactate secretion in ATDC mechanisms of action

A-B. CPD-labeled $CD4^+$ T cells were cultured with allogeneic mature DCs in presence or absence of ATDCs and with increasing concentrations of L-Glutamine (A, n=3 donors) and Pyruvate (B, n=4 donors). T cell proliferation was assessed after 6 days.p values were calculated by 2way ANOVA; ***p<0.001.**C.**Intracellular ATP was measured in ATDCs and MoDCs (n=4 donors). p value was calculated by paired t test; *p<0.05.**D.**ATDC or MoDCs were cultured alone in complete RPMI medium for 6 days. pH was measured in cell supernatants at the end of culture. Δ pH was measured as pH of cell supernatant – pH of control RPMI. (n ≥ 4 donors).p value was calculated by unpaired t test; **p<0.01.**E.**CPD-labeled $CD4^+$ T cells were cultured with allogeneic mature DCs in presence of increasing concentrations of lactic acidor lactic acid neutralized with the same concentration of NaOH. T cell proliferation was assessed after 6 days (n=5 experiments). p values were calculated by 2way ANOVA; ***p<0.001.**F.**CPD-labeled $CD4^+$ T cells were cultured with allogeneic mature DCs in presence of increasing concentrations

of HCland/or increasing concentrations of NaLactate. T cell proliferation was assessed after 6 days (n=6 experiments). p values were calculated by 2way ANOVA; *p<0.05, **p<0.01.**G.**Supernatants from ATDCs or MoDCs cultured alone were transfered to a new MLR in presence of absence of 5mM NaOH. $CD4^+$ T cell proliferation was assessed by CPD dilution. A graph showing cumulative data of >8 independent experiments is depicted on Figure 5H. **H.**Purified $CD4^+CD25^-CD127^{low}$ Tregs were expanded with anti-CD3 and anti-CD28 antibodies for 15 days in presence of absence of ATDC SN. This histogram displays the Foxp3 expression of expanded Tregs treated or not with ATDC SN and is representative of n=8 experiments.

Figure S7

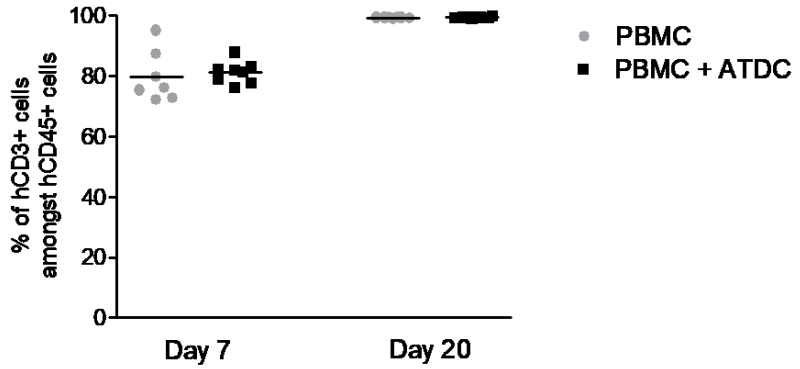


Figure S7: Engraftment of T cells in humanized mice is predominantly CD3 + T cells in both groups of mice treated with PBMCs or PBMC+ATDCs.

The percentage of human $CD3^+$ T cells observed in human $CD45^+$ cells was assessed in the blood of injected mice at day 7 (n=10/11 mice per group pooled from 3 independent experiments) and day 20 (n=7/8 mice per group pooled from 2 independent experiments) post cell infusion.

Table S1

ATDCs	BLOOD							ASCITES	
	classical monocytes	non-classical monocytes	Macrophages	pre-DCs	CD141$^+$ DCs	CD1c$^+$ DCs	pDCs	Inflammatory DCs	Inflammatory Macrophages
MHC class II$^+$	MHC class II$^+$	MHC class II$^+$		MHC class II$^+$	MHC class II$^+$	MHC class II$^+$	MHC class II$^+$	MHC class II$^+$	MHC class II$^+$
CD11b$^+$	CD11b$^+$	CD11b^{low}	CD11b$^+$		CD11b^{low}	CD11b$^+$		CD11b$^+$	CD11b$^+$
CD11c$^+$	CD11c$^+$	CD11c$^+$		CD11c^b	CD11c^b	CD11c$^+$	CD11c$^-$	CD11c$^+$	CD11c$^+$
CD1c$^+$				CD1c$^{-/+}$	CD1c$^-$	CD1c$^+$	CD1c$^-$	CD1c$^+$	CD1c$^-$
CD141$^+$				CD141$^+$	CD141$^+$	CD141$^-$	CD141$^-$	CD141$^+$	CD141$^+$
CD1a$^-$					CD1a$^-$	CD1a$^-$	CD1a$^-$	CD1a$^+$	CD1a$^-$
CD206$^+$			CD206$^+$			CD206$^-$		CD206$^+$	CD206$^+$
CD209$^-$			CD209$^+$					CD209$^-$	CD209$^-$
CD123^{int}				CD123^{II}	CD123$^-$	CD123$^-$	CD123^{II}		
CD14^{int}	CD14^{high}	CD14^{low}	CD14$^+$	CD14$^-$	CD14$^-$	CD14$^-$	CD14$^-$	CD14$^+$	CD14$^+$
FcεR1$^-$							FcεR1γ$^+$	FcεR1$^+$	FcεR1$^-$
CD64$^+$			CD64$^+$					CD64$^+$	CD64$^+$
CX3CR1$^+$	CX3CR1^{low}	CX3CR1^{II}		CX3CR1$^+$	CX3CR1$^+$	CX3CR1$^+$	CX3CR1$^+$		
CCR2^{low}	CCR2^{high}	CCR2^{low}							
ILT7/CD85g$^-$							ILT7/CD85g$^+$		
CD304^{low}				CD304$^+$	CD304$^-$	CD304$^-$	CD304$^+$		
CD163$^-$	CD163$^-$	CD163$^-$	CD163$^+$					CD163$^-$	CD163$^+$
CD16$^{low/+}$	CD16$^-$	CD16$^+$		CD16$^-$	CD16$^-$	CD16$^-$	CD16$^-$	CD16$^-$	CD16$^+$
other markers		TLR7$^+$	CD68$^+$	CD303$^+$	FLT3$^+$	CD172$^+$	CD303$^+$	CD172$^+$	CD172$^+$
		TLR9$^+$	FXIIIA$^+$	CD45RA$^+$	XCR1$^+$	Cd1b$^+$	CD45RA$^+$		
			LYVE-1$^+$		CLEC9A$^+$	CLEC7A$^+$	CD4$^+$		
					NECL2$^+$	CLEC6A$^+$	TLR9$^+$		
					TLR3$^+$	FLT3$^+$			
					CD26$^+$				
					CD4$^+$				
					BTLA$^+$				

Figure S7: Engraftment of T cells in humanized mice is predominantly CD3 + T cells in both groups of mice treated with PBMCs or PBMC+ATDCs.

The percentage of human CD3$^+$ T cells observed in human CD45$^+$ cells was assessed in the blood of injected mice at day 7 (n=10/11 mice per group pooled from 3 independent

experiments) and day 20 (n=7/8 mice per group pooled from 2 independent experiments) post cell infusion.

Table S2: List of genes with expression fold change over 2 in ATDCs compared to MoDCs and rMoMacro

Expression values were log-2 transformed. FC (Fold change) expression between ATDCs and MoDCs or rMoMacro were shown in the last two columns.

Table S2 is provided in separated file.

Table S3: List of anti-human antibodies used in this study.

	Clone	Source	Catalog#
CCR2 (CD192)	48607	R&D systems	FAB151A
CCR5 (CD195)	2D7/CCR5	BD Biosciences	556903
CCR7 (CD197)	3D12	BD Biosciences	557648
CCR9 (CD199)	112509	R&D systems	FAB179A
CD11b	BEAR1	Beckman Coulter	IM0530
CD11c	S-HCL-3	BD Biosciences	333144
CD123	9F5	BD Biosciences	551065
CD127	hIL-7R-M21	BD Biosciences	557938
CD14	M5E2	BD Biosciences	557742
CD141	1A4	BD Biosciences	559781
CD1a	HI149	BD Biosciences	555808
CD1c	L161	Thermo Fisher Scientific	46-0015-42
CD206	19.2	BD Biosciences	551135
CD209	DCN46	BD Biosciences	551265
CD25	M-A251	BD Biosciences	557753
CD25	M-A251	BD Biosciences	555431
CD25	M-A251	BD Biosciences	555434
CD3	UCHT1	BD Biosciences	555332
CD3	UCHT1	BD Biosciences	555333
CD4	RPA-T4	BD Biosciences	555346
CD4	RPA-T4	BD Biosciences	555347
CD4	RPA-T4	BD Biosciences	560650
CD40	5C3	BD Biosciences	555588
CD45	HI30	BD Biosciences	557748
CD64	10.1	BD Biosciences	555527
CD80	L307.4	BD Biosciences	561134
CD83	HB15e	BD Biosciences	551058
CD86	2331 (FUN-1)	BD Biosciences	555660
CX3CR1	2A9.1	Thermo Fisher Scientific	17-6099-42
Fcε R1	AER-37	Thermo Fisher Scientific	25-5899-42
FoxP3	PCH101	Thermo Fisher Scientific	45-7776-42
HLA-DR	G46-6	BD Biosciences	561225
HLA-G	87G	Thermo Fisher Scientific	17-9957-42
IFN-g	B27	BD Biosciences	557995
IL-17ª	eBio64DEC17	Thermo Fisher Scientific	17-7179-42
ILT2 (CD85j)	GHI/75	Miltenyi Biotech	130-101-552
ILT3 (CD85k)	ZM3.8	BD Biosciences	564181
ILT4 (CD85d)	287219	R&D systems	FAB2078N
ILT7 (CD85g)	17G10.2	BD Biosciences	562340
PDL-1 (CD274)	MIH1	BD Biosciences	558065
anti-mouse CD45	30-F11	BD Biosciences	550994

Table S4:List of primers used in this study.

Gene	Forward primer	Reverse primer
IFNG	TCGGTAACTGACTTGAATGTCCA	TCGCTTCCCTGTTTTAGCTGC
TGFB	CTGGCGATACCTCAGCAACC	TGCAGTGTGTTATCCCTGCT
FOXP3	GTGGCCCGGATGTGAGAAG	GGAGCCCTTGTCGGATGATG
CMYC	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
IL1B	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNFA	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL12P40	TGCCCATTGAGGTCATGGTG	CTTGGGTGGGTCAGGTTTGA
IL10	GATCCAGTTTTACCTGGAGGA	CCTGAGGGTCTTCAGGTTCTC
HPRT	CGAGATGTGATGAAGGAGATGG	CCTGTTGACTGGTCATTACAATAGC

Results II: Tolerogenic dendritic cells generated with low dose of GM-CSF display a highly active metabolism

ATDC display a complex and highly oxidative and glycolytic metabolism

As we previously reported, Tol-DC generated with low dose of GM-CSF (ATDC) displayed a highly glycolytic metabolism resulting in a high glucose consumption and high secretion of lactate and protons **(Figure 19A-C)**. On the other hand, we also demonstrated that ATDC also displayed a highly oxidative metabolism and a higher production of ATP comparing to monocyte derived DC (MoDC) **(Figure 19D)**. In order to better understand ATDC immunosuppressive function linked to metabolism, we indeepth into metabolic characterization of these cells. Firstly we investigate glucose metabolism with metabolism assay Seahorse to evaluate the mitochondrial contribution to oxygen consumption and on the other hand the spare capacity **(Figure 19E)**. We observe that after the addition of oligomycin, inhibitor of mitochondrial ATP synthase, there is a decrease of oxygen consumption that impairs totally the oxidative metabolism in MoDC but not in ATDC. This result suggests an alternative pathway in ATDC respiration that keeps a certain oxidative capacity independent from ATP synthase activity **(Figure 19F)**. After adding FCCP, a drug that increases permeability in inner mitochondrial membrane impairing proton gradient and leading to maximal activity of oxygen consumption, we observed that both ATDC and MoDC have similar capacity to increase their OCR from basal to maximal (Spare capacity). Despite the higher oxygen consumption of ATDC, comparing to MoDC, the spare capacity remains the same **(Figure 19G)**. As the effect of the oligomycin does not abrogate totally oxygen consumption in ATDC we use Rotenone and Antinomycin in order to evaluate the contribution of the proton leak, or oxygen consumption independent from ATP synthase. We observed that after adding rotenone, an inhibitor of complex I of respiratory chain, the oxygen consumption is not totally abrogated in ATDC. Interestingly, the inhibition of complex I impaired the capacity to oxydize NADH, in consequence oxygen consumption is derived from oxidation of FADH2 lead by the succinate to fumarate reaction **(Figure 19H)**. Finally, the addition of antinomycin A, inhibitor of complex III, responsible of oxygen reduction, lead to a total impairment of

oxidative capacity in both cells. On the other hand, as we observed an enhanced respiratoy activity in ATDC we interested then in mitochondrial content, mitochondrial ROS and total ROS production. We observed a higher mitochondrial content in ATDC comparing to MoDC. On the other hand, we observed a non significant increase tendence in mtROS production in ATDC and no differences in total ROS production **(Figure 19I-K)**. These results suggest a high oxidative metabolism lead by a high mitochondrial mass and activity. Finally, we were also interested on the glycolytic capacity of ATDC. In order to evaluate maximal glycolytic capacity, we checked the acidification rate in presence of oligomycin with Seahorse. We observed that even displaying a increased ECAR, in presence of oligoycin, ATDC increased rapidly acidification rate but they are not able to maintain. On the other hand MoDC increased ECAR in presence of oligomycin and maintain a stable rate **(Figure 19L-M)**. In conclusion, ATDC display a high oxidative and glycolytic metabolism, a different behavior in ECAR face to oligomycin and an alternative electron source for oxygen consumption in presence of rotenone. These results suggest that ATDC and MoDC display different metabolism and potentially different regulations of metabolism.

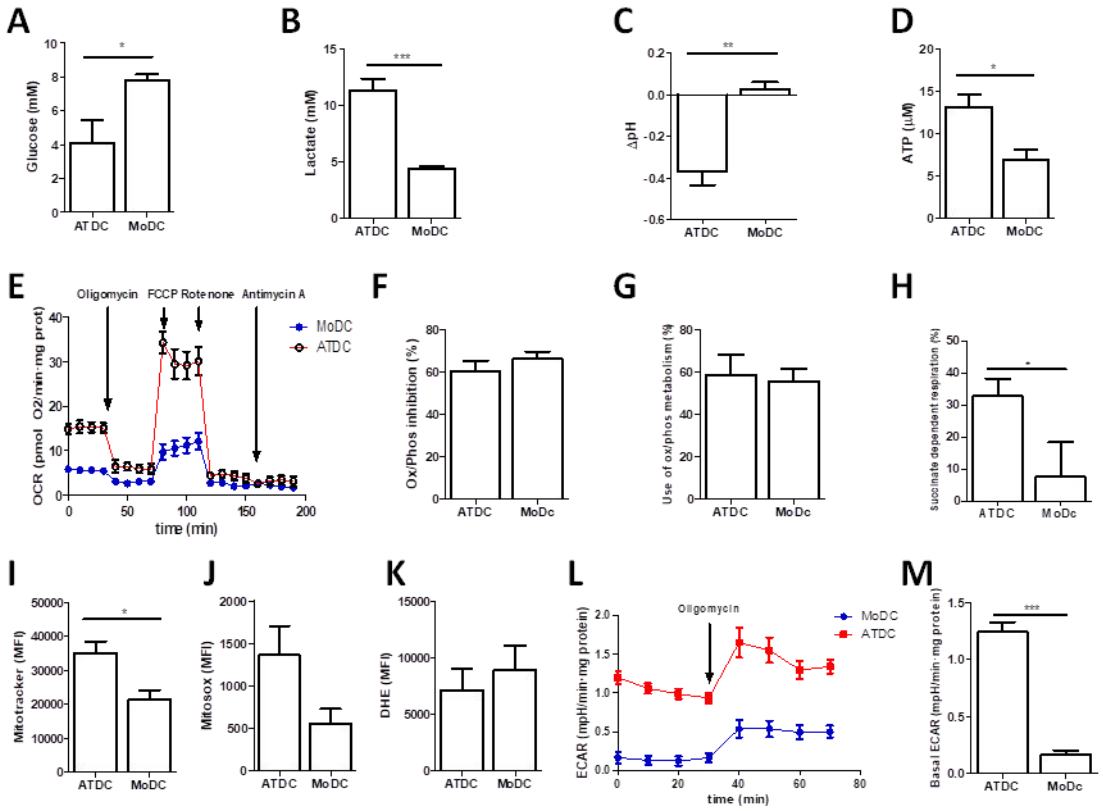


Figure 19. hATDC display a highly glycolytic and oxidative metabolism. **(A-C)** Supernatant glucose, lactate and pH after 6 days of hATDC or MoDC culture A(n=5), B(n=9), C(n=8). **(D)** hATDC and MoDC intracellular ATP after differentiation (n=4). **(E)** hATDC and MoDC OCR sequentially stimulated with oligomycin, FCCP, Rotenone and antinomycin A (n=6). **(F)** hATDC and MoDC ATP production linked respiration, (n=6) **(G)** Spare capacity (n=6) and **(H)** FADH dependent respiration (n=6). **(I)** hATDC and MoDC Mitochondrial mass (n=4), **(J)** mROS (n=4) and **(K)** ROS (n=4). **(L-M)** hATDC and MoDC ECAR stimulated with oligomycin (n=6). Experimental procedures and details are described in material and methods. *, **, *** indicate $p<0,05$, $p<0,01$ and $p<0,001$, respectively (Paired Student's t test). Error bars indicate SEM.

Lipid oxidative metabolism is enhanced in ATDC

Usually, a high mitochondrial and oxidative metabolism is linked to fatty acid oxidation. For this reason we characterize lipid metabolism in ATDC and MoDC. First we investigated the contribution of lipid metabolism to the oxidative capacity of ATDC. In this purpose we checked for the OCR in presence of etomoxir, an inhibitor of lipid transport to mitochondria. We observed that the addition of etomoxir lead to a high decrease in oxidative metabolism in both cells, however, this decrease was more pronounced in ATDC. However this result must be confirmed by additional experiments. This result indicated that lipid oxidation contribute to the 50% of total mitochondrial oxidative activity in ATDC meanwhile in MoDC is around 30% **(Figure 20A and 20B)**. As lipids can be generated de novo or can be uptake from the extracellular media, we first checked for the neutral lipid content of both cells. We observed that after differentiation ATDC have a higher lipid content comparing to MoDC **(Figure 20C)**. On the other hand, higher lipid content observed after differentiation in ATDC comparing to MoDC could be the consequences of the use of different culture media. MoDC are differentiated with RPMI media supplemented with SVF and ATDC are differentiated with AIMV media. However, the addition of lipids during the last 24h of differentiation leads to a higher content of lipids in ATDC but no in MoDC **(Figure 20D and 20E)**. These results suggest that ATDC can uptake lipids from the extracellular medium. Interestingly, 48h after lipid preincubation, lipids were

totally disappeared **(Figure 20F)**. According to literature, lipids can be release, modified or oxidized, however regarding FAO in ATDC we propose that lipids are mostly oxidize during the 48h. Additionally, we checked for the expression of genes related to fatty acid synthesis **(Figure 20G)** and lipid intake **(Figure 20H)** after differentiation. We observed no difference between ATDC and MoDC in lipid biosynthesis enzymes *Fas*, *Acaca* and *Acl*. On the other hand, we observed an increased expression in lipid receptors related to lipid transport and endocytosis *CD36* and *Lox1*. Finally, such as endocytosis pathways can be related to the lipid oxidative metabolism, we performed electronic microscopy in order to detect endocytic structures **(Figure 20I)**. Preliminary results suggest that ATDC present enormous structures closed to cytoplasmic membrane comparing to MoDC. Additionally, the low electronic density around these vesicles suggests that these vesicles could be associated to macropinocytosis **(Figure 20J)**. However, these results must be confirmed. Altogether, these results suggest that ATDC have a highly lipolytic profile and a high capacity to uptake lipids from the extracellular medium.

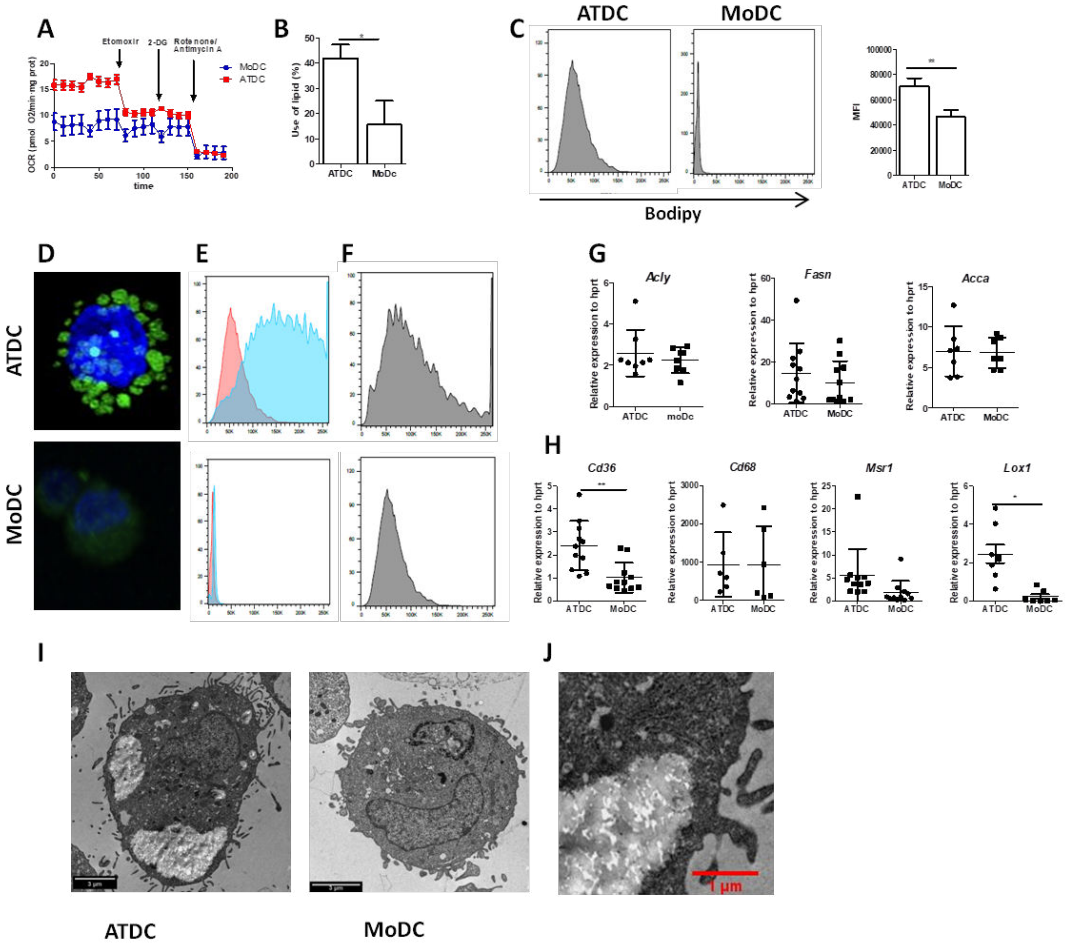


Figure 20. hATDC display a highly lipolytic metabolism. **(A)** hATDC and MoDC OCR sequentially stimulated with etomoxir, 2-DG and rotenone/antimycin A (n=4). **(B)** hATDC and MoDC oxidative capacity depending on lipids (n=4). **(C)** Neutral lipid staining in hATDC and MoDC (n=9). **(D)** Confocal microscopy of hATDC and MoDC after lipid stimulation and stained with bodipy. **(E)** hATDC and MoDC bodipy staining after lipid intake and **(F)** after 48h in fresh medium(D, E and F, representative from 9 experiments). **(G)** Expression of lipid biosynthesis-related enzymes *Acly*, *Fas* and *Acca (n=8-11).* **(H)** Expression of lipid receptors *Cd36*, *Cd68*, *Msr1* and *Lox1(n=8-11).* **(I)** ETM of hATDC and MoDC (n=1). **(J)** Contour detail of hATDC large vesicles (n=1). Experimental procedures and details are described in material and methods. *, **, *** indicate p<0,05, p<0,01 and p<0,001, respectively (Paired Student's t test). Error bars indicate SEM.

High metabolic rate and function of ATDC can be controlled by lipid oxidation

In order to indeepth on the dual lipidic and glycolytic metabolism of ATDC, we wonder about the cause and the regulation. As the ATP production in steady state is the reflection of the energetic homeostasis, we previously demonstrated that intracellular ATP was increased in ATDC **(Figure 21B)**. However, looking for the highly active metabolism, we already expected a major production of ATP in ATDC. About the causes of this high energetic demand we think that cellular size can be an important factor as well the metabolic regulation. Cellular size, mitochondrial content and architecture are determinants in metabolism. It has been already described that biggest cells have a lower oxygen and nutrient diffusion across the cytoplasm and more requirements in order to regenerate organelles. We observed through electronic microscopy images that ATDC are biggest than MoDC **(Figure 21A)**. On the other hand, as a high glycolytic metabolism is usually regulated by different transcription factors and enzymes, we determine the expression of glycolysis related factors such as glycolytic enzymes *Glut1*, *Pfk1*, *Pkm* **(Figure 21B)** and glycolysis related transcription factors *Hifa* and *c-myc* **(Figure 21C)**. We observed no significant differences between the expressions of these genes. On the other hand, as lipid metabolism can blocks the entrance to mitochondria of pyruvate derived from glycolysis we checked for the expression of PDH inhibitors PDKs. We observed that PDH inhibitors *Pdk1* and *Pdk4* are highly expressed on ATDC meanwhile *Pdk2* displayed an increased trend and no differences in *Pdk3* were observed **(Figure 21D)**. These results suggest that ATDC can

potentially impair oxidative use of glucose leading to a pyruvate to lactate derivation. These results support the idea that this highly active metabolism is regulated by an active FAO. In order to better characterize the ATDC metabolism, we determine intracellular ATP, glucose consumption and lactate production after LPS stimulation. Usually, LPS activate glycolytic metabolism and lipid biosynthesis. We observed that in presence of LPS, glucose consumption and lactate production are increased in both cells **(Figure 21E)**. However, ATP production increases in MoDC but decreases in ATDC **(Figure 21F)**. Additionally, LPS stimulation led to an increase in lipidic content **(Figure 21G)**. These results suggest that forcing glycolytic pathway potentially impairs FAO in ATDC leading to a less efficient production of ATP. Following these results, we wondered if the addition of lipids can favour FAO and lead to a decrease in tolerogenic function of ATDC toward $CD4^+$T-cells in MLR context. We observed that the addition of lipids impaired ATDC suppressive potential in a dose dependent manner but not in MoDC. On the other hand, the presence of lipids impairs by itself $CD4^+$T-cell proliferation **(Figure 21H)**. Altogether, these results suggest that highly active metabolism in ATDC is potentially driven by FAO and a highly bioenergetic demands.

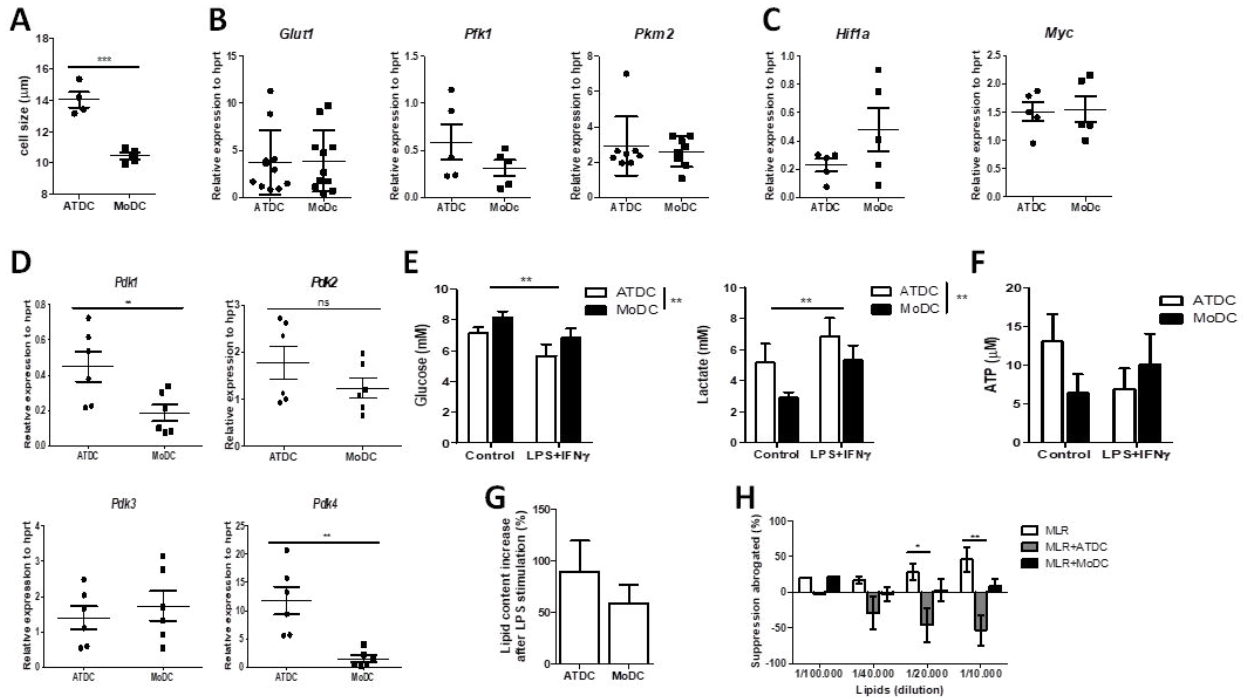


Figure 21. High metabolic rate and function of ATDC can be controlled by lipid oxidation. (A) hATDC and MoDC cell size (4 cells from 1 experiment). **(B)** Expression of glycolytic enzymes *Glut1*, *Pkf1* and *Pkm2 (n=6-11)*. **(C)** Expression of transcription

factors related to aerobic glycolysis *Hif1a* and *Myc (n=6-11)*. **(D)** Expression of PDH inhibitors *pdk1*, *pdk2*, *pdk3* and *pdk4 (n=6)*. **(E)** Supernatant glucose and lactate in hATDC and MoDC culture after 48h of stimulation with LPS and IFNγ (n=3). **(F)** Intracellular ATP in hATDC and MoDC after 48h of stimulation with LPS and IFNγ (n=3). **(G)** Intracellular lipids in hATDC and MoDC after 48h of stimulation with LPS and IFNγ (n=3). **(H)** Abrogation of hATDC and MoDC suppressive activity in suppression assay (n=4). Experimental procedures and details are described in material and methods. *, **, *** indicate $p<0,05$, $p<0,01$ and $p<0,001$, respectively (Paired Student's t test and 2way ANOVA Bonferroni Post-test for figures E, F and H). Error bars indicate SEM.

Experimental procedures.

ATDC and MoDC generation. Dendritic cells were generated from monocytes isolated from healthy volunteer donors (French Blood Service, Nantes, France)**.** ATDC were differentiated following a 6 day-culture of monocytes in AIMV medium CTS (Life Technologies) supplemented with recombinant human GM-CSF (CellGenix) at 100U/ml. MoDC were derived from monocytes cultured in complete RPMI 1640 medium (Life Technologies, France) supplemented with 10% decomplementedFetal Calf serum(Eurobio), recombinant human IL-4 (200U/ml, CellGenix) and recombinant human GM-CSF (1000U/ml, CellGenix) for 6 days.

Suppression assay. Autologous $CD4^+$ T cells were selected using a $CD4^+$ T Cell isolation kit (Miltenyi), labeled with Cell Proliferation Dye (CPD) efluor 450 (ebioscience) and cultured in 96-well plates with DC (ATDC or MoDC) at a 1:1 ratio and with mature DC at a 1:0.1 ratio for 6 days.

Metabolic assay. ATDC or MoDC were plated on Seahorse Bioanalyzer XFe24 culture plates (120.000 cells/well) in Seahorse XF-base medium (Sigma) supplemented with 9mM of glucose, 0,86mM of NaOH, 1mM of pyruvate and 2mM of glutamine. Before the experiment, cells were incubated 20 minutes at 37°C and 0% CO_2. Drugs used during the plate reading were Oligomycin (2,5µM), FCCP (0,5µM), Rotenone (1µM), Antinomycin A (1µM), Etomoxir (100µM) and 2-DG (100mM). OCAR and ECAR readout were obtained using Seahorse XF24 analyzer. Seahorse data analysis was performed using Seahorse Wave 2.4 software.

ATP measurement. ATDC or MoDC were directly used or plated on flat bottom 96 wells plate (50.000 cells/well) and incubated with LPS (Sigma) for 48h. Intracelullar ATP was measured using ATPlite Luminescence ATP Detection Assay System (PerkinElmer) according to manufacturer protocol. Luminescence was measured with a SPARK 10M (TECAN) plate reader.

Measurement of reactive oxygen species. After differentiation, ATDC and MoDC were washed and incubated with DHE (Thermofisher Scientific) according to manufacturer protocol.

Measurement of mitochondrial reactive oxygen species. After differentiation, ATDC and MoDC were washed and incubated with Mitosox (Thermofisher Scientific) according to manufacturer protocol.

Measurement of mitochondrial content. After differentiation, ATDC and MoDC were washed and incubated with Mitotracker (Thermofisher Scientific) according to manufacturer protocol.

Lipid measurement. After differentiation or after LPS stimulation for 48h, ATDC and MoDC were washed and incubated with Bodipy (Thermofisher Scientific) according to manufacturer protocol.

Confocal microscopy. ATDC and MoDC were stained with Bodipy and then fixed with Prolong Gold with DAPI. Confocal microscopy was performed using A1 R Si Confocal Microscope (Nikon, Champigny sur Marne-France)

RNA extraction and real-time quantitative PCR. Total RNA from cell cultures was extracted with RNeasy mini kit (Qiagen) according to manufacturer protocol. mRNA were reverse transcribed into first strand cDNA using poly dT oligonucleotide and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed in a ViiA7 Real-Time PCR system (Thermofisher) Detection System using Fast SYBR Green master mix (Applied Biosystems).

Transmission electronic microscopy. TEM was performed at the Electronic Microscopy Facility of the Federative Institute of Research 26 (IFR26, Nantes, France). Briefly, after differentiation, 5 million ATDC or MoDC were washed and resuspended in the fixative solution (glutaraldehyde 2.5% v/v in Sorensen's Phosphate Buffer at 0.1 M) for two hours and a half at 4°C. Cells were washed and post-fixed in 1% w/v osmium tetroxide for one hour at 4°C, dehydrated in ethanol and embedded in an Epon resin mixture. Ultra-thin sections were double-stained using uranyl acetate and lead citrate. Finally, thin sections (60 to 70 nm) were cut on a Reichert Ultracut E microtome and were doublestained using uranyl acetate and lead citrate. Observation of the contrasted sections was performed at 80 kV under a JEM-1010 transmission electron microscope (JEOL).

Statistical analysis. Statistical analysis was conducted using Graphpad Prism 6 software. Measurements between two groups were performed with a paired Student's t

test. Groups of two or three were analyzed by Two-way ANOVA with Bonferroni Post-test. Statistical parameters for each experiment can be found within the corresponding figure legend.

Primers.

Gene	Forward primer	Reverse primer
Pdk1	CTGTGATACGGATCAGAAACCG	TCCACCAAACAATAAAGAGTGCT
Pdk2	ATGAAAGAGATCAACCTGCTTCC	GGCTCTGGACATACCAGCTC
Pdk3	CGCTCTCCATCAAACAATTCCT	CCACTGAAGGGCGGTTAAGTA
Pdk4	GGAGCATTTCTCGCGCTACA	ACAGGCAATTCTTGTCGCAAA
Lox1	TTGCCTGGGATTAGTAGTGACC	GCTTGCTCTTGTGTTAGGAGGT
Fas	CCGAGACACTCGTGGGCTA	CTTCAGCAGGACATTGATGCC
Acly	ATCGGTTCAAGTATGCTCGGG	GACCAAGTTTTCCACGACGTT
Acac	ATGTCTGGCTTGCACCTAGTA	CCCCAAAGCGAGTAACAAATTCT
Hprt	CGAGATGTGATGAAGGAGATGG	CCTGTTGACTGGTCATTACAATAGC
Glut1	CATTGGCTCCGGTATCGTCA	GGCCACGATGCTCAGATAGG
Msr1	GACACTGATAGCTGCTCCGA	AACTGCAAACACGAGGAGGT
Cd36	CGGCTGCAGGTCAACCTATT	GGCCAAGGAGGTTTATTTTTCCA
Cd64	AGCTGTGAAACAAAGTTGCTCT	GGTCTTGCTGCCCATGTAGA
Myc	GGCTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
Hif1a	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
Pfk1	GGTGCCCGTGTCTTCTTTGT	AAGCATCATCGAAACGCTCTC
Pkm2	ATGTCGAAGCCCCATAGTGAA	TGGGTGGTGAATCAATGTCCA

Discussion

Results discussion

Low dose-induced hATDC are tol-DC

During the last two decades, the therapeutic use of Tol-DC has demonstrated to be a potent strategy to treat autoimmune diseases and to induce tolerance in transplantation in rodent models. Nowadays these cells have been tested in several clinical trials demonstrating the feasibility and safety of the therapy. Among these clinical trials, just one has been performed in the context of kidney transplantation, in the CHU of Nantes as a part of the One study consortium. This study will show for the first time the ATDC effect in humans in the context of transplantation. Tol-DC used for this study were Autologous Tol-DC (ATDC) differentiated from human monocytes in AIMV medium supplemented with low dose of GM-CSF. Based on this low dose-GMCSF protocol, previous studies performed by our group demonstrated that ATDC differentiated from bone marrow, were able to prolong skin, heart and pancreatic islet allograft survival in rodent models. Additionally, studies on NHP demonstrated the safety of this therapy before to move to human. In the translation to human, we first demonstrated that human ATDC (hATDC) similarly to their rodent homologues, displayed an immature dendritic cell phenotype based on a low expression of costimulatory molecules CD40, CD80 and CD86 and HLA-DR comparing to monocyte-derived DC classically differentiated with GM-CSF and IL-4 (MoDC). Moreover, hATDC present resistance to maturation following pro-inflammatory stimuli observed by the lack of upregulation of CD83 and most part of costimulatory molecules. Also, after LPS and IFNγ stimulation, hATDC secreted of IL-10, in similar quantities than MoDC, but lower amounts of pro-inflammatory cytokine IL-12p70. Finally, hATDC, contrary to MoDC, were able to impair $CD3^+$T-cell alloproliferation in mix lymphocyte reaction as well in a suppression assay using $CD4^+$T-cells, allogeneic mature DC and hATDC autologous to $CD4^+$T-cells. All these characteristics are typically observed in Tol-DC differentiated with different protocols.

hATDC differ from MoDC in terms of phenotype, transcriptome and metabolism

In our studies we also demonstrated that hATDC display a unique phenotype based on the expression of myeloid markers such as monocytes, macrophages and DC. We

observed that hATDC express classical MoDC markers such as CD11b, CD11c and CD206 but no DC-Sign (CD209), FceRI nor CD1a. Also, hATDC express different macrophage markers such as CD14, CD64 and CX3CRI. Apart from phenotype, we demonstrated that hATDC display a unique transcriptomic profile comparing to different myeloid populations. Moreover, we characterize hATDC metabolism and we observed a highly glycolytic and oxidative metabolism comparing to MoDC. Interestingly, their glycolytic potential during homeostasis is more close to M1 macrophages, inflammatory macrophages, than DC or M2 macrophages. Additionally we demonstrated that hATDC are highly dependent of lipid oxidative metabolism, supporting the 50% of oxidative metabolism during homeostasis. We propose that hATDC display a high metabolic profile due to the cell size and to a high expression of lipid receptors such as *Lox1* and *Cd36*. Indeed, it has been reported that big cells required more energy and lipids in order to regenerate membrane systems such as mitochondria, ER and Golgi. Additionally, the cell size also influenced the oxygen and nutrient diffusion, leading to a preferential glycolytic metabolism in big cells (279). This glycolytic metabolism is usually related to lipogenesis.

On the other hand, we do not find differences in the expression of lipogenic genes such as *Fas*, *Acly* nor *Acac* but we observed enormous endocytic vesicles near to the cytoplasmic membrane. We propose that these endocytic vesicles, due to their low electronic density could be macropinocytosis vesicles. We consider that from these endocytic pathway hATDC can uptake large amounts of lipids such as the ones we observed in confocal microscopy images. We also propose that the high expression of PDK, inhibitors of PDH, could maintain lipolysis and amino acids (Only those that do not enter in the mitochondria via pyruvate) oxidation preferential face to oxidative use of glucose. Another data that support the high use of FAO is the oxidative capacity in presence of rotenone. In fact, the inhibition of complex I abrogates completely the capacity of mitochondria to oxidize NADH, then the only source of electron to reduce O_2 to water is the $FADH_2$. This molecule is produced during the catalysis of succinate to fumarate in the Krebs cycle but also during β-oxidation (280). Interestingly, we are not the only group to observe a highly active metabolism in Tol-DC. In fact, it has been shown that Tol-DC differentiated with VitD3 and dexamethasone display a highly glycolytic and lypolytic metabolism(175). hATDC and possibly other Tol-DC seem to display a highly complex metabolism comparing to MoDC. Finally, such as lipid

metabolism corresponds to 50% of oxidative capacity and the steachiometry of glucose to lactate is near to 80% **(Equation 1)** we proposed that the other 50% of oxidative capacity can correspond mainly to amino-acid (Only those that integrate Kreb's cycle through pyruvate independent pathways) oxidation rather than glucose oxidation. On the other hand, little is known about Tol-DC TCA enzymatic activity. In fact if these cells are close to macrophages M1, we must consider the possibility that TCA is truncated. Truncation at SDH could lead to an accumulation of succinate supported by glutamine oxidation. As it was previously mentioned, succinate is a metabolite that can stabilize HIF1α, a subunity of a transcription factor related to oxidative glycolysis (165). On the other hand, lipids oxidation has been related to a decrease in HIF1α activity in cardiomyocytes (281). However, if we consider the same truncation than M1 macrophages, the lack of IDH can lead to an accumulation of citrate and an increase of itaconate, an inhibitor of SDH. In this way, lipid and amino acids oxidation could sustain SDH inhibition leading to an accumulation of succinate. Truncation of TCA in Tol-DC could explain the exacerbated glycolysis at steady state.

These results imply that despite the terminology of DC, hATDC display a unique phenotype and transcriptome. Moreover, we suggest that hATDC, Tol-MoDC differentiated with VitD3 and dexametahose and possibliy other Tol-MoDC displayed a highly active metabolism potentially related to their functions.

		Glucose →	2 Lactate	
	initial	9mM	2mM	
Theorical (for yield of 100%)	Final	2-3mM	14-16mM	100%
Observed	Final	2-3mM	12-14mM	80-90%

Equation 1.

hATDC are able to migrate and reach the spleen

In terms of migration skills, we observed that hATDC express the chemokine receptor CCR7 at steady state and can be upregulated after pro-inflammatory stimuli by different TLR ligands. These results suggest that hATDC can sense CCL19 and CCL21 and are potentially able to migrate to secondary lymphoid organs. This migratory capacity was verified in vivo in mice, in a GVHD model. In this model, NSG mice were injected intravenously with 5 millions of human PBMC and treated or not with hATDC autologous to PBMC. The therapeutic use of hATDC in these mice results in a delay of

GVHD leading to an increased survival, confirming the tolerogenic potential of hATDC in vivo. In order to inquire on the effect of hATDC in this model, we first analyzed their migratory skills. In this purpose, we tracked hATDC by labeling them with gold nanoparticles previously to injection. At different time points, mice were sacrificed and gold particles were detected in different tissues by mass spectometry. We observed an increase in gold particles in liver, lung and spleen at 3, 6, 10 and 14 days. The major gold quantification was found in spleen at day 6, suggesting that hATDC have the ability to migrate to spleen and potentially drive their suppressive activity in this organ. These observations are closely similar to the works performed by Mansilla María José et al. using BMDC differentiated with VitD3 and Garrod et al. with BMDC transfected with CCR7 and IL-10 genes.

hATDC impair CD4$^+$T-cell proliferation in vitro but no in vivo and induce regulatory CD4$^+$CD25$^+$FoxP3^{hi} in vivo and in vitro

Apart of the characterization and the validation of the immunoregulatory function of hATDC in vivo, this study also aimed to determine their cellular and molecular immunosuppressive mechanisms. First of all, we noticed differences between the immunosuppressive effects of hATDC in vivo and in vitro. In vitro, hATDC were able to impair CD4$^+$T-cell proliferation and the production of IFNγ and IL17-A. On the other hand in vivo no differences in proliferation were found on the engraftment nor in spleen after 21 days after PBMC injection. These results suggest that hATDC hypoproliferative potential depends of the context. In fact, we propose that strong allostimulating response can bypass the anti-proliferative activity of hATDC. On the other hand, in vivo as well in vitro, an increase in CD4$^+$CD25$^+$FoxP3^{hi} population was observed in presence of hATDC. Interestingly, contrary to proliferation, the induction of regulatory T-cells is not abrogated by a strong allostimulation. Moreover, we also observed in vitro, that hATDC were able to induce regulatory CD4$^+$CD25$^+$FoxP3^{hi} T-cells starting from CD4$^+$CD25$^-$T-cells (**Data not shown**). These results suggest that the increased survival in GVHD mice could be supported by an enhancement and induction of CD4$^+$Treg. On the other hand, the lack of hypoproliferative potential in mice GVHD model do not means that it cannot take place at short scale or even in the autologous context in transplanted patients.

hATDC immunosuppressive potential is driven by non conventional Tol-DC mechanisms

In order to deepen on the immunosuppressive mechanisms of hATDC toward T-cells, we were interested on how they inhibit $CD4^+$T-cell proliferation. The impairment in proliferation can be driven by different mechanisms, usually apoptosis or anergy induction. However, we demonstrated that hATDC led to a little increase of apoptosis and do not induce anergy, meaning that potentially none of these mechanisms are involved. We also demonstrated that in presence of hATDC there is a decrease in $CD4^+$Tcells reaching the S and G2/M phase of cell cycle. Altogether, due to the lack of apoptosis, no anergy and increased population at G1 phase, we propose that mechanism underlying the hypoproliferation lead by hATDC is related a hyporesponse to allogeneic stimuli.

Different mechanisms have been described for the immunosuppressive effect of Tol-DC such as TGFβ and IL-10 production, iNOS, Arg1, HO-1, IDO expression and contact dependent molecules such as ICOS-L, Fas-L among others. However, we demonstrated that the inhibition of these molecules with specific inhibitors or receptor inhibitors do not abrogated the immunosuppressive effects of hATDC. Moreover, by increasing the number of mature DC in allogeneic reaction we do not found any sign of competition suggesting that hATDC do not impaired $CD4^+$T-cell proliferation by contact competition. On the other hand, we demonstrated that the physical separation of hATDC from MLR led to immunosuppression, meaning that contact-dependent mechanisms are not involved. Additionally, supernatant obtained from coculture of hATDC with $CD4^+$T-cell and mature DC or hATDC cultured alone and transferred to a new MLR led to hypoproliferation. Moreover, supernatant transfer also led to an induction of $CD4^+CD25^+FoxP3^{hi}$ T-cells **(Data not shown)**. These results imply two possibilities, hATDC impaired T-cell proliferation by nutrient consumption or they secrete a suppressive factor. In order to reject the first hypothesis, we transfered hATDC-derived supernatant (hATDC-SN) into a new MLR and we added increasing doses of metabolic substrates such as glucose, glutamine and pyruvate. We observed no abrogation of hypoproliferation. Finally, in order to approach to the immunosuppressive factor, we filter the supernatant at 3KDa in order to separate small metabolites from big molecules such as proteins. We observed that the fraction under 3KDa was able to impair $CD4^+$T-cell proliferation but no fraction upon 3KDa. Altogether, these results

suggest that hATDC impair T-cell proliferation and increase regulatory T-cells through the suppressive effect of one or several molecules under 3KDa.

hATDC-derived lactic acid impair T-cell proliferation in vitro

Interestingly, we observed that after 6 days of culture, hATDC acidify medium as a consequence of a highly active metabolism. The acidification of medium is usually related to an increase of aerobic glycolysis in cells, resulting in a high production and exportation of lactate. After measure lactate and glucose in medium after 6 days of culture, we observed that hATDC consume high amounts of glucose and secrete high amounts of lactate comparing to MoDC. Additionally we observed an increased LDH activity in hATDC and an increased expression of the lactate/H symporter MCT1 **(Data not shown)**. Interestingly, during the last 10 years, research focused on lactate effect on the immune system has revealed his role as an immunosuppressive molecule. In fact, it has been shown that lactate can act through different mechanisms. For example, lactate can be uptake by cells by the lactate/H^+ symporter MCT1. This mechanism has been observed in cells able to oxidize lactate. Based on the fact that MCT1 is a bidirectional cotransporter, we assume that the export and import of lactate though $CD4^+$T-cells must be mediated by protons. Therefore, the decrease in proton concentration leads to a decrease in lactate transporter. This hypothesis was supported by the fact that we tested the effect of lactate, lactic acid and HCl at different concentration in MLR context and we observed that only lactic acid was able to impair $CD4^+$T-cell proliferation. Following these results, we demonstrated the neutralization of hATDC-SN with NaOH abrogated partially the proliferation inhibition. Additionally, we demonstrated by mass spectrometry that lactate from SN only can be uptake by $CD4^+$T-cells in acidic conditions but not neutralized with NaOH, as it was already observed by Fischer et al 2007 **(Data not shown)**. Interestingly, the entrance of lactate inside the cells also led to a decrease of glucose uptake. We hypothesize that the uptake of lactate can impair glycolytic flux by different mechanisms based on bibliography. Lactate can modify the LDH equilibrium leading to a decrease in lactate production, or at the same time, be oxydized to pyruvate and enter to Krebs cycle. After the analysis of Krebs metabolites, we observed a not significant increase in citrate **(Data not shown)**. More measures must be considered to validate or reject the significance of this result. Citrate can be used as lipogenic substrate but also can act as an inhibitor of PFK-1, the first irreversible enzyme of glycolysis. Additionally, the decrease in LDH activity can impair

the regeneration of NAD^+ leading to a decrease in glycolytic flux. Also, intracellular acidification affects negatively PFK-1. On the other hand, no differences in intracellular ATP production were found at 30minutes nor 1, 2 or 5h **(Data not shown)**, suggesting that lactate is potentially oxidized by Krebs cycle in order to compensate the decreased glycolytic flux and maintaining energetic requirements, or on the other hand compensating by a decrease in protein synthesis and ATP consuming reactions.

On the other hand, in order to abrogate the immunosuppressive potential of hATDC-derived lactate, other approaches apart from supernatant neutralization have been tested. Firstly, as lactate is synthesized through the LDH, we used two different inhibitors in order to reduce hATDC-derived lactate, the oxamic acid and GSK2837808A. Both inhibitors have been proved to decrease lactate production in several cancer lines (267,282). However, in hATDC both inhibitors have a very small effect on lactate production at low concentrations. At higher concentrations LDH inhibitors led to a dose dependent increase of hATDC apoptosis **(Data not shown)**. This datas suggest that LDH activity is essential for hATDC survival. The rational of the second approach was based on the modification of hATDC metabolism. Indeed, as hATDC are highly lipolytic, we wondered if the addition of lipids could decrease the glycolytic flux and reduce lactate production. Despite the lack or little effect on lactate production **(Data not shown)**, the addition of lipids into an MLR in presence of hATDC autolgous to $CD4^+$T-cells lead to a dose dependent decrease of their suppressive potential. On the other hand, lipid addition impaired $CD4^+$T-cell proliferation. We hypothezised that lipid intake by hATDC could modify the synthesis of immunosuppressive lactate-unrelated molecules. On the other hand, hATDC could intake lipids from media avoiding the lipotoxic effect on $CD4^+$T-cells. However, this mechanism remains hypothetical. Finally, another attempt in order to inhibit lactate effect on T-cells was to inhibit lactate transporters in hATDC. Unfortunately, MCT inhibitors, CHC and phloertin reduced dramatically hATDC survival **(Data not shown)**.

hATDC supernatant neutralization partially rescue Ifng expression and $CD4^+$T-cell allo-proliferation but not Foxp3, Tgfb expression nor $CD4^+CD25^+FoxP3^{hi}$ induction

Apart from proliferation, other functions can be affected by lactate in acid condition, such as gene expression. Interestingly, some gene expressions are affected by hATDC-SN and recovered in neutralizing conditions, meanwhile other genes are unaffected by

neutralization. These results indicate that there are genes that can potentially be inhibited by the increase of intracellular lactate and others that are independent of acidification. We observed that *Ifng* is a gene that can be downregulated by hATDC-SN but recovered partially in neutralizing conditions **(Data not shown)**. Interestingly, this result can be related with the work Peng et al. in 2016 demonstrating that during the $CD4^+$T-cell metabolic shift, LDH activity lead to an increase in acetyl-CoA driving to an increase in H3K9 acetylation and activation of *Ifng* expression. We propose that the impairment of LDH activity by the uptake of lactate can decrease acetyl-CoA cytoplasmic production leading to a decrease in H3K9 acetylaction and then inhibiting *Ifng* expression. On the other hand, it has been shown that the excess of GAPDH can bind *Ifng* mRNA, impairing his expression. As GAPDH is the enzyme that regenerates NADH from NAD^+ we also proposed that the lack of NAD^+ regeneration due to a less active LDH can impair GAPDH cytosolic activity leading to nucleus translocation (283). On the other hand, we observed other genes that are over-expressed in presence of hATDC-SN and lactic acid, but do not recover normal values by neutralization. *Foxp3* and *Tgfb* follow this pattern. In the case of *Tgfb*, we observed even an increased expression in neutralizing conditions. Due to the independence to acidification we proposed that the over-expression of these genes could be regulated differently. We hypothesize that acid independent effect could be lead by the activation of the lactate receptor Gpr81. Additionally, this effect independent from acidification has also been observed in $CD4^+CD25^+FoxP3^{hi}$ differentiation. In parallel, we observed that lactate, lactic acid and HCl, lead to an increase of Treg. Moreover we detected a synergistic effect between acidification and lactate suggesting two different action mechanisms to induce Treg. As it was observed in *FoxP3* and *Tgfb* expression, hATDC neutralization do not abrogate Treg proliferation. In fact, we also demonstrated that hATDC-SN increased Treg expansion rate from $CD4^+CD25^+CD127^-$ natural Treg without affecting to the phenotype or function. These results suggest that ATDC-SN apart from lactate and protons, they secrete other unidentified molecules that can be more involved on the inhibition of proliferation than in Treg induction.

hATDC supernatants impairs ROS production and mitochondriogenesis in $CD4^+$T-cells

Other effects independent from acidification observed during $CD4^+$T-cell activation were a decrease in ROS production and a decrease in mitochondrial content **(Data not shown)**. During $CD4^+$T-cell activation, an increase of ROS is observed due to an

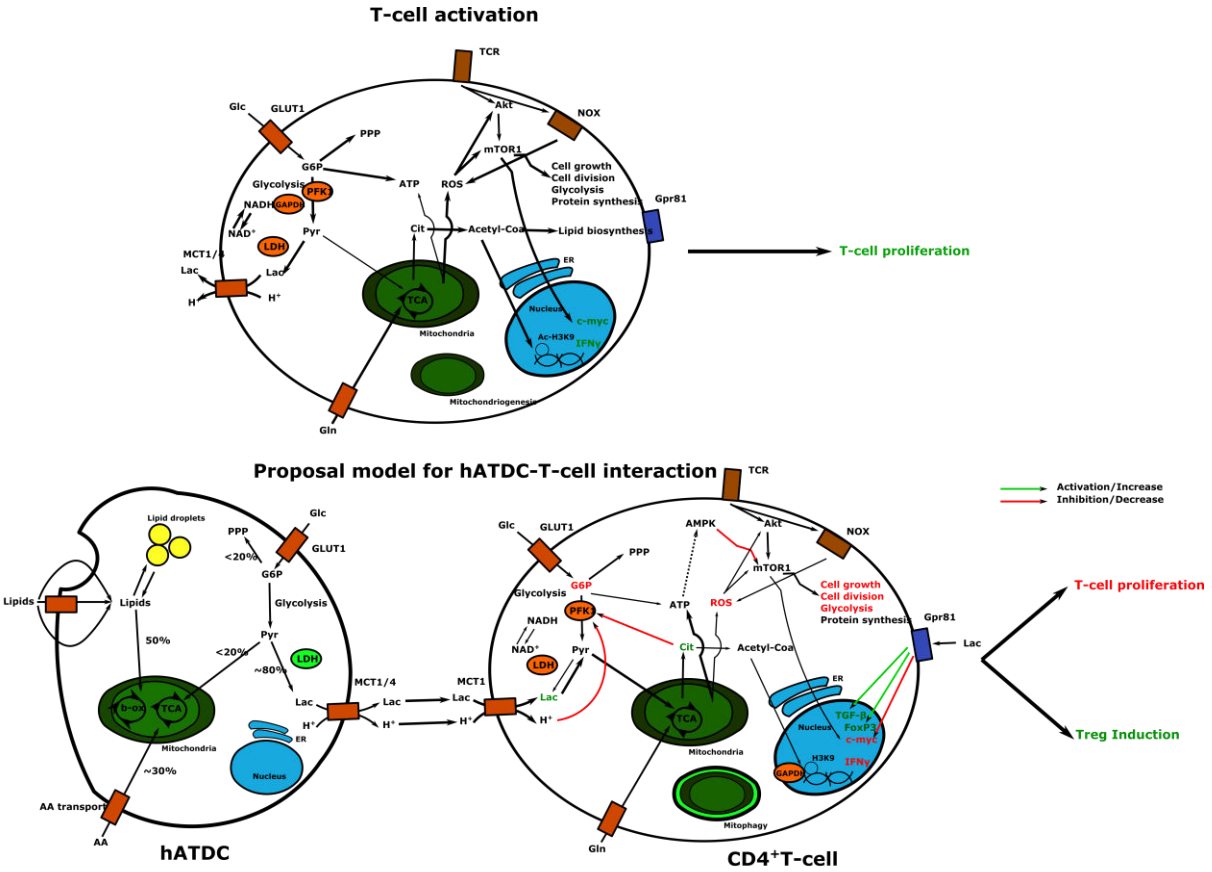
increase of metabolic activity but also due to NOX activation. A decrease of ROS during stimulation has been observed that lead to an impaired T-cell activation. On the other hand, a decrease in mitochondrial content driven by excess of mitophagy can lead to a decrease in T-cell activation as it has been observed in tumour microenvironment (284). Interestingly, there are several metabolic regulators that can lead to an increase in autophagy and mitophagy such as AMPK. We propose that the decrease in glycolytic ATP production can lead to an increase in AMP. AMP can be then sensed by AMPK impairing mTOR1 activation and leading to an increase of mitophagy and a decrase in protein synthesis and cytokines production. On the other hand, we propose that the decrease of ROS during activation can lead to an inefficient activation of Akt resulting in T-cell hyporesponsiveness. However, these hypotheses must be confirmed by checking the phosphorilation state of protein kinases form different pathways.

hATDC increase serum lactate in vivo in NSG mice in GVHD model

On the other hand, in vivo we do not find this immunosuppressive effect mediated by hypoproliferation. However, we observed in vitro as well as in vivo an increase in $CD4^{+}$Treg. As we previously report, lactate can enhance Treg differentiation in a dose dependent response. Interestingly, we demonstrated an increase in serum lactate in mice treated with hATDC. According to the results demonstrated in hATDC migration, we propose that hATDC migrate to different organs and secrete high amounts of lactate that be removed with the same kinetic. Additionally, as hATDC migrate preferentially to the spleen, we propose that the Treg induction is related to a high lactate production in this organ. On the other hand we think that the allogeneic response is enoughtly strong to bypass the hypoproliferative effect in T-cells in vivo. In order to prove this hypothesis, it will be necessary to identify if lactate can enter inside T-cells in spleen or lactate can act through Gpr81. Our results can be related to those observed by Angelin A. et al. in 2017. In their work, the authors demonstrated that the treatment with sodium lactate and rapamycin lead to an increase in cardiac allograft in transplantation model in mice. In contrast with our work, the authors observed hypoproliferation of effector T-cells and increase of Treg in the allograft meanwhile in GVHD model we observed an increase of Treg but not a decrease in T-cell proliferation. Altogether these results suggest that lactate displays an immunosuppressive potential in vivo that invite to be investigated in other mice models.

In conclusion, in this work we demonstrated that hATDC are Tol-DC with an unique phenotype and transcriptome comparing to other myeloid population. Additionally we demonstrated that hATDC display a highly active metabolism dependent from both glucose and lipids that results in a high production and export of lactate and protons. We also demonstrated that lactate derived from hATDC can be uptake by $CD4^{+}$T-cells leading to a decrease in their metabolic flux, activation and proliferation **(Model)**. Additionally, we demonstrated that hATDC enhances the expansion and induces de novo $CD4^{+}CD25^{+}FoxP3^{hi}$ Treg independently from the media acidification. These results imply that the hypoproliferation and Treg expansion are potentially driven by two different mechanisms. On the other hand, as the neutralization of lactic acid entrance just abrogated the 50% of suppressive potential, other factors under 3KDa must be involved. Finally, we demonstrated that hATDC can increase survival in GVHD mice model by producing high amounts of lactic acid in lymphoid organs such as the spleen. In this work we suggest for the first time, a novel mechanism in cell based therapy driven by metabolic interaction between Tol-DC and $CD4^{+}$T-cells.

Graphic proposal model



Model . Proposal model for hATDC-CD4$^+$T-cell interaction through lactate. In this model we propose that hATDC impair T-cell proliferation and induce regulatory T-cell through lactate synthesis. hATDC display a highly oxidative and glycolytic metabolism. Lipid and amino-acids oxidation mainly support the oxidative capacity of these cells meanwhile glucose is mainly derived to aerobic glycolysis. From this aerobic glycolysis, a high amount of lactate is produced. Lactate exported through MCT1 can be uptake by T-cells only in presence of acidic medium by MCT1. Lactate intake lead to changes in LDH activity leading to a decrease in lactate synthesis and consequently to a decrease in glycolytic flux and glucose intake. Also, lactate can be oxidized through mitochondria and be derived to citrate. This citrate can also regulate negatively glycolysis by reducing PFK-1 activity. On the other hand, changes in glycolytic metabolism can affect AMPK activity leading to an inhibition of mTOR1, favoring mitophagy. In consequence inhibition of NOX activity and mitochondrial ROS production lead to a reduced ROS formation. Also the decreased LDH activity and NAD^+ synthesis can lead to a reduced activity of GAPDH. GAPDH can then be translocated to the nucleus and inhibits *Ifng* transcription. On the other hand, the decreased LDH activity also can lead to a decrease in citrate to acetyl-CoA synthesis in cytoplasm and in consequence impair hystone acetylation at the *Ifng* promoter. Finally, Lactate can be sensed by Gpr81. The signaling through this receptor reduces cAMP and lead to an impairment of *c-myc* expression and an increase in *Foxp3* and *Tgfb* expression. On the other hand, Gpr81 is associated to lipolysis inhibition. We propose that Gpr81 signalling can be related to effector T-cell impairment and Treg induction through a decrease in general metabolism supported by lactic acid oxidation.

Therapeutic interest

Tumour microenvironment is able to induce regulatory cells that sustain tumour survival and at the same time is able to inhibit directly anti-tumour response. Despite that lactate is not the only molecule secreted by tumour cells, there are the more and more evidences that make incontestable his contribution in tumour survival. In fact, the inhibition of LDH in several cancer cell lines with small molecules or genetic modification results in a decreased cancer growing and an increase in anti-tumoral response. Considering tumours as dependent tissues with extraordinary abilities to survive and avoid immune response, we contemplate the possibility that cells mimicking tumour mechanisms can be a potent strategy for autoimmune diseases or

transplantation. In this work we demonstrated that hATDC are able to migrate and reach lymphoid organs such as the spleen and to secrete high amounts of lactate in vitro and in vivo without the requierement of pro-inflammatory signals. Additionally, we demonstrated that hATDC supernatant, similarly to lactic acid, was able to inhibit T-cell proliferation and to induce regulatory T-cells. Importantly, in order to disambiguate with tumour cells, we demonstrated in pre-clinical tests that hATDC do not display teratogenic potential and they are not proliferating cells. In conclusion, we consider that this new approach of tumour microenvironment-mimicking cell therapy can be a potent therapeutic strategy to treat autoimmune diseases and to use in transplantation.

Perspectives

The perspectives of this work will try to respond the following questions; Why hATDC are able to produce high amounts of lactate in steady state? Which types of molecules under 3KDa with immunosuppressive activity are released by hATDC apart from lactate? How T-cells and other immune cells can be affected functionally by hATDC immunoregulatory molecules (Lactate and unknown molecules) and through which mechanisms? Can lactate and these unknown molecules be beneficial in vivo in transplantation? Which is the role of Gpr81 in T-cells and regulatory T-cells in humans?

Possible experiments to answer these questions are detailed below:

Why hATDC are able to produce high amounts of lactate in steady state?

- To identify the expression of TCA enzymes and TCA metabolites with MS in order to verfy the integrity or the truncation of TCA.
- To identify potential constitutively activated signaling pathways related to metabolism such as AMPK, Akt, mTOR1, PPAR family, among others. Activation by phosphorylation of these pathways can be detected with western blot or flow cytometry.
- To check mitochondrial architecture and proximity to cytoplasmic membrane and test how fission, fussion, mitochondriogenic and mitophagic stimuli can affect oxidative glycolysis and lipid oxidation. These experiments can be performed using mitotracker and confocal microscopy. To check the role of mitochondria in hATDC can be performed by Seahorse experiments.

- To verify the presence of macropinocytic vesicles and their potential role in lipid oxidation and oxidative glycolysis. Presence of macropinocytic vesicles must be detected by electronic microscopy and confocal microscopy. To check the contribution of this endocytic pathway, endocytosis inhibitors can be used during Seahorse experiments

Which types of molecules under 3KDa with immunosuppressive activity are released by hATDC apart from lactate?

- To fraction and test hypostimulatory activity of hATDC supernatant in MLR context.
- To perform MS in positive fractions of hATDC supernatant

How T-cells and other immune cells can be affected functionally by hATDC immunoregulatory molecules (Lactate and unknown molecules) and through which mechanisms?

- To check the effect of hATDC, lactic acid and lactate in different immune population. The effects can be identified by cytokines secretion, cytotoxicity, mobility, apoptosis, etc…
- To analyze with RNAseq the differential gene expression between different immune populations stimulated in presence of hATDC supernatant, lactic acid and lactate. In this way, we can identify which genes are related to lactate, lactic acid acid and which are related to other potential immunosuppressive molecules.
- To identify signaling pathways in different immune population activated or inhibited in presence of hATDC supernatant, lactate and lactic acid. In this way we can identify which signaling pathways are related to lactate, lactic acid and which are related to other immunosuppressive molecules.
- To identify metabolites from glycolysis and TCA in different immune population stimulated in presence of hATDC supernatant, lactate and lactic acid. In this way we can identify how metabolism can be affected by lactate, lactic acid or other immunosuppressive molecules.

Can lactate and these unknown molecules be beneficial in vivo in transplantation?

As immunosuppressive molecules from ATDC-SN are not known and his effect was only observed in vitro, it is primordial to evaluate his effects and safety in animal models. However, as well as ATDC-SN is obtained from human cells cultured in RPMI medium supplemented with fetal calf serum, several details must be considered. The following experiments will allow validating molecular candidates for human perspectives.

*In order to avoid the injection of fetal calf serum (of unknown composition) it is primordial to detect positive immunosuppressive fractions (previous perspectives) and then to lyophilize.

*It is also essential verfy the hATDC supernatant activity in different types of immune murine cells.

- To check the safety of lactate and fractioned supernatant in untreated mice with different doses and injection route.
- To evaluate the effect of lactate and fractioned supernatant in different models of transplantation in mice (Minor mismatch and allogeneic skin transplantation). The effects can be evaluated by allograft survival, pro-inflammatory cytokines expression in the allograft, skin and lymphnodes, lymphocyte infiltration in the allograft, among other assays.

Which is the role of Gpr81 in T-cells and regulatory T-cells in humans?

- To transfect $CD4^{+}$T-cells and regulatory T-cells with shRNA anti Gpr81 and to perform functional tests.
- To determine the contribution of Gpr81 in T-cells stimulated with lactate, lactic acid and hATDC supernatant. In these experiments, mRNA, ROS, mitochondrial mass and activation of signaling pathways can be performed. Metabolites and metabolism analysis can be interesting too.

Bibliography

1. Barker CF, Markmann JF. Historical Overview of Transplantation. Cold Spring Harbor Perspectives in Medicine. 2013 Apr 1;3(4):a014977–a014977.

2. Merrill JP, Murray JE, Harrison J, Guild WR. Successful homotransplantation of the human kidney between identical twins. JAMA. 1984 May 18;251(19):2566–71.

3. Schwartz R, Eisner A, Dameshek W. The Effect of 6-Mercaptopurine on Primary and Secondary Immune Responses. Journal of Clinical Investigation. 1959 Aug 1;38(8):1394–403.

4. Cooper DKC. A Brief History of Cross-Species Organ Transplantation. Baylor University Medical Center Proceedings. 2012 Jan;25(1):49–57.

5. Dalal P, Shah G, Chhabra D, Gallon L. Role of tacrolimus combination therapy with mycophenolate mofetil in the prevention of organ rejection in kidney transplant patients. International Journal of Nephrology and Renovascular Disease. 2010.

6. Artero M. The role of basiliximab in the evolving renal transplantation immunosuppression protocol. Biologics: Targets & Therapy. 2008 Jun;175.

7. Moreau A, Varey E, Anegon I, Cuturi M-C. Effector Mechanisms of Rejection. Cold Spring Harbor Perspectives in Medicine. 2013 Nov 1;3(11):a015461–a015461.

8. Land WG, Agostinis P, Gasser S, Garg AD, Linkermann A. Transplantation and Damage-Associated Molecular Patterns (DAMPs). American Journal of Transplantation. 2016 Dec;16(12):3338–61.

9. Harper SJF, Ali JM, Wlodek E, Negus MC, Harper IG, Chhabra M, et al. CD8 T-cell recognition of acquired alloantigen promotes acute allograft rejection. Proceedings of the National Academy of Sciences. 2015 Oct 13;112(41):12788–93.

10. Smyth L, Barroso O, Golshayan D, Lombardi G, Lechler R. A Novel Pathway of Antigen Presentation by Dendritic and Endothelial Cells: Implications for Allorecognition and Infectious Diseases. Transplantation. 2006.

11. Karahan GE, Claas FHJ, Heidt S. B Cell Immunity in Solid Organ Transplantation. Frontiers in Immunology. 2017

12. Libby P, Pober JS. Chronic Rejection. Immunity. 2001 Apr 1;14(4):387–97.

13. Farkona S, Diamandis EP, Blasutig IM. Cancer immunotherapy: the beginning of the end of cancer? BMC Medicine. 2016

14. Gliwiński M, Iwaszkiewicz-Grześ D, Trzonkowski P. Cell-Based Therapies with T Regulatory Cells. BioDrugs. 2017 Aug;31(4):335–47.

15. Skuljec J, Chmielewski M, Happle C, Habener A, Busse M, Abken H, et al. Chimeric Antigen Receptor-Redirected Regulatory T Cells Suppress Experimental Allergic Airway Inflammation, a Model of Asthma. Frontiers in Immunology. 2017

16. Dahlberg CIM, Sarhan D, Chrobok M, Duru AD, Alici E. Natural Killer Cell-Based Therapies Targeting Cancer: Possible Strategies to Gain and Sustain Anti-Tumor Activity. Frontiers in Immunology. 2015

17. Mandal A, Viswanathan C. Natural killer cells: In health and disease. Hematology/Oncology and Stem Cell Therapy. 2015 Jun;8(2):47–55.

18. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nature Reviews Immunology. 2005 Apr;5(4):296–306.

19. Marín E, Cuturi MC, Moreau A. Tolerogenic Dendritic Cells in Solid Organ Transplantation: Where Do We Stand? Frontiers in Immunology. 2018

20. Wei X, Yang X, Han Z, Qu F, Shao L, Shi Y. Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacologica Sinica. 2013 Jun;34(6):747–54.

21. Fleming BD, Mosser DM. Regulatory macrophages: Setting the Threshold for Therapy. European Journal of Immunology. 2011 Sep;41(9):2498–502.

22. Franquesa M, Hoogduijn MJ, Reinders ME, Eggenhofer E, Engela AU, Mensah FK, et al. Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Fourth Meeting: Lessons Learned from First Clinical Trials. Transplantation Journal. 2013 Aug;96(3):234–8.

23. Tan J, Wu W, Xu X, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: A randomized controlled trial. JAMA. 2012 Mar 21;307(11):1169–77.

24. Hutchinson JA, Riquelme P, Sawitzki B, Tomiuk S, Miqueu P, Zuhayra M, et al. Cutting Edge: Immunological Consequences and Trafficking of Human Regulatory Macrophages Administered to Renal Transplant Recipients. The Journal of Immunology. 2011 Sep 1;187(5):2072–8.

25. Riquelme P, Haarer J, Kammler A, Walter L, Tomiuk S, Ahrens N, et al. TIGIT+ iTregs elicited by human regulatory macrophages control T cell immunity. Nature Communications. 2018

26. Todo S, Yamashita K, Goto R, Zaitsu M, Nagatsu A, Oura T, et al. A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. Hepatology. 2016 Aug;64(2):632–43.

27. Geissler EK. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. Transplant Res. 2012;3.

28. Steinman RM. Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice: I. Morpholog, Quantitation, Tissue Distribution. Journal of Experimental Medicine. 1973 May 1;137(5):1142–62.

29. Steinman RM. Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice: II. Functional Properties in Vitro. Journal of Experimental Medicine. 1974 Feb 1;139(2):380–97.

30. Hubo M, Trinschek B, Kryczanowsky F, Tuettenberg A, Steinbrink K, Jonuleit H. Costimulatory Molecules on Immunogenic Versus Tolerogenic Human Dendritic Cells. Frontiers in Immunology. 2013

31. Mbongue J, Nicholas D, Firek A, Langridge W. The Role of Dendritic Cells in Tissue-Specific Autoimmunity. Journal of Immunology Research. 2014;2014:1–17.

32. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nature Reviews Immunology. 2012 Jul 13;12:557.

33. Mildner A, Jung S. Development and Function of Dendritic Cell Subsets. Immunity. 2014 May;40(5):642–56.

34. Bluestone JA. Mechanisms of tolerance: Mechanisms of Tolerance. Immunological Reviews. 2011 May;241(1):5–19.

35. Ohnmacht C, Pullner A, King SBS, Drexler I, Meier S, Brocker T, et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. The Journal of Experimental Medicine. 2009 Mar 16;206(3):549–59.

36. Chang S-Y, Ko H-J, Kweon M-N. Mucosal dendritic cells shape mucosal immunity. Experimental & Molecular Medicine. 2014 Mar;46(3):e84–e84.

37. Suter T, Biollaz G, Gatto D, Bernasconi L, Herren T, Reith W, et al. The brain as an immune privileged site: dendritic cells of the central nervous system inhibit T cell activation. European Journal of Immunology. 2003 Nov;33(11):2998–3006.

38. Gaudreau S, Guindi C, Menard M, Besin G, Dupuis G, Amrani A. Granulocyte-Macrophage Colony-Stimulating Factor Prevents Diabetes Development in NOD Mice by Inducing Tolerogenic Dendritic Cells that Sustain the Suppressive Function of CD4+CD25+ Regulatory T Cells. The Journal of Immunology. 2007 Sep 15;179(6):3638–47.

39. Tisch R. Immunogenic Versus Tolerogenic Dendritic Cells: A Matter of Maturation. International Reviews of Immunology. 2010 Mar;29(2):111–8.

40. Arellano. Clinical uses of GM-CSF, a critical appraisal and update. Biologics: Targets & Therapy. 2008 Mar;13.

41. Shiomi A, Usui T, Mimori T. GM-CSF as a therapeutic target in autoimmune diseases. Inflammation and Regeneration. 2016

42. Lutz MB, Suri RM, Niimi M, Ogilvie ALJ, Kukutsch NA, Rößner S, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survivalin vivo. European Journal of Immunology. 2000 Jul;30(7):1813–22.

43. Yoo S, Ha S-J. Generation of Tolerogenic Dendritic Cells and Their Therapeutic Applications. Immune Network. 2016;16(1):52.

44. Gadani SP, Cronk JC, Norris GT, Kipnis J. IL-4 in the Brain: A Cytokine To Remember. The Journal of Immunology. 2012 Nov 1;189(9):4213–9.

45. Maroof A, Penny M, Kingston R, Murray C, Islam S, Bedford PA, et al. Interleukin-4 can induce interleukin-4 production in dendritic cells. Immunology. 2006 Feb;117(2):271–9.

46. La Flamme AC, Kharkrang M, Stone S, Mirmoeini S, Chuluundorj D, Kyle R. Type II-Activated Murine Macrophages Produce IL-4. Bereswill S, editor. PLoS ONE. 2012 Oct 5;7(10):e46989.

47. Sehra S, Yao Y, Howell MD, Nguyen ET, Kansas GS, Leung DYM, et al. IL-4 Regulates Skin Homeostasis and the Predisposition toward Allergic Skin Inflammation. The Journal of Immunology. 2010 Mar 15;184(6):3186–90.

48. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. Nature Reviews Immunology. 2007 Jul 13;7:610.

49. Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory Activity of Autocrine IL-10 on Dendritic Cell Functions. The Journal of Immunology. 2001 Apr 1;166(7):4312–8.

50. Strobl H, Knapp W. TGF-β1 regulation of dendritic cells. Microbes and Infection. 1999 Dec 1;1(15):1283–90.

51. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. Blood. 2010 Aug 12;116(6):935–44.

52. Torres-Aguilar H, Sánchez-Torres C, Jara LJ, Blank M, Shoenfeld Y. IL-10/TGF-β-Treated Dendritic Cells, Pulsed with Insulin, Specifically Reduce the Response to Insulin of CD4+ Effector/Memory T Cells from Type 1 Diabetic Individuals. Journal of Clinical Immunology. 2010 Sep;30(5):659–68.

53. Penna G, Adorini L. 1 ,25-Dihydroxyvitamin D3 Inhibits Differentiation, Maturation, Activation, and Survival of Dendritic Cells Leading to Impaired Alloreactive T Cell Activation. The Journal of Immunology. 2000 Mar 1;164(5):2405–11.

54. Unger WWJ, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D_3 or dexamethasone: Differential role for PD-L1. European Journal of Immunology. 2009 Nov;39(11):3147–59.

55. Trabanelli S, Lecciso M, Salvestrini V, Cavo M, Očadlíková D, Lemoli RM, et al. PGE_2-Induced IDO1 Inhibits the Capacity of Fully Mature DCs to Elicit an *In Vitro* Antileukemic Immune Response. Journal of Immunology Research. 2015;2015:1–10.

56. Naranjo-Gómez M, Raïch-Regué D, Oñate C, Grau-López L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. Journal of Translational Medicine. 2011;9(1):89.

57. Jauregui-Amezaga A, Cabezón R, Ramírez-Morros A, España C, Rimola J, Bru C, et al. Intraperitoneal Administration of Autologous Tolerogenic Dendritic Cells for Refractory Crohn's Disease: A Phase I Study. Journal of Crohn's and Colitis. 2015 Dec;9(12):1071–8.

58. Machen J, Harnaha J, Lakomy R, Styche A, Trucco M, Giannoukakis N. Antisense Oligonucleotides Down-Regulating Costimulation Confer Diabetes-Preventive Properties

to Nonobese Diabetic Mouse Dendritic Cells. The Journal of Immunology. 2004 Oct 1;173(7):4331–41.

59. Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (Safety) Study of Autologous Tolerogenic Dendritic Cells in Type 1 Diabetic Patients. Diabetes Care. 2011 Sep 1;34(9):2026–32.

60. Chitta S, Santambrogio L, Stern LJ. GMCSF in the absence of other cytokines sustains human dendritic cell precursors with T cell regulatory activity and capacity to differentiate into functional dendritic cells. Immunology Letters. 2008 Feb 15;116(1):41–54.

61. Moreau A, Varey E, Bouchet-Delbos L, Cuturi M-C. Cell therapy using tolerogenic dendritic cells in transplantation. Transplantation Research. 2012;1(1):13.

62. Ingulli E. Mechanism of cellular rejection in transplantation. Pediatric Nephrology. 2010 Jan;25(1):61–74.

63. Rainienė T, Izvolskaja N, Dainys B, Kučinskis G, Razukas V. Donor-specific transfusions as a way of tolerance induction to living donor kidney transplant. Biologija. 2009 Jan 1;55(3):99–104.

64. Yabu JM, Anderson MW, Kim D, Bradbury BD, Lou CD, Petersen J, et al. Sensitization from transfusion in patients awaiting primary kidney transplant. Nephrology Dialysis Transplantation. 2013 Nov 1;28(11):2908–18.

65. A DePaz H, O Oluwole O, O Adeyeri A, Witkowski P, Jin M-X, Hardy M, et al. Immature rat myeloid dendritic cells generated in low-dose granulocyte macrophage-colony stimulating factor prolong donor-specific rat cardiac allograft survival. Transplantation. 2003.

66. Divito SJ, Wang Z, Shufesky WJ, Liu Q, Tkacheva OA, Montecalvo A, et al. Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. Blood. 2010 Oct 14;116(15):2694–705.

67. Smyth LA, Ratnasothy K, Moreau A, Alcock S, Sagoo P, Meader L, et al. Tolerogenic Donor-Derived Dendritic Cells Risk Sensitization In Vivo owing to Processing and Presentation by Recipient APCs. The Journal of Immunology. 2013 May 1;190(9):4848–60.

68. Peche H, Trinite B, Martinet B, Cuturi MC. Prolongation of Heart Allograft Survival by Immature Dendritic Cells Generated from Recipient Type Bone Marrow Progenitors. American Journal of Transplantation. 2005 Feb;5(2):255–67.

69. Bériou G, Pêche H, Guillonneau C, Merieau E, Cuturi M. Donor-Specific Allograft Tolerance by Administration of Recipient-Derived Immature Dendritic Cells and Suboptimal Immunosuppression. Transplantation. 2005.

70. Chiffoleau E, Beriou G, Dutartre P, Usal C, Soulillou J-P, Cuturi MC. Induction of Donor-Specific Allograft Tolerance by Short-Term Treatment with LF15-0195 After Transplantation. Evidence for a Direct Effect on T-Cell Differentiation. American Journal of Transplantation. 2002 Sep;2(8):745–57.

71. Chatenoud L. CD3-specific antibody-induced active tolerance: from bench to bedside. Nature Reviews Immunology. 2003 Feb 1;3:123.

72. You S, Zuber J, Kuhn C, Baas M, Valette F, Sauvaget V, et al. Induction of Allograft Tolerance by Monoclonal CD3 Antibodies: A Matter of Timing: Transplant Tolerance by CD3 Antibodies. American Journal of Transplantation. 2012 Nov;12(11):2909–19.

73. Baas MC, Kuhn C, Valette F, Mangez C, Duarte MS, Hill M, et al. Combining Autologous Dendritic Cell Therapy with CD3 Antibodies Promotes Regulatory T Cells and Permanent Islet Allograft Acceptance. The Journal of Immunology. 2014 Nov 1;193(9):4696–703.

74. Segovia M, Louvet C, Charnet P, Savina A, Tilly G, Gautreau L, et al. Autologous Dendritic Cells Prolong Allograft Survival Through *Tmem176b* -Dependent Antigen Cross-Presentation: Immunoregulatory Mechanisms of Autologous DCs. American Journal of Transplantation. 2014 May;14(5):1021–31.

75. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-Conditioned Dendritic Cells Are Poor Stimulators of Allogeneic CD4+ T Cells, but Enrich for Antigen-Specific Foxp3+ T Regulatory Cells and Promote Organ Transplant Tolerance. The Journal of Immunology. 2007 Jun 1;178(11):7018–31.

76. Ezzelarab MB, Zahorchak AF, Lu L, Morelli AE, Chalasani G, Demetris AJ, et al. Regulatory Dendritic Cell Infusion Prolongs Kidney Allograft Survival in Nonhuman Primates: Dendritic Cell Therapy of Graft Rejection. American Journal of Transplantation. 2013 Aug;13(8):1989–2005.

77. Ezzelarab MB, Raich-Regue D, Lu L, Zahorchak AF, Perez-Gutierrez A, Humar A, et al. Renal Allograft Survival in Nonhuman Primates Infused With Donor Antigen-Pulsed Autologous Regulatory Dendritic Cells. American Journal of Transplantation. 2017 Jun;17(6):1476–89.

78. Moreau A, Vandamme C, Segovia M, Devaux M, Guilbaud M, Tilly G, et al. Generation and in vivo evaluation of IL10-treated dendritic cells in a nonhuman primate model of AAV-based gene transfer. Molecular Therapy - Methods & Clinical Development. 2014;1:14028.

79. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype–positive rheumatoid arthritis patients. Science Translational Medicine. 2015 Jun 3;7(290):290ra87.

80. Bell GM, Anderson AE, Diboll J, Reece R, Eltherington O, Harry RA, et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. Annals of the Rheumatic Diseases. 2017 Jan;76(1):227–34.

81. Alvarez D, Vollmann EH, von Andrian UH. Mechanisms and Consequences of Dendritic Cell Migration. Immunity. 2008 Sep;29(3):325–42.

82. Mansilla María José, Sellès-Moreno Carla, Fàbregas-Puig Sara, Amoedo Joan, Navarro-Barriuso Juan, Teniente-Serra Aina, et al. Beneficial Effect of Tolerogenic Dendritic Cells Pulsed with MOG Autoantigen in Experimental Autoimmune Encephalomyelitis. CNS Neuroscience & Therapeutics. 2014 Nov 18;21(3):222–30.

83. Garrod KR, Chang CK, Liu F-C, Brennan TV, Foster RD, Kang S-M. Targeted Lymphoid Homing of Dendritic Cells Is Required for Prolongation of Allograft Survival. The Journal of Immunology. 2006 Jul 15;177(2):863–8.

84. Svajger U, Rozman P. Tolerogenic dendritic cells: molecular and cellular mechanisms in transplantation. Journal of Leukocyte Biology. 2014 Jan 1;95(1):53–69.

85. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature Reviews Immunology. 2013 Apr;13(4):227–42.

86. Tuettenberg A, Huter E, Hubo M, Horn J, Knop J, Grimbacher B, et al. The Role of ICOS in Directing T Cell Responses: ICOS-Dependent Induction of T Cell Anergy by Tolerogenic Dendritic Cells. The Journal of Immunology. 2009 Mar 15;182(6):3349–56.

87. Sandner SE, Clarkson MR, Salama AD, Sanchez-Fueyo A, Domenig C, Habicht A, et al. Role of the Programmed Death-1 Pathway in Regulation of Alloimmune Responses In Vivo. The Journal of Immunology. 2005 Mar 15;174(6):3408–15.

88. Tanaka K, Albin MJ, Yuan X, Yamaura K, Habicht A, Murayama T, et al. PDL1 Is Required for Peripheral Transplantation Tolerance and Protection from Chronic Allograft Rejection. The Journal of Immunology. 2007 Oct 15;179(8):5204–10.

89. Peng W, Ran B, Ma Y, Huang X, Chang Q, Wang X. Dendritic cells transfected with PD-L1 recombinant adenovirus induces T cell suppression and long-term acceptance of allograft transplantation. Cellular Immunology. 2011 Jan 1;271(1):73–7.

90. Min W-P, Gorczynski R, Huang X-Y, Kushida M, Kim P, Obataki M, et al. Dendritic Cells Genetically Engineered to Express Fas Ligand Induce Donor-Specific Hyporesponsiveness and Prolong Allograft Survival. The Journal of Immunology. 2000 Jan 1;164(1):161–7.

91. Wu J, Horuzsko A. Expression and function of immunoglobulin-like transcripts on tolerogenic dendritic cells. Human Immunology. 2009 May;70(5):353–6.

92. Amodio G, Gregori S. Human tolerogenic DC-10: perspectives for clinical applications. Transplantation Research. 2012;1(1):14.

93. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med. 1996 Jul 1;184(1):19.

94. Wan YY, Flavell RA. TGF-β and Regulatory T Cell in Immunity and Autoimmunity. Journal of Clinical Immunology. 2008 Nov;28(6):647–59.

95. Horton C, Shanmugarajah K, Fairchild PJ. Harnessing the properties of dendritic cells in the pursuit of immunological tolerance. Biomedical Journal. 2017 Apr;40(2):80–93.

96. Texier LL, Thebault P, Carvalho-Gaspar M, Vignard V, Merieau E, Usal C, et al. Immunoregulatory Function of IL-27 and TGF-β1 in Cardiac Allograft Transplantation. Transplantation Journal. 2012 Aug;94(3):226–33.

97. Dixon KO, van der Kooij SW, Vignali DAA, van Kooten C. Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members: Immunomodulation. European Journal of Immunology. 2015 Jun;45(6):1736–47.

98. Goriely S, Goldman M. The Interleukin-12 Family: New Players in Transplantation Immunity? American Journal of Transplantation. 2007 Feb;7(2):278–84.

99. Morishima N, Owaki T, Asakawa M, Kamiya S, Mizuguchi J, Yoshimoto T. Augmentation of Effector CD8+ T Cell Generation with Enhanced Granzyme B Expression by IL-27. The Journal of Immunology. 2005 Aug 1;175(3):1686–93.

100. Hill M, Thebault P, Segovia M, Louvet C, Bériou G, Tilly G, et al. Cell Therapy With Autologous Tolerogenic Dendritic Cells Induces Allograft Tolerance Through Interferon-Gamma and Epstein-Barr Virus-Induced Gene 3: Cell Therapy With TolDC. American Journal of Transplantation. 2011 Oct;11(10):2036–45.

101. Durante W. Role of Arginase in Vessel Wall Remodeling. Frontiers in Immunology. 2013

102. Rath M, Müller I, Kropf P, Closs EI, Munder M. Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages. Frontiers in Immunology. 2014

103. Bhatt S, Qin J, Bennett C, Qian S, Fung J, Hamilton T, et al. All-Trans Retinoic Acid Induces Arg-1 and iNOS Producing Dendritic Cells With T Cell Inhibitory Function. Transplantation. 2014 Jul;98:19.

104. Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, et al. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. European Journal of Immunology. 2007 Nov;37(11):3054–62.

105. Terness P, Bauer TM, Röse L, Dufter C, Watzlik A, Simon H, et al. Inhibition of Allogeneic T Cell Proliferation by Indoleamine 2,3-Dioxygenase–expressing Dendritic Cells: Mediation of Suppression by Tryptophan Metabolites. The Journal of Experimental Medicine. 2002 Aug 19;196(4):447–57.

106. Li C, Liu T, Zhao N, Zhu L, Wang P, Dai X. Dendritic cells transfected with indoleamine 2,3-dioxygenase gene suppressed acute rejection of cardiac allograft. International Immunopharmacology. 2016 Jul 1;36:31–8.

107. Araujo JA, Zhang M, Yin F. Heme Oxygenase-1, Oxidation, Inflammation, and Atherosclerosis. Frontiers in Pharmacology. 2012.

108. Amano M, Camara NOS. The immunomodulatory role of carbon monoxide during transplantation. Medical Gas Research. 2013;3(1):1.

109. Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol D-W, Flavell RA, et al. Carbon Monoxide Inhibits T Lymphocyte Proliferation via Caspase-Dependent Pathway. The Journal of Immunology. 2004 Jan 15;172(2):1220–6.

110. Wang H, Lee SS, Gao W, Czismadia E, McDaid J, Ollinger R, et al. Donor Treatment With Carbon Monoxide Can Yield Islet Allograft Survival and Tolerance. Diabetes. 2005 May 1;54(5):1400–6.

111. Chauveau C. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. Blood. 2005 Sep 1;106(5):1694–702.

112. Moreau A, Hill M, Thébault P, Deschamps JY, Chiffoleau E, Chauveau C, et al. Tolerogenic dendritic cells actively inhibit T cells through heme oxygenase-1 in rodents and in nonhuman primates. The FASEB Journal. 2009 May 6;23(9):3070–7.

113. Torres-Aguilar H, Aguilar-Ruiz SR, González-Pérez G, Munguía R, Bajaña S, Meraz-Ríos MA, et al. Tolerogenic Dendritic Cells Generated with Different Immunosuppressive Cytokines Induce Antigen-Specific Anergy and Regulatory Properties in Memory CD4 $^{+}$ T Cells. The Journal of Immunology. 2010 Feb 15;184(4):1765–75.

114. Raker VK, Domogalla MP, Steinbrink K. Tolerogenic Dendritic Cells for Regulatory T Cell Induction in Man. Frontiers in Immunology. 2015.

115. Graca L, Cobbold SP, Waldmann H. Identification of Regulatory T Cells in Tolerated Allografts. The Journal of Experimental Medicine. 2002 Jun 17;195(12):1641–6.

116. de Goër de Herve M-G, Gonzales E, Hendel-Chavez H, Décline J-L, Mourier O, Abbed K, et al. CD25 Appears Non Essential for Human Peripheral Treg Maintenance In Vivo. Unutmaz D, editor. PLoS ONE. 2010 Jul 30;5(7):e11784.

117. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation–mediated apoptosis of effector CD4+ T cells. Nature Immunology. 2007 Nov 4;8:1353.

118. Schmitt EG, Williams CB. Generation and Function of Induced Regulatory T Cells. Frontiers in Immunology. 2013

119. White AM, Wraith DC. Tr1-Like T Cells – An Enigmatic Regulatory T Cell Lineage. Frontiers in Immunology. 2016

120. Gagliani N, Jofra T, Valle A, Stabilini A, Morsiani C, Gregori S, et al. Transplant Tolerance to Pancreatic Islets Is Initiated in the Graft and Sustained in the Spleen: Foxp3 $^{+}$ Treg and Tr1 Cells in Transplant Tolerance. American Journal of Transplantation. 2013 Aug;13(8):1963–75.

121. García-González P, Ubilla-Olguín G, Catalán D, Schinnerling K, Aguillón JC. Tolerogenic dendritic cells for reprogramming of lymphocyte responses in autoimmune diseases. Autoimmunity Reviews. 2016 Nov 1;15(11):1071–80.

122. Pletinckx K, Vaeth M, Schneider T, Beyersdorf N, Hünig T, Berberich-Siebelt F, et al. Immature dendritic cells convert anergic nonregulatory T cells into Foxp3 $^{-}$ IL-10 $^{+}$ regulatory T cells by engaging CD28 and CTLA-4: Immunomodulation. European Journal of Immunology. 2015 Feb;45(2):480–91.

123. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, et al. IL-35-mediated induction of a potent regulatory T cell population. Nature Immunology. 2010 Dec;11(12):1093–101.

124. Li S, Xie Q, Zeng Y, Zou C, Liu X, Wu S, et al. A naturally occurring CD8+CD122+ T-cell subset as a memory-like Treg family. Cellular & Molecular Immunology. 2014 Jul;11(4):326–31.

125. Dai H, Wan N, Zhang S, Moore Y, Wan F, Dai Z. Cutting Edge: Programmed Death-1 Defines CD8+CD122+ T Cells as Regulatory versus Memory T Cells. The Journal of Immunology. 2010 Jul 15;185(2):803–7.

126. Ezzelarab MB, Lu L, Guo H, Zahorchak AF, Shufesky WF, Cooper DKC, et al. Eomesoderminlo CTLA4hi Alloreactive CD8+ Memory T Cells Are Associated With Prolonged Renal Transplant Survival Induced by Regulatory Dendritic Cell Infusion in CTLA4Ig-Treated Non- Human Primates. Transplantation. 2017.

127. Dhodapkar MV, Steinman RM. Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans. Blood. 2002;100(1):5.

128. Durand J. B cells with regulatory properties in transplantation tolerance. World Journal of Transplantation. 2015;5(4):196.

129. Niimi M, Pearson TC, Larsen CP, Alexander DZ, Hollenbaugh D, Aruffo A, et al. The Role of the CD40 Pathway in Alloantigen-Induced Hyporesponsiveness In Vivo. J Immunol. 1998 Nov 15;161(10):5331.

130. Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. Proceedings of the National Academy of Sciences. 1995 Oct 10;92(21):9560–4.

131. Durand J, Huchet V, Merieau E, Usal C, Chesneau M, Remy S, et al. Regulatory B Cells with a Partial Defect in CD40 Signaling and Overexpressing Granzyme B Transfer Allograft Tolerance in Rodents. The Journal of Immunology. 2015 Nov 15;195(10):5035–44.

132. Moreau A, Blair PA, Chai J-G, Ratnasothy K, Stolarczyk E, Alhabbab R, et al. Transitional-2 B cells acquire regulatory function during tolerance induction and contribute to allograft survival: Immunomodulation. European Journal of Immunology. 2015 Mar;45(3):843–53.

133. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. Journal of Clinical Investigation. 2010 Jun 1;120(6):1836–47.

134. Pallier A, Hillion S, Danger R, Giral M, Racapé M, Degauque N, et al. Patients with drug-free long-term graft function display increased numbers of peripheral B cells with a memory and inhibitory phenotype. Kidney International. 2010 Sep;78(5):503–13.

135. Chesneau M, Michel L, Dugast E, Chenouard A, Baron D, Pallier A, et al. Tolerant Kidney Transplant Patients Produce B Cells with Regulatory Properties. Journal of the American Society of Nephrology. 2015 Oct 1;26(10):2588–98.

136. Di Caro V, Phillips B, Engman C, Harnaha J, Trucco M, Giannoukakis N. Involvement of Suppressive B-Lymphocytes in the Mechanism of Tolerogenic Dendritic Cell Reversal of Type 1 Diabetes in NOD Mice. Fiorina P, editor. PLoS ONE. 2014 Jan 17;9(1):e83575.

137. Mukherji B, Chakraborty NG, Yamasaki S, Okino T, Yamase H, Sporn JR, et al. Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with

synthetic peptide-pulsed autologous antigen presenting cells. Proceedings of the National Academy of Sciences. 1995 Aug 15;92(17):8078–82.

138. Dhodapkar MV, Steinman RM, Sapp M, Desai H, Fossella C, Krasovsky J, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. Journal of Clinical Investigation. 1999 Jul 15;104(2):173–80.

139. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-Specific Inhibition of Effector T Cell Function in Humans after Injection of Immature Dendritic Cells. The Journal of Experimental Medicine. 2001 Jan 15;193(2):233–8.

140. Thomson AW, Humar A, Lakkis FG, Metes DM. Regulatory dendritic cells for promotion of liver transplant operational tolerance: Rationale for a clinical trial and accompanying mechanistic studies. Human Immunology. 2018 May;79(5):314–21.

141. Nafarrate IZ, Florez G, Vila G, Cabezón R, España C, Benitez D, et al. Phase 1b clinical trial with antigen-specific tolerogenic dendritic in Multiple Sclerosis and Neuromyelitis optica: safety and immunological effects (P2.330). Neurology. 2017 Apr 18;88

142. Metallo CM, Vander Heiden MG. Understanding Metabolic Regulation and Its Influence on Cell Physiology. Molecular Cell. 2013 Feb;49(3):388–98.

143. Brewer PD, Habtemichael EN, Romenskaia I, Mastick CC, Coster ACF. Insulin-regulated Glut4 Translocation: MEMBRANE PROTEIN TRAFFICKING WITH SIX DISTINCTIVE STEPS. Journal of Biological Chemistry. 2014 Jun 20;289(25):17280–98.

144. Long YC. AMP-activated protein kinase signaling in metabolic regulation. Journal of Clinical Investigation. 2006 Jul 3;116(7):1776–83.

145. Navale AM, Paranjape AN. Glucose transporters: physiological and pathological roles. Biophysical Reviews. 2016 Mar;8(1):5–9.

146. Massa ML, Gagliardino JJ, Francini F. Liver glucokinase: An overview on the regulatorymechanisms of its activity. IUBMB Life. 2011 Jan;63(1):1–6.

147. Stincone A, Prigione A, Cramer T, Wamelink MMC, Campbell K, Cheung E, et al. The return of metabolism: biochemistry and physiology of the pentose phosphate pathway: The pentose phosphate pathway. Biological Reviews. 2015 Aug;90(3):927–63.

148. Rotondo F, Ho-Palma AC, Remesar X, Fernández-López JA, Romero M del M, Alemany M. Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acyl-glycerol turnover. Scientific Reports. 2017.

149. Zheng J. Energy metabolism of cancer: Glycolysis versus oxidative phosphorylation (Review). Oncology Letters. 2012;4(6):1151–7.

150. Tarling EJ, Vallim TQ de A, Edwards PA. Role of ABC transporters in lipid transport and human disease. Trends in Endocrinology & Metabolism. 2013 Jul;24(7):342–50.

151. Wilkinson K, El Khoury J. Microglial Scavenger Receptors and Their Roles in the Pathogenesis of Alzheimer's Disease. International Journal of Alzheimer's Disease. 2012;2012:1–10.

152. Lee C-H, Olson P, Evans RM. Minireview: Lipid Metabolism, Metabolic Diseases, and Peroxisome Proliferator-Activated Receptors. Endocrinology. 2003 Jun;144(6):2201–7.

153. Ren W, Liu G, Yin J, Tan B, Wu G, Bazer FW, et al. Amino-acid transporters in T-cell activation and differentiation. Cell Death & Disease. 2017 Mar;8(3):e2655–e2655.

154. Puchalska P, Crawford PA. Multi-dimensional Roles of Ketone Bodies in Fuel Metabolism, Signaling, and Therapeutics. Cell Metabolism. 2017 Feb;25(2):262–84.

155. Garcia D, Shaw RJ. AMPK: Mechanisms of Cellular Energy Sensing and Restoration of Metabolic Balance. Molecular Cell. 2017 Jun;66(6):789–800.

156. Cantó C, Jiang LQ, Deshmukh AS, Mataki C, Coste A, Lagouge M, et al. Interdependence of AMPK and SIRT1 for Metabolic Adaptation to Fasting and Exercise in Skeletal Muscle. Cell Metabolism. 2010 Mar;11(3):213–9.

157. Zhao Y, Hu X, Liu Y, Dong S, Wen Z, He W, et al. ROS signaling under metabolic stress: cross-talk between AMPK and AKT pathway. Molecular Cancer. 2017

158. Grygiel-Górniak B. Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications - a review. Nutrition Journal. 2014

159. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017 Mar;168(6):960–76.

160. Jeoung NH. Pyruvate Dehydrogenase Kinases: Therapeutic Targets for Diabetes and Cancers. Diabetes & Metabolism Journal. 2015;39(3):188.

161. Zhang S, Hulver MW, McMillan RP, Cline MA, Gilbert ER. The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. Nutrition & Metabolism. 2014;11(1):10.

162. Mor I, Cheung EC, Vousden KH. Control of Glycolysis through Regulation of PFK1: Old Friends and Recent Additions. Cold Spring Harbor Symposia on Quantitative Biology. 2011 Jan 1;76(0):211–6.

163. Wahl DR, Byersdorfer CA, Ferrara JLM, Opipari AW, Glick GD. Distinct metabolic programs in activated T cells: opportunities for selective immunomodulation. Immunological Reviews. 2012 Sep;249(1):104–15.

164. Galván-Peña S, O'Neill LAJ. Metabolic Reprograming in Macrophage Polarization. Frontiers in Immunology. 2014;5:420.

165. Geeraerts X, Bolli E, Fendt S-M, Van Ginderachter JA. Macrophage Metabolism As Therapeutic Target for Cancer, Atherosclerosis, and Obesity. Frontiers in Immunology. 2017

166. Zaccagnino P, Saltarella M, Maiorano S, Gaballo A, Santoro G, Nico B, et al. An active mitochondrial biogenesis occurs during dendritic cell differentiation. The International Journal of Biochemistry & Cell Biology. 2012 Nov 1;44(11):1962–9.

167. Pearce EJ, Everts B. Dendritic cell metabolism. Nature Reviews Immunology. 2015 Jan;15(1):18–29.

168. Everts B, Pearce EJ. Metabolic Control of Dendritic Cell Activation and Function: Recent Advances and Clinical Implications. Frontiers in Immunology. 2014;5:203.

169. Švajger U, Obermajer N, Jeras M. Dendritic cells treated with resveratrol during differentiation from monocytes gain substantial tolerogenic properties upon activation: Resveratrol-induced dendritic cell tolerogenicity. Immunology. 2010 Apr;129(4):525–35.

170. Haidinger M, Poglitsch M, Geyeregger R, Kasturi S, Zeyda M, Zlabinger GJ, et al. A Versatile Role of Mammalian Target of Rapamycin in Human Dendritic Cell Function and Differentiation. The Journal of Immunology. 2010 Oct 1;185(7):3919–31.

171. Hwang J-T, Wook Kwak D, Kyo Lin S, Min Kim H, Kim Y, Jin Park O. Resveratrol Induces Apoptosis in Chemoresistant Cancer Cells via Modulation of AMPK Signaling Pathway. Vol. 1095. 2007. 441 p.

172. García-González PA, Schinnerling K, Sepúlveda-Gutiérrez A, Maggi J, Mehdi AM, Nel HJ, et al. Dexamethasone and Monophosphoryl Lipid A Induce a Distinctive Profile on Monocyte-Derived Dendritic Cells through Transcriptional Modulation of Genes Associated With Essential Processes of the Immune Response. Frontiers in Immunology. 2017.

173. Metabolic control of dendritic cell activation and function: recent advances and clinical implications. Frontiers in Immunology. 2014

174. Ferreira GB, Vanherwegen A-S, Eelen G, Gutiérrez ACF, Van Lommel L, Marchal K, et al. Vitamin D3 Induces Tolerance in Human Dendritic Cells by Activation of Intracellular Metabolic Pathways. Cell Reports. 2015 Feb;10(5):711–25.

175. Malinarich F, Duan K, Hamid RA, Bijin A, Lin WX, Poidinger M, et al. High Mitochondrial Respiration and Glycolytic Capacity Represent a Metabolic Phenotype of Human Tolerogenic Dendritic Cells. The Journal of Immunology. 2015 Jun 1;194(11):5174–86.

176. Michalek RD, Rathmell JC. The metabolic life and times of a T-cell: T-cell metabolism and death. Immunological Reviews. 2010 Jun 15;236(1):190–202.

177. Bantug GR, Galluzzi L, Kroemer G, Hess C. The spectrum of T cell metabolism in health and disease. Nature Reviews Immunology. 2017 Sep 25;18(1):19–34.

178. O'Sullivan D, Pearce EL. Fatty acid synthesis tips the TH17-Treg cell balance. Nature Medicine. 2014 Nov 6;20:1235.

179. Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, et al. Mitochondria Are Required for Antigen-Specific T Cell Activation through Reactive Oxygen Species Signaling. Immunity. 2013 Feb;38(2):225–36.

180. Previte DM, O'Connor EC, Novak EA, Martins CP, Mollen KP, Piganelli JD. Reactive oxygen species are required for driving efficient and sustained aerobic glycolysis during CD4+ T cell activation. Tan M, editor. PLOS ONE. 2017 Apr 20;12(4):e0175549.

181. Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang C-H, Sanin DE, et al. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. Cell. 2016 Jun;166(1):63–76.

182. Chao T, Wang H, Ho P-C. Mitochondrial Control and Guidance of Cellular Activities of T Cells. Frontiers in Immunology. 2017

183. Peng M, Yin N, Chhangawala S, Xu K, Leslie CS, Li MO. Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. Science. 2016 Oct 28;354(6311):481–4.

184. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting Edge: Distinct Glycolytic and Lipid Oxidative Metabolic Programs Are Essential for Effector and Regulatory CD4+ T Cell Subsets. The Journal of Immunology. 2011 Mar 15;186(6):3299–303.

185. Procaccini C, Carbone F, Di Silvestre D, Brambilla F, De Rosa V, Galgani M, et al. The Proteomic Landscape of Human Ex Vivo Regulatory and Conventional T Cells Reveals Specific Metabolic Requirements. Immunity. 2016 Feb;44(2):406–21.

186. Kishore M, Cheung KCP, Fu H, Bonacina F, Wang G, Coe D, et al. Regulatory T Cell Migration Is Dependent on Glucokinase-Mediated Glycolysis. Immunity. 2017 Nov;47(5):875-889.e10.

187. Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. mTORC1 couples immune signals and metabolic programming to establish Treg-cell function. Nature. 2013 Jul;499(7459):485–90.

188. Stallone G, Infante B, Di Lorenzo A, Rascio F, Zaza G, Grandaliano G. mTOR inhibitors effects on regulatory T cells and on dendritic cells. Journal of Translational Medicine. 2016

189. Barbi J, Pardoll D, Pan F. Metabolic control of the Treg/Th17 axis. Immunological Reviews. 2013 Mar;252(1):52–77.

190. He N, Fan W, Henriquez B, Yu RT, Atkins AR, Liddle C, et al. Metabolic control of regulatory T cell (Treg) survival and function by Lkb1. Proceedings of the National Academy of Sciences. 2017 Nov 21;114(47):12542–7.

191. Chang C-H, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. Cell. 2015 Sep;162(6):1229–41.

192. Ho P-C, Bihuniak JD, Macintyre AN, Staron M, Liu X, Amezquita R, et al. Phosphoenolpyruvate Is a Metabolic Checkpoint of Anti-tumor T Cell Responses. Cell. 2015 Sep;162(6):1217–28.

193. Angelin A, Gil-de-Gómez L, Dahiya S, Jiao J, Guo L, Levine MH, et al. Foxp3 Reprograms T Cell Metabolism to Function in Low-Glucose, High-Lactate Environments. Cell Metabolism. 2017 Jun;25(6):1282-1293.e7.

194. Lyssiotis CA, Kimmelman AC. Metabolic Interactions in the Tumor Microenvironment. Trends in Cell Biology. 2017 Nov;27(11):863–75.

195. Lee GK, Park HJ, Macleod M, Chandler P, Munn DH, Mellor AL. Tryptophan deprivation sensitizes activated T cells to apoptosis prior to cell division. Immunology. 2002 Dec;107(4):452–60.

196. Fletcher M, Ramirez ME, Sierra RA, Raber P, Thevenot P, Al-Khami AA, et al. L-Arginine Depletion Blunts Antitumor T-cell Responses by Inducing Myeloid-Derived Suppressor Cells. Cancer Research. 2015 Jan 15;75(2):275–83.

197. Kremer JC, Prudner BC, Lange SES, Bean GR, Schultze MB, Brashears CB, et al. Arginine Deprivation Inhibits the Warburg Effect and Upregulates Glutamine Anaplerosis and Serine Biosynthesis in ASS1-Deficient Cancers. Cell Reports. 2017 Jan;18(4):991–1004.

198. Amersfoort J, Kuiper J. T cell metabolism in metabolic disease-associated autoimmunity. Immunobiology. 2017 Oct;222(10):925–36.

199. Gerriets VA, MacIver NJ. Role of T Cells in Malnutrition and Obesity. Frontiers in Immunology. 2014

200. Kompanje EJO, Jansen TC, Hoven B Van Der, Bakker J. The first demonstration of lactic acid in human blood in shock by Johann Joseph Scherer (1814 – 1869) in January 1843. 2007;1967–71.

201. Russell CA. H. Benninga. A History of Lactic Acid Making: A Chapter in the History of Biotechnology. The British Journal for the History of Science. 2009.

202. Rumbo M. Is lactate an undervalued functional component of fermented food products ? Front Microbiol. 2015;6(June):1–5.

203. Earnshaw RG. The Antimicrobial Action of Lactic Acid Bacteria : Natural Food Preservation Systems. The Lactic Acid Bacteria Volume 1. Springer, Boston, MA 1992;1:211–2.

204. Wang C, Chang T, Yang H, Cui M. Antibacterial mechanism of lactic acid on physiological and morphological properties of Salmonella Enteritidis , Escherichia coli and Listeria monocytogenes. Food Control. 2015;47:231–6.

205. Oren A, Gurevich P. Production of d-lactate, acetate, and pyruvate from glycerol in communities of halophilic archaea in the Dead Sea and in saltern crystallizer ponds. FEMS Microbiology Ecology. 1994;14(2):147–55.

206. Dolferus R, Scientific TC, Carroll RT, Ismond KP, Good AG. Functional analysis of lactate dehydrogenase during hypoxic stress in Functional analysis of lactate dehydrogenase during hypoxic stress in Arabidopsis. Functional Plant Biology. 2008;(May 2014).

207. Saarela M. Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. Appl Environ Microbiol 2005;66(5):2001–5.

208. Yiu S, Choi C, Collins CC, Gout PW, Wang Y. Cancer-generated lactic acid : a regulatory , immunosuppressive metabolite? J Pathol. 2013;350–5.

209. Valvona CJ, Fillmore HL, Nunn PB, Pilkington GJ. The Regulation and Function of Lactate Dehydrogenase A : Therapeutic Potential in Brain Tumor. Brain Pathol. 2015;

210. Odet F, Duan C, Willis WD, Goulding EH, Kung A, Eddy EM, et al. The expression of *Ldh-c* in the skeletal muscle of plateau pika (*Ochotona curzoniae*) enhances adaptation to a hypoxic environment. Biol Open. 2009;79(1):26–34.

211. Zakhartsev M, Johansen T, Pörtner HO, Blust R. Effects of temperature acclimation on lactate dehydrogenase of cod (Gadus morhua): genetic , kinetic and thermodynamic aspects. J Exp Biol. 2004;95–112.

212. Bishop MJ, Everse J, Kaplan N. Identification of Lactate Dehydrogenase Isoenzymes by Rapid Kinetics. Proc Natl Acad Sci USA. 1972;69(7):1761–5.

213. Quistorff B, Grunnet N. The isoenzyme pattern of LDH does not play a physiological role ; except perhaps during fast transitions in energy metabolism. Aging. 3(5):457–60.

214. Eggert MW, Byrne ME, Chambers RP. Impact of High Pyruvate Concentration on Kinetics of Rabbit Muscle Lactate Dehydrogenase. Appl Biochem Biotechnol. 2011;

215. Wang Y, Wei L, Wei D, Li X, Xu L, Wei L. Enzymatic Kinetic Properties of the Lactate Dehydrogenase Isoenzyme C 4 of the Plateau Pika (Ochotona curzoniae). Biol Open. 2016

216. Feldman-salit A, Hering S, Messiha H, Veith N, Cojocaru V, Sieg A, et al. Regulation of lactate dehydrogenases activity. JBC. 2013;

217. Matoba Y, Miyasako M, Matsuo K, Oda K, Noda M, Higashikawa F, et al. An alternative allosteric regulation mechanism of an acidophilic L -lactate dehydrogenase from Enterococcus mundtii 15-1A. FEBS OPEN BIO. 2014;4:834–47.

218. Fan J, Hitosugi T, Chung T, Xie J, Ge Q, Gu T, et al. Tyrosine Phosphorylation of Lactate Dehydrogenase A Is Important for NADH / NAD ƨ Redox Homeostasis in Cancer Cells. Mol Cell Biol. 2011;31(24):4938–50.

219. Zhao D, Zou S-W, Liu Y, Zhou X, Mo Y, Wang P, et al. Lysine-5 Acetylation Negatively Regulates Lactate Dehydrogenase A and Is Decreased in Pancreatic Cancer. Cancer cell. 2013 Apr 15;23(4):10.1016/j.ccr.2013.02.005.

220. Shahriari A, Dawson NJ, Bell RA V, Storey KB. Stable Suppression of Lactate Dehydrogenase Activity during Anoxia in the Foot Muscle of Littorina littorea and the Potential Role of Acetylation as a Novel Posttranslational Regulatory Mechanism. Enzyme Res. 2013;2013.

221. Dawson NJ, Bell RA V, Storey KB. Purification and Properties of White Muscle Lactate Dehydrogenase from the Anoxia-Tolerant Turtle , the Red-Eared Slider , Trachemys scripta elegans. Enzyme Res. 2013;2013(c).

222. Su Y, Yu Q, Wang X, Yu L, Wang Z, Cao Y, et al. JMJD2A promotes the Warburg effect and nasopharyngeal carcinoma progression by transactivating LDHA expression. BMC Cancer. 2017;1–13.

223. Weidemann A, Johnson RS. Biology of HIF-1a. Cell Deat Differ. 2008;621–7.

224. Jones S. An overview of the basic helix-loop-helix proteins. Genome Biol. 2004;1–6.

225. Zhou Z-Q, Hurlin PJ. The interplay between Mad and Myc in proliferation and differentiation. Trends in Cell Biology. 2001 Nov 1;11(11):S10–4.

226. Miller DM, Thomas SD, Islam A, Muench D, Sedoris K. c-Myc and Cancer Metabolism. Clin Cancer Res. 2012;18(20):5546–54.

227. Boxer LM, Dang C V. Translocations involving c- myc and c- myc function. Oncogene. 2001;

228. Cui J, Shi M, Xie D, Wei D, Jia Z, Zheng S, et al. FOXM1 Promotes the Warburg Effect and Pancreatic Cancer Progression via Transactivation of LDHA Expression. Clin Cancer Res. 2014;2595–607.

229. Chang C, Zhang C, Zhang Q, Sahin O, Wang H. Upregulation of lactate dehydrogenase a by 14-3-3ζ leads to increased glycolysis critical for breast cancer initiation and progression Upregulation of lactate dehydrogenase a by 14-3-3ζ leads to increased glycolysis critical for breast cancer initiation and progression. Oncotarget. 2016;(May).

230. Brown NJ, Higham SE, Perunovic B, Arafa M, Balasubramanian S, Rehman I. Lactate Dehydrogenase-B Is Silenced by Promoter Methylation in a High Frequency of Human Breast Cancers. PLoS One. 2013;8(2):4–11.

231. Liang X, Liu L, Fu T, Zhou Q, Zhou D, Xiao L, et al. Exercise Inducible Lactate Dehydrogenase B Regulates Mitochondrial Function in Skeletal Muscle. The Journal of Biological Chemistry. 2016 Dec 2;291(49):25306–18.

232. Halestrap AP. The monocarboxylate transporter family-Structure and functional characterization. IUBMB Life. 2012 Jan;64(1):1–9.

233. Halestrap AP. The SLC16 gene family – Structure, role and regulation in health and disease. Molecular Aspects of Medicine. 2013 Apr;34(2–3):337–49.

234. Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates across mammalian plasma membranes. American Journal of Physiology-Cell Physiology. 1993 Apr;264(4):C761–82.

235. Halestrap AP, Wilson MC. The monocarboxylate transporter family-Role and regulation. IUBMB Life. 2012 Feb;64(2):109–19.

236. Xu D, Hemler ME. Metabolic Activation-related CD147-CD98 Complex. Mol Cell Proteomics. 2005.

237. Walters DK, Arendt BK, Jelinek DF. CD147 regulates the expression of MCT1 and lactate export in multiple myeloma cells. Cell Cycle. 2013 Oct;12(19):3364–72.

238. Kobayashi M, Itagaki S, Hirano T, Iseki K. Mechanism of L-lactic Acid Transport in L6 Skeletal Muscle Cells. Drug Metabolism and Pharmacokinetics. 2004;19(5):363–8.

239. Ganapathy V, Thangaraju M, Gopal E, Martin PM, Itagaki S, Miyauchi S, et al. Sodium-coupled Monocarboxylate Transporters in Normal Tissues and in Cancer. The AAPS Journal. 2008 Mar;10(1):193–9.

240. Haas R, Smith J, Rocher-Ros V, Nadkarni S, Montero-Melendez T, D'Acquisto F, et al. Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration and Effector Functions. Marrack P, editor. PLOS Biology. 2015 Jul 16;13(7):e1002202.

241. Coady MJ, Chang M-H, Charron FM, Plata C, Wallendorff B, Sah JF, et al. The human tumour suppressor gene *SLC5A8* expresses a Na^+-monocarboxylate cotransporter: *SLC5A8* encodes a Na^+-monocarboxylate cotransporter. The Journal of Physiology. 2004 Jun;557(3):719–31.

242. Ichida K. What lies behind serum urate concentration? Insights from genetic and genomic studies. Genome Medicine. 2009;1(12):118.

243. Liu C, Wu J, Zhu J, Kuei C, Yu J, Shelton J, et al. Lactate Inhibits Lipolysis in Fat Cells through Activation of an Orphan G-protein-coupled Receptor, GPR81. Journal of Biological Chemistry. 2009 Jan 30;284(5):2811–22.

244. Phan LM, Yeung S-CJ, Lee M-H. Cancer metabolic reprogramming: importance, main features, and potentials for precise targeted anti-cancer therapies. Cancer Biol Med. 2014;11(1):19.

245. Shestov AA, Liu X, Ser Z, Cluntun AA, Hung YP, Huang L, et al. Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step. eLife. 2014.

246. Kane DA. Lactate oxidation at the mitochondria: a lactate-malate-aspartate shuttle at work. Frontiers in Neuroscience. 2014

247. Latham T, Mackay L, Sproul D, Karim M, Culley J, Harrison DJ, et al. Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. Nucleic Acids Research. 2012 Jun;40(11):4794–803.

248. Réminiac F, Saint-Etienne C, Runge I, Ayé DY, Benzekri-Lefevre D, Mathonnet A, et al. Are Central Venous Lactate and Arterial Lactate Interchangeable? A Human Retrospective Study: Anesthesia & Analgesia. 2012 Jun;1.

249. Adeva-Andany M, López-Ojén M, Funcasta-Calderón R, Ameneiros-Rodríguez E, Donapetry-García C, Vila-Altesor M, et al. Comprehensive review on lactate metabolism in human health. Mitochondrion. 2014 Jul;17:76–100.

250. Facey A, Irving R, Dilworth L. Overview of Lactate Metabolism and the Implications for Athletes. American Journal of Sports Science and Medicine. 2013;5.

251. Brooks GA. The lactate shuttle during exercise and recovery: Medicine & Science in Sports & Exercise. 1986 Jun;18(3):360–8.

252. Rakus D, Gizak A, Wiśniewski JR. Proteomics Unveils Fibroblast–Cardiomyocyte Lactate Shuttle and Hexokinase Paradox in Mouse Muscles. Journal of Proteome Research. 2016 Aug 5;15(8):2479–90.

253. Newington JT, Harris RA, Cumming RC. Reevaluating Metabolism in Alzheimer's Disease from the Perspective of the Astrocyte-Neuron Lactate Shuttle Model. Journal of Neurodegenerative Diseases. 2013;2013:1–13.

254. Bellomo R. Bench-to-bedside review: Lactate and the kidney. Crit Care. 2002;6(4):5.

255. Jin ES, Sherry AD, Malloy CR. Metabolism of Glycerol, Glucose, and Lactate in the Citric Acid Cycle Prior to Incorporation into Hepatic Acylglycerols. Journal of Biological Chemistry. 2013 May 17;288(20):14488–96.

256. Palacin M. Utilization of glucose, alanine, lactate, and glycerol as lipogenic substrates by periuterine adipose tissue in situ in fed and starved rats. Journal lipid res. 1988;7.

257. Choi CS, Kim Y-B, Lee FN, Zabolotny JM, Kahn BB, Youn JH. Lactate induces insulin resistance in skeletal muscle by suppressing glycolysis and impairing insulin signaling. American Journal of Physiology-Endocrinology and Metabolism. 2002 Aug;283(2):E233–40.

258. Ahmed K, Tunaru S, Tang C, Müller M, Gille A, Sassmann A, et al. An Autocrine Lactate Loop Mediates Insulin-Dependent Inhibition of Lipolysis through GPR81. Cell Metabolism. 2010 Apr;11(4):311–9.

259. Sun J, Ye X, Xie M, Ye J. Induction of triglyceride accumulation and mitochondrial maintenance in muscle cells by lactate. Scientific Reports. 2016.

260. Morland C, Andersson KA, Haugen ØP, Hadzic A, Kleppa L, Gille A, et al. Exercise induces cerebral VEGF and angiogenesis via the lactate receptor HCAR1. Nature Communications. 2017 May 23;8:15557.

261. Morrison BM, Tsingalia A, Vidensky S, Lee Y, Jin L, Farah MH, et al. Deficiency in monocarboxylate transporter 1 (MCT1) in mice delays regeneration of peripheral nerves following sciatic nerve crush. Experimental Neurology. 2015 Jan;263:325–38.

262. Reddy AJ, Lam SW, Bauer SR, Guzman JA. Lactic acidosis: Clinical implications and management strategies. Cleveland Clinic Journal of Medicine. 2015 Sep 1;82(9):615–24.

263. Wallenius K, Thalén P, Björkman J-A, Johannesson P, Wiseman J, Böttcher G, et al. Involvement of the metabolic sensor GPR81 in cardiovascular control. JCI Insight. 2017.

264. Puig-Kröger A, Muñiz-Pello O, Selgas R, Criado G, Bajo M-A, Sánchez-Tomero JA, et al. Peritoneal dialysis solutions inhibit the differentiation and maturation of human monocyte-derived dendritic cells: effect of lactate and glucose-degradation products. Journal of Leukocyte Biology. 2003 Apr;73(4):482–92.

265. Hirschhaeuser F, Sattler UGA, Mueller-Klieser W. Lactate: A Metabolic Key Player in Cancer. Cancer Research. 2011 Nov 15;71(22):6921–5.

266. Gottfried E. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. Blood. 2006 Mar 1;107(5):2013–21.

267. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, et al. Inhibitory effect of tumor cell-derived lactic acid on human T cells. Blood. 2007 May 1;109(9):3812–9.

268. Khatib-Massalha E, Kumari A, Golan K, Massalha H, Avemaria F, Gur-Cohen S, et al. Lactate Release By Bone Marrow Neutrophils Promotes Their Inflammatory Mobilization Via Endothelial GPR81 Signaling. Blood. 2017 Dec 7;130(Suppl 1):446.

269. Dietl K, Renner K, Dettmer K, Timischl B, Eberhart K, Dorn C, et al. Lactic Acid and Acidification Inhibit TNF Secretion and Glycolysis of Human Monocytes. The Journal of Immunology. 2010 Feb 1;184(3):1200–9.

270. Nasi A, Fekete T, Krishnamurthy A, Snowden S, Rajnavolgyi E, Catrina AI, et al. Dendritic Cell Reprogramming by Endogenously Produced Lactic Acid. The Journal of Immunology. 2013 Sep 15;191(6):3090–9.

271. Martinez D, Vermeulen M, von Euw E, Sabatte J, Maggini J, Ceballos A, et al. Extracellular Acidosis Triggers the Maturation of Human Dendritic Cells and the Production of IL-12. The Journal of Immunology. 2007 Aug 1;179(3):1950–9.

272. Ranganathan P, Shanmugam A, Swafford D, Suryawanshi A, Bhattacharjee P, Hussein MS, et al. GPR81, a Cell-Surface Receptor for Lactate, Regulates Intestinal Homeostasis and Protects Mice from Experimental Colitis. The Journal of Immunology. 2018 Jan 31;ji1700604.

273. Errea A, Cayet D, Marchetti P, Tang C, Kluza J, Offermanns S, et al. Lactate Inhibits the Pro-Inflammatory Response and Metabolic Reprogramming in Murine Macrophages in a GPR81-Independent Manner. Bayry J, editor. PLOS ONE. 2016 Nov 15;11(11):e0163694.

274. Ramanathan S, Jagannathan N. Tumor Associated Macrophage: A Review on the Phenotypes, Traits and Functions. Iranian Journal of Cancer Prevention. 2014;7(1):8.

275. Colegio OR, Chu N-Q, Szabo AL, Chu T, Rhebergen AM, Jairam V, et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. Nature. 2014 Sep;513(7519):559–63.

276. Ohashi T, Aoki M, Tomita H, Akazawa T, Sato K, Kuze B, et al. M2-like macrophage polarization in high lactic acid-producing head and neck cancer. Cancer Science. 2017 Jun;108(6):1128–34.

277. Brand A, Singer K, Koehl GE, Kolitzus M, Schoenhammer G, Thiel A, et al. LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells. Cell Metabolism. 2016 Nov;24(5):657–71.

278. Husain Z, Huang Y, Seth P, Sukhatme VP. Tumor-Derived Lactate Modifies Antitumor Immune Response: Effect on Myeloid-Derived Suppressor Cells and NK Cells. The Journal of Immunology. 2013 Aug 1;191(3):1486–95.

279. Miettinen T, Bjorklund M. Mitochondrial Function and Cell Size: An Allometric Relationship. Trends Cell Biol. 2017.

280. Leverve X, Batandier C, Fontaine E. Choosing the Right Substrate. Novartis Found Symp. 2007.

281. Dodd MS, Sousa Fialho M da L, Montes Aparicio CN, Kerr M, Timm KN, Griffin JL, et al. Fatty Acids Prevent Hypoxia-Inducible Factor-1α Signaling Through Decreased Succinate in Diabetes. JACC: Basic to Translational Science. 2018 Aug;3(4):485–98.

282. Xie H, Hanai J, Ren J-G, Kats L, Burgess K, Bhargava P, et al. Targeting Lactate Dehydrogenase-A Inhibits Tumorigenesis and Tumor Progression in Mouse Models of

Lung Cancer and Impacts Tumor-Initiating Cells. Cell Metabolism. 2014 May;19(5):795–809.

283. Chang C-H, Curtis JD, Maggi LB, Faubert B, Villarino AV, O'Sullivan D, et al. Posttranscriptional Control of T Cell Effector Function by Aerobic Glycolysis. Cell. 2013 Jun;153(6):1239–51.

284. Scharping NE, Menk AV, Moreci RS, Whetstone RD, Dadey RE, Watkins SC, et al. The Tumor Microenvironment Represses T Cell Mitochondrial Biogenesis to Drive Intratumoral T Cell Metabolic Insufficiency and Dysfunction. Immunity. 2016 Aug;45(2):374–88.

Annex I. Potential of tolerogenic dendritic cells

Curr Transpl Rep
DOI 10.1007/s40472-016-0109-6

Potential of Tolerogenic Dendritic Cells in Transplantation

Eros Marín[1,2,3] · **Maria Cristina Cuturi**[1,2,3] · **Aurélie Moreau**[1,2,3]

Abstract Dendritic cells (DC) are the most important subset of antigen presenting cells (APC) that are able to polarize the immune response to pro-inflammatory or anti-inflammatory response. This duality places DC in the axis between tolerance and immunogenicity. The regulation of polarization is the key point in autoimmune diseases and organ transplantation. In order to manipulate this duality, DC have been generated ex vivo with a tolerogenic or immunogenic profile since several years. During the last decade, cell therapy using tolerogenic DC (TolDC) has been shown to be safe and effective both in autoimmune diseases and transplantation models in animals. Since 2011, recipient TolDC has been tested in clinical trials in type 1 diabetes, rheumatoid arthritis and Crohn's diseases with favourable results in terms of safety. Indeed, other clinical trials are ongoing including a phase I/II clinical assay in kidney transplantation. In this review, we will discuss the potential of TolDC that has been demonstrated in animal models and used in clinical trials.

Keywords Autologous tolerogenic dendritic cells · Transplantation · Cell therapy · Clinical trial · Safety · Autoimmune diseases

This article is part of the Topical Collection on *Cellular Transplants*

✉ Aurélie Moreau
aurelie.moreau@univ-nantes.fr

[1] INSERM UMR1064, Center for Research in Transplantation and Immunology, Nantes, France

[2] CHU de Nantes, Institut de Transplantation Urologie Nephrologie (ITUN), Nantes, France

[3] Université de Nantes, Nantes, France

Published online: 15 June 2016

Abbreviations

GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL	Interleukin
TGF-β	Transforming growth factor-beta
CD	Cluster of differentiation
DEC	Dendritic and epithelial cells
MHC	Major histocompatibility complex
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
AAV	Adeno-associated virus
PD-1	Programmed cell death protein-1
PDL-1	Programmed cell death ligand-1
IFN-γ	Interferon gamma
HLA	Human leukocyte antigen
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
LAG-3	lymphocyte-activation gene-3
Th	T-cell helper
MOG	Myelin oligodendrocyte glycoprotein

Introduction

The identification in 1973 by Steinman and Cohn of an unusual immune cell in mouse spleen called dendritic cells (DC), demonstrated an essential link between innate and adaptive immunity [1, 2]. This revolutionary finding brought a new perspective to the clearer understanding of immunology. Over following decades, the enormous interest generated by DC led to the identification of different DC subsets with different phenotypes, functionality and ontogeny that can be classified as conventional DC (cDC), plasmacytoid DC (pDC), Langerhans cells and monocyte derived-DC (MoDC) [3]. Despite specific features of each cell, several subsets are potential candidates for cell therapies. This is not only because they link the innate and

adaptive response but they also have the capacity to induce tolerance or immunogenicity for a specific antigen [4]. This duality is essential for the correct regulation of immune response [5]. In fact, it has been demonstrated that ablation of cDC and pDC in the murine model leads to severe autoimmunity [6]. This characteristic to prime the T-cell response or generate tolerance for a specific antigen bestows a special interest on DC for cell therapy in cancer (immunogenicity induction for a cancer antigen) [7], allergy (tolerance induction to harmless substances) [8], autoimmunity (tolerance induction to a self-antigen) [3] and transplantation (tolerance induction to allograft) [5]. Among the different subtypes of DC, autoimmunity and transplantation research are focused on tolerogenic DC (TolDC) as a potential tool for cell therapy [9].

From Tolerance to Autologous Tolerogenic Dendritic Cells

Immune tolerance is understood as a reduction or complete inhibition of immunogenic response against a specific antigen in order to delimit the immune response to foreign and possibly pathogenic organisms or toxins. Tolerance mechanisms depend on the lymphocyte maturation state and could be distinguished in terms of central tolerance and peripheral tolerance. Central tolerance mechanisms occur in the thymus and involve mainly clonal deletion of self-reactive thymocytes and natural regulatory T-cell (nTreg) generation. Peripheral tolerance acts on naive self-reactive T-cells in peripheral tissues by mechanisms such as anergy and apoptosis induction, suppression and induced regulatory T-cell (iTreg) generation [10]. In both cases, tolerance is directed through antigen presentation to the T-cell by an antigen presenting cells (APC) [11].

Several approaches describe different DC populations with tolerogenic characteristics in vivo [12, 13]. In fact, the maintained tolerance in immune-privileged and mucosal sites harbours DC with tolerogenic characteristics. DC regulation is essential to avoid continuous inflammation generated by constant exposure to foreign molecules and peptides [14]. Furthermore, it has been shown that cDC could be manipulated in vivo to induce tolerance. A study performed in NOD (non-obese diabetic) mice models treated with GM-CSF demonstrated diabetes prevention. In this study, the authors showed an increase in splenic $CD8\alpha^{-}CD11c^{+}$ DC in GM-CSF treated mice. After purification, these cells were less immunogenic than the $CD8\alpha^{+}CD11c^{+}$population to induce proliferation of diabetogenic $CD8^{+}$ T-cells [15]. However, the interest in medical research is centred on TolDC generated ex vivo. There is no general consensus for a unique TolDC generation protocol neither in animal models nor in human derived cells. Conceptually, TolDC are defined by several characteristics such as resistance to maturation, production of anti-inflammatory cytokines such as IL-10 and reduced induction of alloantigen T-cell proliferation in mixed lymphocyte reactions (MLR) [16, 17].

Despite obvious differences between autoimmunity and organ transplantation, the strategy to treat both diseases converges in the induction of tolerance. In order to bring TolDC to clinical trials, experimental models demonstrated the efficacy of these cells in autoimmune diseases and transplantation.

Generation of Tolerogenic Dendritic Cells

Several protocols to generate TolDC have been tested in animal models in recent years and have led to similar conclusions. Most of these protocols begin with bone marrow cells (in animals) or monocytes (in humans) cultured with GM-CSF and IL-4. At this point, cytokine cocktails such as IL-10 or TGF-β or drugs are used to generate TolDC. IL-10 stimulated DC (DC10) has been shown to expand Treg in OVA-asthmatic mice [18]. Stimulation with IL-10 or in combination with TGF-β in human monocyte cultures generates TolDC that are able to induce anergy of allogeneic CD4+ memory T-cells [19]. Moreover, immunomodulatory agents such as Vit-D3 [20], rapamycin [21] or dexametasone [16] have been used. In the same way, genetic tools have been used to induce tolerogenic characteristics to DC such as antisense-oligonucleotides or gene transfer. DC treated with CD40, CD80 and CD86 antisense-oligonucleotides (AS-ODN) have been used in the NOD mouse model. Results showed a delaying diabetes onset following DC injection [22]. Another strategy used in the small intestine transplantation model in rats focused on the transfection of human IL-10 gene in GM-CSF/IL-4-induced splenic DC. The results of this study showed prolonged small intestine allograft survival [23]. Our group has adopted a low dose GM-CSF protocol without any other cytokine or drug to generate TolDC in murine models and human cell culture. This protocol was previously described by Lutz in 2000. In this study, the authors showed that CBA mice receiving fully allogeneic B10 fully vascularized heterotropic cardiac allograft and pre-treated with donor low dose GM-CSF-DC induced allograft prolongation. This protocol leads to the generation of immature/maturation-resistant DC with reduced major histocompatibility complex (MHC)II, CD40, CD80, CD86 and absence of DEC205. These TolDC also induce an unresponsiveness of alloantigen-specific T-cell in vitro [24].

Tolerogenic Dendritic Cells in Transplantation

Donor and Recipient Tolerogenic Dendritic Cells in Transplantation Models

Immunosuppression by immunosuppressive drugs (IS) is essential to avoid graft-versus-host and host-versus-graft

diseases. Nevertheless, the immune system in the immunosuppressive state is more prone to develop cancer and infections [25]. This is where more specific therapies are required, such as the use of TolDC therapy to minimize IS therapy and consequently to minimize the risk of IS derived-diseases.

The aim in organ transplantation is to avoid allograft rejection due to donor antigen allorecognition. Allorecognition could take place via three pathways, direct, indirect and semi-direct. On the direct pathway, donor DC presents MHC molecules to recipient T-cells leading to an acute rejection. On the indirect pathway, recipient DC presents donor peptides to T-cells leading to chronic rejection. On the semi-direct pathway, recipient DC presents MHC donor molecules to T-cells [26]. TolDC origin for tolerance induction has been discussed for several years. Additionally, another essential point to evaluate the safety of donor/recipient TolDC therapy is the specificity of tolerance. Tolerance could be induced non-specifically (i.e. inhibiting the expansion of all recipient T-cells) or specifically (i.e. inhibiting the expansion of concrete clones of recipient T-cells). Several approaches have been adopted in order to determine the specificity of donor and recipient TolDC.

In transplantation models, it has been demonstrated over several years that intravenously unpulsed donor-DC injection with deficient costimulatory molecules (CD80 and CD86), similar to TolDC, prolongs cardiac allograft survival in mice. In addition, MLR performed using T-cells from recipient mice with syngeneic, allogeneic and third party alloantigens showed a hyporesponsiveness in a donor-specific manner [27]. Nonetheless, in the absence of immunosuppressive therapy, the injected DC upregulated CD80 and CD86 molecule in lymphoid tissues and the cardiac allograft was finally rejected [27]. Years later, donor TolDC generated with low dose GM-CSF has been seen to prolong cardiac allograft survival and even induce tolerance in the 50 % of recipients in the rat model treated 7 days before transplantation. In this study, $CD4^+$ T-cells purified from the spleen of recipient mice with permanent allograft tolerance showed hyporesponsiveness to the donor antigens [28]. In both cases, donor DC injection was seen to lead to donor-specific tolerance. Furthermore, it has been shown in the murine model that after being injected, donor TolDC have a low viability but tolerance still occurs. In fact, apoptotic bodies are scavenged by recipient DC, processed and peptides presented in a tolerance context to CD4 cells [29]. These results could explain improve graft survival after donor-specific transfusion (DST) still used in clinical kidney transplants [30]. In contrast, the use of donor TolDC increases the risk of injecting slightly contaminant donor cell products. These contaminants could lead to sensitization against donor antigens and consequently to an anti-allograft immune response. Also, donor DC may be recognized by the recipient immune system and deleted by NK cells [31].

Conceptually, the injection of autologous cells has a greater safety profile. The use of TolDC derived from recipient monocytes circumvents the allorecognition problem. In addition to the relevance for minimizing the allorecognition risk, we have demonstrated that the adherent population of recipient DC generated ex vivo with IL-4 and GM-CSF injected the day before transplantation induce longer graft survival compared to donor-DC injection in rats. Two essential facts were retained from this study. Firstly, the clinical relevance of this study is the option to inject DC the day before transplantation. The waiting time between kidney availability and transplantation procedure is only a matter of hours; hence, preventive therapy must be administered as soon as possible. Secondly, these results showed that the use of recipient TolDC could minimize the allorecognition risk without losing the tolerogenic potential [32]. Moreover, in order to consider a possible combined immunosuppressive post-transplantation therapy to align this model with the clinical setting, another study was performed with low doses of the immunosuppressive drug. A suboptimal dose of LF15-0195, an NF-kB blocking agent that inhibits DC maturation, was tested in the transplantation model in rats treated with recipient immature DC. Total allograft acceptance was achieved in 92 % of recipients. This model showed that tolerance induced by recipient TolDC was donor-specific. To demonstrate this fact, tolerant rats received skin grafts from syngeneic, allogeneic and third party donors at 100 days post-heart transplantation. Third party skin was the only rejected graft [33]. More recently, our group demonstrated prolonged graft survival in minor mismatch skin allografts and fully mismatched pancreatic islet transplantations in mice models following the administration of recipient TolDC combined with anti-CD3 treatment. In fact anti-CD3 treatment acts in synergy with TolDC. This concomitantly response enhances $CD4^+$ Treg proliferation in pancreatic islets and $CD8^+$ Treg in skin [34].

For a clearer understanding as to how recipient-TolDC induce specific tolerance, several approaches using pulsed and unpulsed recipient TolDC were performed in mice. The use of rapamycin-induced TolDC pulsed with splenocytes lysate has been seen to induce specific tolerance in the heart allograft transplantation model. In this model, the authors observed that, after adoptive transfer, DC migrate to splenic T-cell areas and suppress AlloAg-specific response leading to survival and even heart allograft tolerance [35]. Strategies using donor-pulsed and unpulsed TolDC have been seen to share similar results. Nevertheless, the use of unpulsed recipient TolDC is enough to induce specific tolerance in transplantation models [32].

Finally, in order to promote the safety of these therapies in humans, TolDC injections were performed in non-human primates. This study demonstrated the tolerance and safety of intradermal and intravenous bone marrow-derived DC injection. At the same time, both administration routes showed a different modulation of host immunity against an immunogenic protein expressed by the rAAV-derived vector. In more concrete terms, IV was shown to be less overwhelming than ID injection [36, 37].

Therefore, studies performed on animal models have demonstrated that TolDC therapy is safe and efficient.

Tolerogenic Dendritic Cells Mechanisms in Transplantation Models

In order to characterize the efficacy of TolDC, several mechanisms have been proposed. To summarize, these mechanisms involve the induction of T-cell anergy, deletion, suppression and the enhancing of regulatory populations. T-cell anergy could be induced directly by DC through the surface molecule PDL-1 in contact with the PD-1 surface molecule in activated T-cells [38, 39]. Mechanisms based on T-cell deletion are mediated through the expression of FasL in the TolDC membrane in contact with the activated T-cell Fas receptor [40]. Several soluble factors secreted by TolDC have been described in relation to maintaining tolerance such as IL-10, TGF-β, IFN −γ [41] and IL-27 [42]. These cytokines have an essential role to play in the expansion of Treg and IL-10-producing T-cells. Other soluble factors are more associated with effector T-cells such as the enzyme indoleamine 2,3-dioxygenase (IDO) and heme-oxygenase-1 (HO-1). IDO is a soluble factor that leads to deletion of L-triptophane in the microenvironment necessary for T-cell proliferation [43]. HO-1 degrades the heme-group to carbon monoxide (CO) leading to inhibition of the effector T-cell [44]. Moreover, it has been shown that TolDC express the Epstein-Barr virus-induced gene 3, a member of IL-12 family essential for the expression of IFN-γ by double negative Treg that plays an important role in avoiding allograft rejection [45, 46]. In addition, TolDC surface proteins involved in alloresponse inhibition could be linked to the generation of regulatory populations. ILT3/4 in relation with HLA-G has been shown to decrease T-cell alloproliferation, T-cell and natural killer cell (NK) functions and to increase the Tr1 population [47]. Tolerance could be induced directly by TolDC through surface molecules or soluble factors but also indirectly by enhancing regulatory populations [48].

The most important role of TolDC in graft tolerance in the long term is its role in expanding regulatory T-cells (Treg) [49, 50]. Moreover, a tolerogenic feedback is generated between TolDC and Treg. In fact, expanded Treg deliver signals to DC to maintain the tolerogenic state [51]. Several subtypes of TolDC-stimulated regulatory lymphocytes have been described such as classical $CD4^+CD25^+FoxP3^+$ Treg [18], regulatory T-cell type 1 (Tr-1) [47], $CD8^+$Treg [52] as well as regulatory B-cells [53]. The more documented Treg populations induced by TolDC are natural or induced Treg $CD4^+CD25^+FoxP3^+$ and Tr-1. In transplantation model, it has been showed that DC producing TGF-β and IL-2 induce naive $CD4^+$ differentiation to alloantigen specific-$CD4^+CD25^+FoxP3^+$ Treg [54]. These populations have a protective role in graft-versus-host-disease [55]. Another widely described regulatory population induced by TolDC is $LAG\text{-}3^+CD49b^+CD25^+FoxP3^{-/+}$ Tr-1 [56]. Tr-1 have been shown to express IL-10, IL-4, IL-13 and IFN-γ and display suppressive activity in MLR [56, 57]. Tr-1 have been linked to long term-tolerance in pancreatic islet transplantation [58]. Other lesser described DC-induced regulatory populations are CD8 Treg and Breg. DC-induced CD8 Treg have been described in vivo over several years. In a study published in 2002, two healthy volunteers were injected with DC pulsed with influenza matrix peptide (MP). Samples obtained 7 days after the injection showed a $CD8^+$ T-cell fraction with specific suppressive activity for MP-specific effectors cells [59]. A more recent study performed in non-human primates has highlighted an increase in $Eomesodermin^{lo}CTLA4^{hi}$ $CD8^+$ alloreactive memory T-cells that is, in fact, a population with a specific suppressive profile. This $CD8^+$ population is associated with renal transplant survival [60]. Otherwise, much less is known about the TolDC-Breg axis in transplantation. In the first clinical trial with TolDC in type 1 diabetes, the authors showed that TolDC are able to regulate B-cell functions and expand the regulatory $B220^+CD11c^-$ B-cells population [61•]. This Breg corresponds to a population characterized by the production of anti-inflammatory cytokines as IL-10, increased phagocytic activity and are effective in MLR suppression [53].

Tolerogenic Dendritic Cells in Autoimmune Diseases

Autoimmune disease models support the results observed in transplantation models. In the collagen-induced arthritis model, the administration of TolDC generated with dexametasone and Vit-D3 and pulsed with type II collagen has been seen to significantly reduce disease severity and progression in mice. The authors also observed an increase in IL-10-producing T-cells and a decrease in Th17 T-cells in treated mice. In contrast to other studies, they did not find an increase in $CD4^+FoxP3^+$ Treg [62]. In hypercholesterolemic mice, treatment with TolDC generated with GM-CSF, IL-10 and pulsed with the apolipoprotein B-100 has been shown to inhibit the autoimmune T-cell response against ApoB100 and leads to a reduction in the size of atherosclerotic lesion. Moreover, they observed a reduction in IFN-γ in plasma, a decrease in CD4 infiltration in the atherosclerotic lesion and an increase in $CD4^+FoxP3^+$ Treg [63]. In experimental immune myasthenia gravis, the administration of TolDC has been seen to lead to disease alleviation with an increase in Treg and Breg. For this study, TolDC were generated from bone marrow cultured with GM-CSF, IL-4 and atorvastatin [64]. Recently, a study reported the beneficial effect of TolDC in the experimental autoimmune encephalomyelitis (EAE) model. In this study, TolDC were generated from murine bone marrow cultured with GM-CSF, stimulated with 1α,25-dihidroxyvitamin D3 (Vit D3) and pulsed with MOG peptide. An evaluation of the preventive, pre-clinical and therapeutic use of TolDC in EAE-induced mice showed that the preventive treatment revokes

EAE induction in 75 % of treated mice and pre-clinical and therapeutic treatments improves the clinical course and clinical signs [20].

Clinical Trials with Autologous Tolerogenic Dendritic Cells

DC vaccines are not new to clinical trials. The first study using DC in humans dates back to 1995. In this study, three immunocompetent patients with advanced melanoma were injected intradermally with autologous DC pulsed with MAGE-1 nonapeptide. The results did not revealed any therapeutic response but it seemed reasonably safe [65]. The first evidence of DC-generated biological effect was observed in 1999. In this study, nine healthy subjects were injected subcutaneously with DC pulsed with keyhole limpet hemocyanin (KLH), HLA-A*0201-positive restricted influenza matrix peptide (MP) and tetanus toxoid (TT). The results showed a CD4 response to KLH and TT and CD8 response to MP in most subjects [66]. These data led to the first study using TolDC. In 2001, two subjects were injected with immature DC pulsed with MP and KLH. The results demonstrated MP-specific CD8 inhibition and an increase in MP-specific IL-10 producing cells [67]. In subsequent years, DC therapies were mainly focused on cancer research [7, 68] and anti-viral immunotherapy [69]. Although clinical trials for these conditions have not always led to beneficial treatments for the disease, a lack of severe adverse reactions has been established. Nevertheless, TolDC therapy is now being tested in clinical trials. At present, data have been published from three phase I clinical trials in type 1 diabetes, rheumatoid arthritis and Crohn's disease. Moreover, other clinical trials are ongoing (Table 1). The first phase I clinical trial using TolDC therapy in type 1 diabetic patients was conducted at the University of Pittsburg Medical and Translational Research Center. In this double-blind study, 10 healthy insulin-requiring type I diabetic patients were monitored for 1 year following intradermal injection of ten million cells in the abdomen once every 2 weeks for a total of four administrations. Three of the 10 patients were injected with recipient DC without manipulation meanwhile the other seven patients received autologous DC treated ex vivo with antisense oligonucleotides targeting CD40, CD80 and CD86 primary transcripts. Any adverse effect was recorded. Autologous DC injection was safe and well tolerated. During the study, the unique parameter with a statistically significant variation was the percentage of $B220^{+}$ $CD11c^{-}$ B-cells during the first 6 weeks of treatment [61•]. This corresponds to a regulatory B-cell population [70]. The second phase I clinical trial with TolDC was performed in rheumatoid arthritis (RA) patients. In this study, 18 human leukocyte antigen (HLA)-positive RA patients with minimal disease activity were treated with a single dose of DC. Patients were treated with two doses of DC (1 million or 5 million cells) modified with Bay11-7082, an NF-kB inhibitor and pulsed and exposed to four citrulinated peptide antigens named "rheumavax". The results of this study demonstrated the safety of DC injection and a significant biological activity. The authors described an increase in circulating regulatory T-cells, a decrease in serum cytokines IL-15, IL-29, CX3CL1 and CXCL11 and a decrease in T-cell IL-6 response against $vimentin_{447\text{-}455}$-Cit450 [71••]. The third published phase I study was performed in refractory Crohn's disease patients. In this clinical trial, 12 patients were divided into six cohorts (two patients per cohort) [72••]. Three cohorts received a single injection of TolDC administrated by sonography-guided intraperitoneal injection at escalating doses (2, 5 or 10 million cells) and the other three received biweekly injections. During the study, three patients withdrew due to a disease worsening. The TolDC used for this study were stimulated with dexamethasone [73]. The study was followed up for 1 year. During the study, the authors described an increase in circulating regulatory T-cells and a decrease in IFN-γ production. Nevertheless, no correlation with the clinical response was found. The study demonstrated the safety of TolDC injection [72••]. Apart from these three clinical trials, TolDC were also studied in patients with multiple sclerosis. In this report, TolDC were generated from monocytes of relapsing-remitting multiple sclerosis (RR-MS) or healthy patients cultured with GM-CSF, IL-4, VitD3 and loaded with myelin peptides. Results showed that both healthy and RR-MS TolDC are maturation-resistant and induce hyporesponsiveness to allogeneic T-cells. Furthermore, the authors described hyporesponsiveness to autologous memory T-cells in RR-MS patients in an antigen-specific manner [74].

At present, these are the only clinical trials with published data. Nevertheless, other clinical trials are ongoing, and others are about to begin, asthma studies in Massachusetts General Hospital (NCT01711593), multiple sclerosis and neuromyelitis optica at the Hospital Clinic of Barcelona (NCT02283671) and University Hospital, Antwerp (NCT02618902), rheumatoid and inflammatory arthritis at the Newcastle University (NCT01352858) and kidney transplantation, the first phase I/II clinical trial in transplantations in humans, at the Nantes University Hospital (NCT02252055) as part of the ONE Study consortum [75].

Tolerogenic Dendritic Cells in Kidney Transplantation

The ONE study project aims to develop and trial various immunoregulatory cell products in organ transplantation recipients such as Treg, regulatory macrophages (Mreg), Tr1 and TolDC. The results of all these studies will allow direct comparison of the safety, clinical practicality and therapeutic efficacy of each immunoregulatory cell type (www.onestudy.org)

Table 1 Clinical trials of TolDC immunotherapy

Clinical trial	Number of patients	Conditions	Injection/administration route	Number of injected cells	Protocol	Properties	Safety profile
Type 1 diabetes	10 healthy insulin-requiring type 1 diabetes	3 patients injected with TolDC nonmanipulated 7 patients injected with TolDC manipulated	•Biweekly •Intradermal	10 million	Recipient TolDC modified with ODN anti-CD40/80/86	•Increase in B220 during the first 6 weeks of treatment	No adverse effects observed
Rheumatoid arthritis	18 HLA-positive RA patients with minimal disease activity	9 patients treated with a high dose and 9 with a low dose of TolDC	•Single dose •Intradermal	Low dose: 1 million TolDC; high dose: 5 million TolDC	TolDC modified with NF-kB inhibitor and pulsed with 4 citrullinated peptides	•Increase in circulating regulatory T cells •Decrease in serum cytokines IL-15, IL-29, CX3CL1, and CXCL11 •Decrease in T cell IL-6 response against vimentin$_{447\text{-}455}$-Cit450	No adverse effects observed
Refractory Crohn's disease	12 refractory Crohn's disease patients	3 cohorts receiving 2, 5, or 10 million TolDC in a single dose and 3 cohorts biweekly; 2 patients in each cohort	•Single dose or biweekly •Intraperitoneal	2, 5, or 10 million	TolDC stimulated with dexamethasone and cytokine cocktail (IL-6, TNF-α, IL-1β, prostaglandin E_2)	•Increase in circulating regulatory T cells •Decrease in IFN-γ in blood	3 patients withdrew because of a disease worsening. No adverse effects observed in the other 6 patients
Asthma (NCT01711593)	Estimated enrolment of 20 allergic asthmatic patients and 10 healthy controls				Immune-tolerant DC (it-DC)	•No results yet	Ongoing
Multiple sclerosis and neuromyelitis optica (NCT02283671)	Estimated enrolment of 12 patients with MS or neuromyelitis optica		•Biweekly •Intravenous		TolDC loaded with myelin peptides	•No results yet	Ongoing
Multiple sclerosis (NCT02618902)	Estimated enrolment of 9 patients with MS		•Intradermal	5, 10, or 15 million cells	Myelin-derived peptide-pulsed TolDC	•No recruitment yet	No recruitment yet
Rheumatoid and inflammatory arthritis (NCT01352858)	13 patients with inflammatory arthritis and an inflamed knee	10 participants in 3 cohorts receiving 1, 3, or 10 million TolDC and 3 placebo	•Single dose •Intra-articular	1, 3, or 10 million cells	TolDC loaded with autologous synovial fluid	•Ongoing	Ongoing
Kidney transplantation (NCT02252055)	Estimated enrolment of 16 living-donor renal transplant patients with renal insufficiency		•A single dose before transplantation •Intravenous	1 million cells/kg body weight	Low-dose GM-CSF TolDC	•No results yet	Ongoing

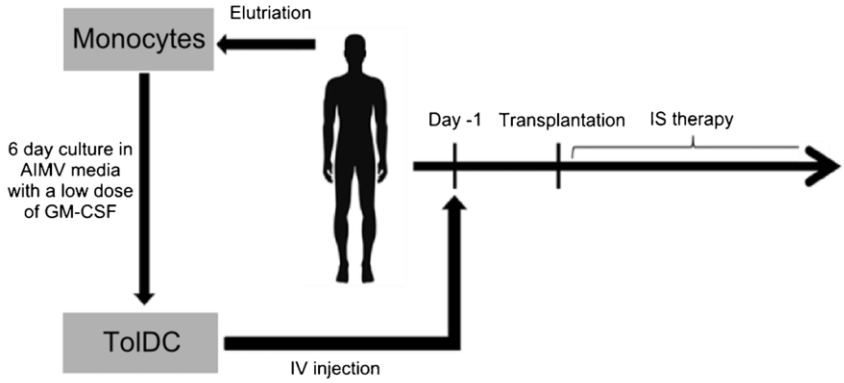


Fig. 1 Recipient TolDC therapy in kidney transplantation. DC therapy used in ongoing kidney transplantation clinical trial. TolDC are generated ex vivo with low dose GM-CSF and injected the day before the transplantation. After the procedure, patients are treated with IS drugs

[76]. As part of this international project (ONE study), we developed a phase I/II clinical trial in kidney transplantation. The clinical protocol for this trial consists in the intravenous injection of 10^6TolDC/kg body weight to patients the day before kidney transplantation. Individuals enrolled for this study are patients with renal insufficiency receiving as a first transplantation a kidney transplant from a living donor. The transplantation patients are subsequently treated with IS therapy, prednisolone, mycophenolate mofetil and tacrolimus (Fig. 1). The TolDC used for this phase I/II clinical trial was generated from monocytes elutriated from the recipient blood. Monocytes are cultured for 6 days in AIMV media with low dose of GM-CSF. We expect the results of this ongoing clinical trial to corroborate safety observed in the autoimmune disease clinical trials.

Conclusion

There is a vast evidence to demonstrate the potential of TolDC in cell therapy. Animal models have shown that TolDC injection suppresses the development of several autoimmune diseases and leads to tolerance in allograft transplantation. In humans, only three phase I clinical trials have reported results in TolDC therapy, and several others are ongoing. At present, despite the small number of clinical trials, all of them have confirmed the safety of TolDC. Furthermore, increased populations of regulatory cells have also been highlighted in blood of RA patients and type-1 diabetes. Other clinical trials are required in order to investigate the potency of TolDC. Moreover, future clinical trials must focus on identifying the mechanisms used by TolDC therapy in humans.

Acknowledgments The work performed in the INSERM Unit 1064 and presented in this review was funded by IMBIO-DC, Fondation Progreffe, DHU Oncogreffe, The ONE Study (FP7-260687) and BIODRIM (FP7-305147) European Union 7th Framework Programs. The work of INSERM U1064 was also supported by funds from IHU-CESTI (Investissement d'Avenir ANR-10-IBHU-005, Région Pays de la Loire and Nantes Métropole) and the Labex IGO project (n° ANR-11-LABX-0016-01).

Compliance with Ethical Standards

Conflict of Interest Maria Cristina Cuturi, Eros Marín and Aurélie Moreau declare no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Papers of particular interest, published recently, have been highlighted as:
•O f importance
•Of major importance

1. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med. 1973;137:1142–62.
2. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. J Exp Med. 1974;139:380–97.
3. Mbongue J, Nicholas D, Firek A, Langridge W. The role of dendritic cells in tissue-specific autoimmunity. J Immunol Res. 2014;2014:857143.
4. Hubo M, Trinschek B, Kryczanowsky F, Tuettenberg A, Steinbrink K, Jonuleit H. Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. Front Immunol. 2013;4:1–14.
5. Ezzelarab M, Thomson AW. Tolerogenic dendritic cells and their role in transplantation. Semin Immunol. 2011;23:252–63.
6. Ohnmacht C, Pullner A, King SBS, Drexler I, Meier S, Brocker T, et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. J Exp Med. 2009;206:549–59.
7. Butterfield LH. Dendritic cells in cancer immunotherapy clinical trials: are we making progress? Front Immunol. 2013;4:452.
8. Maldonado RA, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. Adv Immunol. 2010;108:111-65.
9. Yang J, Bernier SM, Ichim TE, Li M, Xia X, Zhou D, et al. LF15-0195 generates tolerogenic dendritic cells by suppression of NF-kB signaling through inhibition of IKK activity that inhibited Th1 polarization and increased Th2. J Leukoc Biol. 2003;74:438–47.

10. Bluestone JA. Mechanisms of tolerance. Immunol Rev. 2011;241:5–19.
11. Thompson AG, Thomas R. Induction of immune tolerance by dendritic cells: implications for preventative and therapeutic immunotherapy of autoimmune disease. Immunol Cell Biol. 2002;80:509–19.
12. Faivre V, Lukaszewicz AC, Alves A, Charron D, Payen D, Haziot A. Human monocytes differentiate into dendritic cells subsets that induce anergic and regulatory T cells in sepsis. PLoS One. 2012;7:e47209.
13. Louvet C, Chiffoleau E, Heslan M, Tesson L, Heslan J-M, Brion R, et al. Identification of a new member of the CD20/FceRIb family overexpressed in tolerated allografts. Am J Transplant. 2005;5:2143–53.
14. Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. Blood. 2006;108:1435–40.
15. Gaudreau S, Guindi C, Ménard M, Besin G, Dupuis G, Amrani A. Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of $CD4^+CD25^+$ regulatory T cells. J Immunol. 2007;179:3638–47.
16. Naranjo-Gómez M, Raïch-Regué D, Oñate C, Grau-López L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. J. Transl. Med. 2011;9:89.
17. Moreau A, Varey E, Bouchet-Delbos L, Cuturi MC. Cell therapy using tolerogenic dendritic cells in transplantation. Transplant Res. 2012;1:13.
18. Huang H, Dawicki W, Zhang X, Town J, Gordon JR. Tolerogenic dendritic cells induce $CD4^+CD25^{hi}Foxp3^+$ regulatory T cell differentiation from $CD4^+CD25^{-/lo}Foxp3^-$ effector T cells. J Immunol. 2010;185:5003–10.
19. Torres-Aguilar H, Aguilar-Ruiz SR, González-Pérez G, Munguía R, Bajaña S, Meraz-Ríos M a, et al. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory $CD4^+$ T cells. J. Immunol. 2010;184:1765–75.
20. Mansilla MJ, Sellès-Moreno C, Fàbregas-Puig S, Amoedo J, Navarro-Barriuso J, Teniente-Serra A, et al. Beneficial effect of tolerogenic dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. CNS Neurosci Ther. 2015;21:222–30.
21. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic $CD4^+$ T Cells, but enrich for antigen-specific $Foxp3^+$ T regulatory cells and promote organ transplant tolerance. J Immunol. 2007;178:7018–31.
22. Machen J, Harnaha J, Lakomy R, Styche A, Trucco M, Giannoukakis N. Antisense oligonucleotides down-regulating costimulation confer diabetes-preventive properties to nonobese diabetic mouse dendritic cells. J Immunol. 2004;173:4331–41.
23. Zhu M, Wei MF, Liu F, Shi HF, Wang G. Interleukin-10 modified dendritic cells induce allo-hyporesponsiveness and prolong small intestine allograft survival. World J Gastroenterol. 2003;9:2509–12.
24. Lutz MB, Suri RM, Niimi M, Ogilvie ALJ, Kukutsch NA, Rössner S, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. Eur J Immunol. 2000;30:1813–22.
25. Gallagher MP, Kelly PJ, Jardine M, Perkovic V, Cass A, Craig JC, et al. Long-term cancer risk of immunosuppressive regimens after kidney transplantation. J Am Soc Nephrol. 2010;21:852–8.
26. Herrera OB, Golshayan D, Tibbott R, Ochoa FS, James MJ, Marelli-Berg FM, et al. A novel pathway of alloantigen presentation by dendritic cells. J Immunol. 2004;173:4828–37.
27. Fu F, Li Y, Qian S, Lu L, Chambers F, Starzl TE, et al. Costimulatory molecule-deficient dendritic cell progenitors (MHC class II+, CD80dim, CD86-) prolong cardiac allograft survival in nonimmunosuppressed recipients. Transplantation. 1996;62:659–65.
28. DePaz H a, Oluwole OO, Adeyeri AO, Witkowski P, Jin MX, Hardy MA, et al. Immature rat myeloid dendritic cells generated in low-dose granulocyte macrophage-colony stimulating factor prolong donor-specific rat cardiac allograft survival. Transplantation. 2003;75:521–8.
29. Divito SJ, Wang Z, Shufesky WJ, Liu Q, Tkacheva OA, Montecalvo A, et al. Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. Blood. 2010;116:2694–705.
30. Rainienė T, Izvolskaja N, Dainys B, Kučinskis G, Razukas V. Donor-specific transfusions as a way of tolerance induction to living donor kidney transplant. Biologija. 2009;55:99–104.
31. Yu G, Xu X, Vu MD, Kilpatrick ED, Li XC. NK cells promote transplant tolerance by killing donor antigen-presenting cells. J Exp Med. 2006;203:1851–8.
32. Pêche H, Trinité B, Martinet B, Cuturi MC. Prolongation of heart allograft survival by immature dendritic cells generated from recipient type bone marrow progenitors. Am J Transplant. 2005;5:255–67.
33. Bériou G, Pêche H, Guillonneau C, Merieau E, Cuturi MC. Donor-specific allograft tolerance by administration of recipient-derived immature dendritic cells and suboptimal immunosuppression. Transplantation. 2005;79:969–72.
34. Baas MC, Kuhn C, Valette F, Mangez C, Duarte MS, Hill M, et al. Combining autologous dendritic cell therapy with CD3 antibodies promotes regulatory T cells and permanent islet allograft acceptance. J Immunol. 2014;193:4696–703.
35. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce Ag-specific T cell regulation and prolong graft survival. Am J Transplant. 2005;5:228–36.
36. Moreau A, Vandamme C, Segovia M, Devaux M, Guilbaud M, Tilly G, et al. Generation and in vivo evaluation of IL10-treated dendritic cells in a nonhuman primate model of AAV-based gene transfer. Mol Ther — Methods Clin Dev. 2014;1:14028.
37. Moreau A, Chiffoleau E, Beriou G, Deschamps J-Y, Heslan M, Ashton-Chess J, et al. Superiority of bone marrow-derived dendritic cells over monocyte-derived ones for the expansion of regulatory T cells in the Macaque. Transplantation. 2008;85:1351–6.
38. Chikuma S, Terawaki S, Hayashi T, Nabeshima R, Yoshida T, Shibayama S, et al. PD-1-mediated suppression of IL-2 production induces $CD8^+$ T cell anergy in vivo. J Immunol. 2009;182:6682–9.
39. Huang H, Ma Y, Dawicki W, Zhang X, Gordon JR. Comparison of induced versus natural regulatory T cells of the same TCR specificity for induction of tolerance to an environmental antigen. J Immunol. 2013;191:1136–43.
40. Baatar D, Olkhanud P, Sumitomo K, Taub D, Gress R, Biragyn A. Human peripheral blood T regulatory cells (Tregs), functionally primed $CCR4^+$ Tregs and unprimed $CCR4^-$ Tregs, regulate effector T cells using FasL. J Immunol. 2007;178:4891–900.
41. Schinnerling K, García-González P, Aguillón JC. Gene expression profiling of human monocyte-derived dendritic cells—searching for molecular regulators of tolerogenicity. Front Immunol. 2015;6:1–10.
42. Awasthi A, Carrier Y, Peron JPS, Bettelli E, Kamanaka M, Flavell RA, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol. 2007;8:1380–9.
43. Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, et al. IDO expands human $CD4^+CD25^{high}$ regulatory T cells by promoting maturation of LPS-treated dendritic cells. Eur J Immunol. 2007;37:3054–62.
44. Moreau a, Hill M, Thébault P, Deschamps JY, Chiffoleau E, Chauveau C, et al. Tolerogenic dendritic cells actively inhibit T cells through heme oxygenase-1 in rodents and in nonhuman primates. FASEB J. 2009;23:3070–7.
45. Hill M, Thebault P, Segovia M, Louvet C, Bériou G, Tilly G, et al. Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and Epstein-Barr virus-induced gene 3. Am J Transplant. 2011;11:2036–45.
46. Dixon KO, van der Kooij SW, Vignali DAA, van Kooten C. Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. Eur J Immunol. 2015;45:1736–47.

47. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10 – dependent ILT4 / HLA-G pathway. Blood. 2010;116:935–44.
48. Svajger U, Rozman P. Tolerogenic dendritic cells: molecular and cellular mechanisms in transplantation. J Leukoc Biol. 2014;95:53–69.
49. Zou T, Caton AJ, Koretzky GA, Kambayashi T. Dendritic cells induce regulatory T cell proliferation through antigen-dependent and -independent interactions. J Immunol. 2010;185:2790–9.
50. Mahnke K, Johnson TS, Ring S, Enk AH. Tolerogenic dendritic cells and regulatory T cells: a two-way relationship. J Dermatol Sci Elsevier. 2016;46:159–67.
51. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3^{+} natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc Natl Acad Sci U S A. 2008;105:10113–8.
52. Hsu SM, Mathew R, Taylor AW, Stein-Streilein J. Ex-vivo tolerogenic F4/80+ antigen-presenting cells (APC) induce efferent CD8^{+} regulatory T cell-dependent suppression of experimental autoimmune uveitis. Clin Exp Immunol. 2014;176:37–48.
53. Qian L, Qian C, Chen Y, Bai Y, Bao Y, Lu L, et al. Regulatory dendritic cells program B cells to differentiate into CD19^{hi} FcgIIb hi regulatory B cells through IFN-b and CD40L. Blood. 2012;120:581–91.
54. Yang H, Cheng EY, Sharma VK, Lagman M, Chang C, Song P, et al. Dendritic cells with TGF-b1 and IL-2 differentiate naïve CD4^{+} T cells into alloantigen specific and allograft protective FoxP3 regulatory T cells. Transplantation. 2012;93:580–8.
55. Beres AJ, Drobyski WR. The role of regulatory T cells in the biology of graft versus host disease. Front Immunol. 2013;4:163.
56. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat Med. 2013;19:739–46.
57. Vicente R, Quentin J, Mausset-Bonnefont AL, Chuchana P, Martire D, Cren M, et al. Nonclassical CD4^{+}CD49b^{+} regulatory T cells as a better alternative to conventional CD4^{+}CD25^{+} T cells to dampen arthritis severity. J Immunol. 2016;196:298–309.
58. Gagliani N, Jofra T, Valle A, Stabilini A, Morsiani C, Gregori S, et al. Transplant tolerance to pancreatic islets is initiated in the graft and sustained in the spleen. Am J Transplant. 2013;13:1963–75.
59. Dhodapkar MV, Steinman RM. Antigen-bearing immature dendritic cells induce peptide-specific CD8^{+} regulatory T cells in vivo in humans. Blood. 2002;100:174–7.
60. Ezzelarab MB, Lu L, Guo H, Zahorchak AF, Shufesky WF, Cooper DKC, et al. Eomesodermin^{lo} CTLA4^{hi} alloreactive CD8^{+} memory T cells are associated with prolonged renal transplant survival induced by regulatory dendritic cell infusion in CTLA4 immunoglobulin-treated nonhuman primates. Transplantation. 2016;100:91–102.
61.• Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (Safety) Study of autologous tolerogenic dendritic cells in Type 1 diabetic patients. Diabetes Care. 2011;34:2026–32. **First clinical study using TolDC. This study showed the safety of TolDC in type-1 diabetes patients**.
62. Stoop JN, Harry RA, Von Delwig A, Isaacs JD, Robinson JH, Hilkens CMU. Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses. Arthritis Rheum. 2010;62:3656–65.
63. Hermansson A, Johansson DK, Ketelhuth DFJ, Andersson J, Zhou X, Hansson GK. Immunotherapy with tolerogenic apolipoprotein B-100-loaded dendritic cells attenuates atherosclerosis in hypercholesterolemic mice. Circulation. 2011;123:1083–91.
64. Li H, Wang C-C, Zhang M, Li X-L, Zhang P, Yue L-T, et al. Statin-modified dendritic cells regulate humoral immunity in experimental autoimmune myasthenia gravis. Mol. Cell. Neurosci. 2015;68:284–92.
65. Mukherji B, Chakraborty NG, Yamasaki S, Okino T, Yamase H, Sporn JR, et al. Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. Proc Natl Acad Sci U S A. 1995;92:8078–82.
66. Dhodapkar MV, Steinman RM, Sapp M, Desai H, Fossella C, Krasovsky J, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest. 1999;104:173–80.
67. Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med. 2001;193:233–8.
68. Palmer DH, Midgley RS, Mirza N, Torr EE, Ahmed F, Steele JC, et al. A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. Hepatology. 2009;49:124–32.
69. Gowans EJ, Roberts S, Jones K, Dinatale I, Latour PA, Chua B, et al. A phase i clinical trial of dendritic cell immunotherapy in HCV-infected individuals. J. Hepatol. 2010;53:599–607.
70. Di Caro V, Phillips B, Engman C, Harnaha J, Trucco M, Giannoukakis N. Involvement of suppressive B-lymphocytes in the mechanism of tolerogenic dendritic cell reversal of type 1 diabetes in NOD mice. PLoS One. 2014;9:e83575.
71.•• Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype–positive rheumatoid arthritis patients. Sci. Transl. Med. 2015;7:290ra87–290ra87. **First clinical trial using TolDC in remitting-relapsing multiple sclerosis. This study showed the safety of TolDC in patients with RR-MS disease. The authors observed biological activity**.
72.•• Jauregui-Amezaga A, Cabezón R, Ramírez-Morros A, España C, Rimola J, Bru C, et al. Intraperitoneal administration of autologous tolerogenic dendritic cells for refractory Crohn's disease: a phase I study. J. Crohns. Colitis. 2015;9:1071–8. **First clinical trial using TolDC in Crohn's disease. This study showed the safety of TolDC in patients with refractory Crohn's disease**.
73. Cabezón R, Ricart E, España C, Panés J, Benitez-Ribas D. Gram-negative enterobacteria induce tolerogenic maturation in dexamethasone conditioned dendritic cells. PLoS One. 2012;7:e52456.
74. Raïch-Regué D, Grau-López L, Naranjo-Gómez M, Ramo-Tello C, Pujol-Borrell R, Martínez-Cáceres E, et al. Stable antigen-specific T-cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients. Eur J Immunol. 2012;42:771–82.
75. Moreau A, Varey E, Bériou G, Hill M, Bouchet-Delbos L, Segovia M, et al. Tolerogenic dendritic cells and negative vaccination in transplantation: from rodents to clinical trials. Front Immunol. 2012;3:218.
76. Geissler EK, The ONE. Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. Transplant Res. 2012;1:11.

Annex II. Autologous Tolerogenic dendritic cells in solid organ transplantation: Where do we stand?

frontiers in Immunology

REVIEW
published: 19 February 2018
doi: 10.3389/fimmu.2018.00274

Tolerogenic Dendritic Cells in Solid Organ Transplantation: Where Do We Stand?

*Eros Marín[1,2], Maria Cristina Cuturi[1,2] and Aurélie Moreau[1,2]**

[1] Centre de Recherche en Transplantation et Immunologie UMR1064, INSERM, Université de Nantes, Nantes, France, [2] Institut de Transplantation Urologie Nephrologie (ITUN), CHU Nantes, Nantes, France

Over the past century, solid organ transplantation has been improved both at a surgical and postoperative level. However, despite the improvement in efficiency, safety, and survival, we are still far from obtaining full acceptance of all kinds of allograft in the absence of concomitant treatments. Today, transplanted patients are treated with immunosuppressive drugs (IS) to minimize immunological response in order to prevent graft rejection. Nevertheless, the lack of specificity of IS leads to an increase in the risk of cancer and infections. At this point, cell therapies have been shown as a novel promising resource to minimize the use of IS in transplantation. The main strength of cell therapy is the opportunity to generate allograft-specific tolerance, promoting in this way long-term allograft survival. Among several other regulatory cell types, tolerogenic monocyte-derived dendritic cells (Tol-MoDCs) appear to be an interesting candidate for cell therapy due to their ability to perform specific antigen presentation and to polarize immune response to immunotolerance. In this review, we describe the characteristics and the mechanisms of action of both human Tol-MoDCs and rodent tolerogenic bone marrow-derived DCs (Tol-BMDCs). Furthermore, studies performed in transplantation models in rodents and non-human primates corroborate the potential of Tol-BMDCs for immunoregulation. In consequence, Tol-MoDCs have been recently evaluated in sundry clinical trials in autoimmune diseases and shown to be safe. In addition to autoimmune diseases clinical trials, Tol-MoDC is currently used in the first phase I/II clinical trials in transplantation. Translation of Tol-MoDCs to clinical application in transplantation will also be discussed in this review.

Keywords: autologous tolerogenic dendritic cells, transplantation, cell therapy, clinical trial, safety, mechanisms

OPEN ACCESS

Edited by:
Daniel Hawiger, Saint Louis University, United States

Reviewed by:
Alain Le Moine, Université libre de Bruxelles, Belgium
Bruce Milne Hall, University of New South Wales, Australia

****Correspondence:***
Aurélie Moreau
aurelie.moreau@univ-nantes.fr

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *31 October 2017*
Accepted: *30 January 2018*
Published: *19 February 2018*

Citation:
Marín E, Cuturi MC and Moreau A (2018) Tolerogenic Dendritic Cells in Solid Organ Transplantation: Where Do We Stand? Front. Immunol. 9:274. doi: 10.3389/fimmu.2018.00274

INTRODUCTION

More than half a century has passed since the first successful renal transplantation at the Peter Bent Brigham Hospital in Boston. The procedure performed by Joseph Murray's team showed for the first time the surgical feasibility of solid organ transplantation, at least between identical twins (1). Parallel to this achievement, research on immunosuppressive drugs (IS) demonstrated that 6-mercaptopurine (6-MP), a drug already used to treat acute lymphocytic leukemia, was able to impair immune response (2). These novel concepts of feasibility of solid organ transplantation and immunosuppressive treatment to avoid graft-versus host disease opened the doors for unrelated organ transplantation. Over the following years, advances in IS research led to the replacement of

6-MP, which is highly toxic, by cyclosporine, leading to an increase in one-year graft survival (3). Nowadays, more specific IS are being used to treat post-transplanted patients, such as mophetil mycophenolate, a B and T-cell proliferation inhibitor; tacrolimus, a B and T-cell activation inhibitor (4), and monoclonal antibodies, such as basiliximab, an IL2Rα (CD25) blocking antibody (5). However, although IS treatments favor allograft survival, these treatments are also associated with an increased risk of cancer and infections associated to the immunosuppressive state (6). Moreover, IS primarily prevents the acute rejection of allografts, whereas their efficacy in chronic rejection remains difficult to predict (7). A novel and promising strategy to minimize drugs treatment and control of chronic rejection is to combine reduced amounts of IS with immunoregulatory cell therapy in solid organ transplantation.

Cell therapy for solid organ transplantation could be performed with mesenchymal stem cells (MSC), regulatory macrophages (Mreg), tolerogenic monocyte-derived dendritic cells (Tol-MoDCs), and regulatory T (Treg) and B (Breg) cells (8). The common characteristic between these different cells is that they have been already tested in transplantation models in animals showing a benefit in terms of safety and graft survival. For example, MSC have been shown to delay heart allograft rejection (9). In humans, several clinical trials with MSC have been performed in kidney and liver transplantation (10). Among them, a large trial was carried out to compare MSC to anti-IL2Rα therapy. In this study, the authors showed a lower incidence of acute rejection and a better estimated renal function at 1 year compared to the anti-IL2Rα receiving cohort (11). On the other hand, Mreg have been shown to increase fully allogeneic allograft survival in non-immunosuppressed mice (12). Additionally, Mreg were tested in a clinical trial in living donor renal transplantation. In this study, two patients were treated with Mreg prior to transplantation followed by low doses of tacrolimus. The outcomes of this trial showed that Mreg-treated patients displayed a stable graft function after tacrolimus weaning (13). Tolerogenic bone marrow-derived DCs (Tol-BMDCs) have demonstrated to increase heart, skin, and pancreatic islet allograft survival in combination with IS (14–16). Regarding lymphoid cells, Treg therapy has been shown to be safe and effective in a pilot study in living donor liver transplantation. Indeed, 6 from 10 initial patients in this study were able to stop the immunosuppressive therapy (17). In the context of the ONE study consortium, clinical trials with Treg, Mreg, type 1 Treg cells (Tr1), and Tol-MoDCs are currently performed in living donor kidney transplantation in order to evaluate and compare the safety of these cells in transplantation (www.onestudy.org) (18). In this review, we will focus on both Tol-MoDCs and Tol-BMDCs and their translation to the clinical trial with an emphasis on their characteristics, mechanisms, and safety.

DENDRITIC CELLS

Dendritic cells were discovered by Steinman and Cohn back in 1973 (19, 20). However, the first clinical trial with DC therapy was carried out in 1995 in advanced melanoma patients (21). The reason to use these cells in cell therapy resides in their capacity to present antigens to T cells and to polarize the immune response; in other words, to link the innate and adaptive response (22). DCs are potent antigen presenting cells (APC), able to induce either immunity or tolerance. The first studies about the functions and characteristics of DCs demonstrated that DCs were strong stimulators of T cell response in allogeneic MLR. Additionally, the authors demonstrated the capacity of these cells to induce antigen-specific proliferation (23). Over the following years, different subsets with different ontogenies and functions have been characterized in DCs, such as conventional DC (cDC), plasmacytoid DC (pDC), Langerhans cells (LC), and inflammatory DCs. cDC commonly located in lymphoid tissues and nonlymphoid tissues are able to present antigen through major histocompatibility complex class II (MHC class II) in rodent and humans. Moreover, cDC can cross-present antigens *via* MHC class I (24). pDC, located usually in peripheral organs, are able to induce T-cell proliferation. However, pDCs are usually known to secrete high amounts of type I interferon (IFN) upon viral infection. Inflammatory DCs, also named MoDCs are derived from monocytes that infiltrate lymphoid and nonlymphoid organs as a consequence of inflammation or infection. Finally, LCs are DC skin-resident cells with the capacity to migrate to skin-draining lymph nodes. Unlike cDC, pDC, and MoDC that share the same precursor (monocyte-DC common precursor), the ontogeny of LC go back to the prenatal origin (25).

Nowadays, it has been demonstrated that the orchestration of all these DC subsets is essential for an adequate physiological response against threats, but also for the preservation of self-tolerance. In fact, it has been demonstrated that the ablation of cDC, pDC, and LCs in a model of transgenic CD11c-CRE mice, leads to a spontaneous autoimmunity (26).

Ex Vivo Generated Tolerogenic DCs

Nowadays, rodent DCs are derived from bone marrow cells, whereas human DCs are derived from monocytes for both immunosuppressive and other therapies. Monocytes are used in humans for convenient reasons as they are more abundant than other DC precursors, and can be also manipulated *ex vivo*. From a pragmatic point of view, DCs can be differentiated *in vitro* as immunogenic or tolerogenic cells depending on the protocol. Immunogenic DCs are characterized by a high expression of costimulatory molecules, such as CD80 and CD86, a production of pro-inflammatory cytokines, such as IL1β, IL-12, and tumor necrosis factor-α (TNFα) and the ability to stimulate T-cell proliferation. In counterpart, tolerogenic DCs weakly express costimulatory molecules, are resistant to maturation, produce immunomodulatory cytokines, such as IL-10 and transforming growth factor-β (TGFβ) and impair T-cell proliferation (**Figure 1**). Both DCs are known to express common markers, such as CD11c, CD11b, or MHC Class I and Class II molecules (27).

As it has been previously mentioned, *in vitro* derived DC can be manipulated *ex vivo* in order to design more accurate therapies. For example, these cells can be loaded with target peptides, such as synthetic nanopeptides of MAGE-1 protein in order to direct immune response against human melanoma cells (21). On the other hand, they can be treated with inhibiting

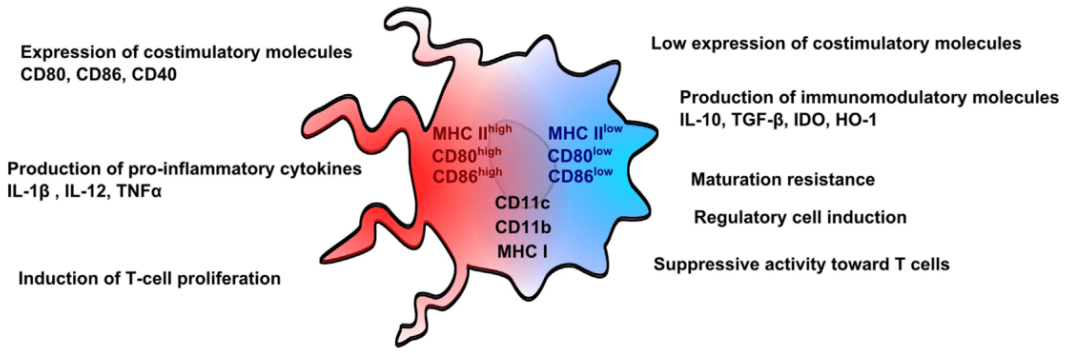


FIGURE 1 | Pro-inflammatory and tolerogenic dendritic cell profile. Pro-inflammatory dendritic cells (DCs) are characterized by a high expression of costimulatory molecules (CD80 and CD86) and pro-inflammatory cytokines and by an ability to stimulate T-cell proliferation. Tolerogenic DCs display a low expression of costimulatory molecules, which are resistant to maturation, and express immunomodulatory molecules. Tolerogenic DCs have also suppressive activity toward T cells and promote regulatory T cells. Both pro-inflammatory and tolerogenic DCs express CD11b, CD11c, and MHCI.

molecules associated to antigen presentation, in order to prevent pro-inflammatory response (28).

Due to this versatility and functional duality, *in vitro* derived DCs have already been used in immunogenic therapy, such as in infections (29) and cancer therapy (30), and immunosuppressive therapy, such as in allergy (31), autoimmunity (32), immunization (33), and more recently in transplantation (34).

GM-CSF is a growth factor related with bone marrow precursor mobilization and DC differentiation (35). However, the role of GM-CSF in tolerance remains unclear as its administration improves some diseases, such as myasthenia gravis, type 1 diabetes (T1D), and colitis, but its depletion improves experimental autoimmune encephalomyelitis (EAE), arthritis, nephritis, and psoriasis in rodent models (36). GM-CSF is a cytokine indispensable for *in vitro* DC generation, which is used both for immunogenic or tolerogenic DC differentiation. This dual role of GM-CSF is dichotomized by the concentration of the cytokine. Indeed, low doses of GM-CSF are associated to tolerogenic phenotypes, whereas high amounts of GM-CSF lead to immunogenic phenotypes (37).

Moreover, there is not a single standardized method to generate Tol-MoDC from monocytes in humans or Tol-BMDCs in rodents apart from GM-CSF and IL-4. Protocols to induce human and rodent tolerogenic DCs usually include several other factors, such as cytokine cocktails, organic molecules, or even clinically approved and experimental drugs (38). For example, IL-10 and TGF-β, two well-known immunomodulatory molecules, have been shown to maintain the immature phenotype of DCs (39, 40). Human Tol-MoDCs generated with IL-10 spontaneously secrete high amounts of IL-10 and are able to impair T-cell proliferation and induce Tr1 cells (41). Similarly, Tol-MoDCs generated with IL-10 and TGF-β from monocytes obtained from T1D patients was able to induce tolerance to insulin antigens. These cells express several DC markers, such as CD83, CD1a, MHC II, but not CD14 (42). Regarding small organic molecules, such as 1α,25-dihydroxyvitamin D3 (1α,25$(OH)_2D_3$ Vit D3), and prostaglandin E2 (PGE2) have been shown to induce Tol-MoDCs (38). Immature DCs treated with Vit D3 are resistant to maturation upon lipopolysaccharide (LPS) stimulation and impair allogeneic T-cell proliferation. In this study, the authors showed that Vit D3 treated MoDCs downregulated CD1a and CD14 markers (43). However, another study demonstrated that Vit D3 differentiated Tol-MoDCs express DC-SIGN (CD209), CD14, but not CD1a (44). PGE2 induces the expression of indoleamine 2,3 dioxygenase (IDO) by DC leading to a production of kynurenine that plays a role in Treg generation and allogeneic response inhibition (45). Tol-MoDCs can also be differentiated in the presence of dexamethasone (Dex) and rapamycin (Rapa). A comparative study determined that both Dex-DCs and Rapa-DCs were able to impair T-cell proliferation, but unlike Dex-DCs, Rapa-DCs displayed a mature DCs phenotype and were not able to produce IL-10 upon LPS stimulation (46). Phenotypically, it has been shown that Dex-DCs have a low expression of CD1a and CD14 and they express CD209 (44). On the other hand, it has been shown that Tol-BMDCs differentiated with Rapa are phenotypically characterized by the expression of CD11b, CD11c, CCR7, and have a low expression of MHC ClassII (47). Furthermore Dex-DCs stimulated with a cytokine cocktail (IL-6, TNFα, IL-1β, and PGE2) have been administered in patients suffering from refractory Crohn's disease. An increase of Treg cells and a decrease of interferon-γ (IFN-γ) in blood were observed following DC injection (48). Other protocols to generate TolDCs, include genetic tools, concretely antisense oligonucleotides (AS-ODN). A study performed in nonobese diabetic (NOD)-mice showed that the injection of TolDCs modified using AS-ODN anti-CD40, CD80, and CD86 delayed diabetes onset (28).

Among these different methods, our group has adopted a protocol to generate tolerogenic DCs from mouse bone marrow cells with low doses of GM-CSF, excluding IL-4 from the classic protocol (16). This protocol, previously described by Lutz et al. (49), allowed obtaining Tol-BMDCs expressing low levels of MHCII, CD40, CD80, and CD86, and displaying resistance to maturation upon LPS stimulation. Furthermore, these Tol-BMDCs impaired allogeneic T-cell proliferation. Lutz et al. demonstrated that these cells were able to increase graft survival following a fully allogeneic vascularized heterotopic cardiac

allograft, whereas we highlighted the potential of Tol-BMDCs in minor antigen skin graft survival. Alternatively, this protocol was adopted in human to generate Tol-MoDCs from blood monocytes, resulting in an equivalent profile (49). Nowadays, we are performing a first phase I/II clinical trial in kidney transplantation using Tol-MoDCs generated with low doses of GM-CSF as described previously (50). Altogether, the common phenotypical observation after tolerogenic DC differentiation showed that due to the heterogeneity of differentiation protocols it is not possible to describe a unique phenotype for these cells. However, the most common markers observed on tolerogenic DCs are CD11c and low expression of MHCII. On the other hand the expression of DC markers CD209 and CD1a, monocyte/macrophage marker CD14, and macrophage marker CD11c are variable.

TOLEROGENIC DC SOURCE

Unlike other diseases or conditions, transplantation involves the allorecognition between the two parts, the graft and the host. Allorecognition refers to an immune response against allogeneic peptides or against MHC molecules (51). The alloresponse could be differentiated depending on the nature of the interaction by direct, indirect, and semi-direct pathways. In the direct pathway, recipient T cells are activated following presentation of allogeneic MHC molecules by donor DCs and this pathway is associated with acute rejection. Indirect pathway refers to the processed allopeptides presentation by recipient DCs to autologous T cells and is usually associated to chronic rejection. On the semi-direct pathway, intact donor MHC molecules are transferred to recipient DCs through cell-to-cell contacts; the cells are then able to stimulate autologous T cells (52). Therefore, in order to avoid these types of rejection two strategies were considered: the infusion of donor-specific antigens in order to generate antigen-specific regulatory cells or in contrast, the minimization of the risk of transfer allogeneic molecules in order to avoid sensitization.

The first alternative is currently used clinically in kidney transplantation. Indeed, donor-specific transfusion (DST) is a procedure in which recipients receive a donor-specific blood transfusion in order to generate tolerance to donor antigens. A study performed in living donor kidney transplantation comparing recipients receiving DST or not, in addition to immunosuppressive therapy, showed a reduction in patients with acute rejection and an increase in patients with optimal renal function at 1 and 10 years after transplantation in the DST group (53). On the other hand, the presence of allogeneic molecules in transplantation is unavoidable and even if the efficacy of DST has been demonstrated, sensitization against HLA can occur and appears as a risk for allograft rejection (54). For this reason, the safety and efficiency of donor and recipient DCs have been discussed in DC-based therapy in transplantation.

As it has been previously mentioned, the work performed by Lutz et al. showed that Tol-BMDCs generated with low dose of GM-CSF induced an increase in allograft survival in recipient CBA mice receiving a cardiac allograft from donor B10 mice and pretreated with donor Tol-BMDCs for 7 days before the transplantation. This prolongation of allograft survival was achieved until day 100 for 70% of mice, meanwhile the mice pretreated with donor Tol-BMDCs receiving a third-party allograft from NZW mice or DC generated with GM-CSF and IL-4 increased graft survival only in 20% of mice. Moreover, in this study the authors showed that T cells cultured with allogeneic Tol-BMDCs remained unresponsive after polyclonal restimulation. These results implied that this unresponsiveness was specific (55). Another study performed by DePaz et al. in rats using donor BMDCs generated with low doses of GM-CSF showed that Tol-BMDC therapy in combination with antilymphocyte serum (ALS) was able to increase rat cardiac allograft survival in 50% of rats up to 200 days. In the same way as the previous work, the authors showed that T cells purified from transplanted mice receiving Tol-BMDCs therapy and ALS were unresponsive to donor antigens, indicating an induction of antigen-specific tolerance (56). Nevertheless, a later study using donor Tol-BMDCs or apoptotic bodies from donor Tol-BMDCs, showed that tolerance was mediated by the presentation of donor peptides (from donor cells or apoptotic bodies) by recipient DC, that inhibits $CD4^+$T-cell activation and favors Treg expansion (57). Altogether these studies demonstrate the similarities of donor Tol-BMDC therapy with DST therapy. Both therapies have been shown to be partly efficient, but on the other hand, the risk of sensitization (including the development of alloantibodies) still remains. Therefore, the use of autologous tolerogenic DCs appears as a better alternative at least in terms of safety because it avoids the risk of sensitization.

In order to determine if autologous tolerogenic DCs shared a closer efficacy with donor tolerogenic DCs in transplantation, several studies have been performed. In 2005, a study performed by our team demonstrated that rat Tol-BMDCs (corresponding to the adherent fraction of rat BMDCs generated with GM-CSF and IL-4) displayed an immature phenotype were maturation resistant and were able to prolong cardiac allograft survival. Interestingly, autologous Tol-BMDCs were more efficient than donor DCs in delaying graft rejection. In this study, autologous Tol-BMDCs were injected the day before the transplantation suggesting that this time of administration was sufficient to pre-treat patients before the intervention (58). We then demonstrated that rats receiving heart allograft and treated with autologous Tol-BMDCs in combination with suboptimal doses of LF15-0195, an nuclear factor-κB (NF-κB) inhibitor, achieved definitive allograft acceptance. Moreover, we demonstrated that this tolerance was donor-specific (59). These results combined demonstrated that autologous Tol-BMDCs are even more efficient than donor Tol-BMDCs and due to its source, conceptually safer.

PROFILING TOLEROGENIC DC THERAPY

Combined Therapy

Previous results have shown that tolerogenic DC therapy could be improved by the addition of a complementary treatment such as ALS. However, more specific drugs have been used showing an improvement of tolerogenic DC therapy.

LF15-0195 is a NF-κB blocking agent that was previously reported to increase cardiac allograft survival in rats in

short-term treatment (60). Moreover, this compound impairs the maturation of DCs (31). In combination, autologous Tol-BMDCs with a suboptimal dose of LF15-0195 induced tolerance to cardiac allograft in 92% of treated rats compared to autologous Tol-BMDCs alone, LF15-0195 alone, or rats treated with Rapa with or without autologous Tol-BMDCs. In order to determine whether this tolerance was specific, donor, recipient, and third-party skin transplantations were performed in tolerant rats. Our results showed that tolerant rats do not reject donor skin graft, but reject third-party skin graft for 16–18 days after transplantation (59). Another efficient combined therapy in transplantation was anti-CD3 antibody. Indeed, it has been demonstrated that the use of monoclonal antibody anti-CD3 leads to an increase of pancreatic islet, skin, and cardiac allograft survival in transplantation models and led to remission in T1D in autoimmune disease models (61, 62). Our results show that the combination of anti-CD3 antibody and autologous Tol-BMDCs therapy led to an increase of pancreatic islet allograft survival, associated with a decrease in $CD4^+$/$CD8^+$ T cell frequency, and an increase in Treg frequency. The relevance of this increased $CD4^+CD25^+FoxP3^+$ Treg frequency and its contribution to allograft survival in this model was demonstrated by the depletion of $CD25^+$ T cells with anti-CD25 antibody (15). We then confirmed the strong potential of autologous Tol-BMDCs and anti-CD3 therapy to prolong allograft survival in a model of minor antigen mismatch skin transplantation. In this model, our group found an increase in regulatory $CD8^+CD11c^+$ T cells associated with this combined therapy (16). Rapa is another drug that demonstrated an improvement of efficacy in collaboration with Tol-BMDCs in transplantation. Indeed, the injection of donor Tol-BMDCs generated with Rapa and pulsed with donor antigens followed by post-operative low doses of Rapa in heart transplantation mouse model demonstrated an increase of allograft survival. This allograft survival was related to an increase in donor-specific $CD4^+CD25^+FoxP3^+$ Treg in the graft. To ensure the specific regulatory activity of these induced Treg, the authors performed an adoptive transfer of purified $CD4^+$ T cells from treated mice to naïve mice receiving heart allograft. Adoptive Treg transfer resulted in an increase in allograft survival, indicating that tolerance was induced by this combined therapy (47). These results altogether demonstrated that autologous Tol-BMDC therapy in combination with specific drugs increased its potency.

Administration Route and Efficacy in Non-Human Primates (NHP)

In terms of therapeutic effects, Tol-BMDCs have been shown to be efficient and safe in rodents. To ensure its safety profile for clinical trial, several works have been performed in NHP. The first study using tolerogenic DCs therapy, performed in a kidney transplantation model in NHP, showed an increase of median graft survival compared to the control group. In this study, rhesus macaques were co-treated with CTLA4-Ig and donor Tol-BMDCs generated with Vit D3 and IL-10, 7 days before the transplantation. This study demonstrated for the first time the safety and the efficiency of intravenously (IV) injected Tol-MoDCs in transplantation in NHP (63). More recently, the same authors demonstrated similar results in kidney transplantation models in NHP using autologous Tol-MoDCs pulsed with allogeneic cell membranes from donor monocytes. In this study, the authors showed an increase of graft median survival in the group treated with pulsed Tol-MoDCs compared to unpulsed Tol-MoDCs group. This improvement in allograft survival was associated with the hyporesponsiveness of T cells to donor antigens resulting in a decrease in systemic IL-17 (64). In addition, other studies have demonstrated the safety and efficacy of Tol-BMDCs in NHP notably in gene therapy. Indeed, we demonstrated the benefits of autologous Tol-BMDCs therapy to reduce immune response against a transgene product in NHP. In this study, autologous Tol-BMDCs were injected IV or intradermally (ID) in order to determine the best administration route. Our results highlighted the superiority of IV route to favor immune tolerance (65). Furthermore, several clinical trials have already been performed, confirming that ID (32, 66), intraperitoneal (48), and IV administration routes were safe and well tolerated in humans.

TOLEROGENIC DC CELLULAR AND MOLECULAR MECHANISMS

Once it has been confirmed that tolerogenic DCs improve allograft survival in rodent models and are safe in humans, the remaining question is to determine the cellular and molecular mechanisms of these cells in transplantation. To understand tolerogenic DC mechanisms (in Tol-BMDC and Tol-MoDC), it is first crucial to define the complexity of solid organ transplantation. Due to the invasiveness of the surgical procedure and the implantation of a foreign organ, even from a close source, different types of immune and non-immune cells are involved in the physiological response following transplantation. This physiological response against allograft will lead in some cases, to three expected types of rejection. The earliest one is the hyperacute rejection, in which, pre-existing recipient antidonor antibodies will react against allograft over the hours following the transplantation. This type of rejection is rare thanks to the control of HLA donor/recipient compatibility. The acute rejection is led by cellular and humoral response against allograft. This type of rejection is usually bypassed by the use of IS. Finally, the chronic rejection is led by cellular and humoral response and associated with memory cells. Chronic rejection is nowadays the main cause of rejection (67). Due to the complexity of the different types of rejection, TolDC therapy in transplantation has been evaluated on these different parameters: the migration to graft and lymphoid organs, the capacity to induce specific regulatory cells, and the ability to impair cellular and humoral response.

Migration

It is well known that DCs have migratory skills that allow reaching different organs in order to exert different functions, depending on the maturation state. At the immature state, DCs express chemokine receptors, such as CCR2, CCR5, CCR6, CXCR4, and CXCR3 and are attracted by inflamed tissues expressing

chemokines, such as CCL2, CCL5, and CCL20. At the inflammation site, DCs become mature due to the stimuli provided by the microenvironment and the antigen intake. Following their maturation, DCs overexpress CCR7 allowing them to migrate to the lymphatic system and reach the lymph nodes through CCL19 and CCL21 chemoattraction, where they present antigens to T cells. In the lymph nodes, a certain percentage of DCs will migrate to other lymphoid organs, such as spleen, thymus, and bone marrow (68). In a recent study performed in an EAE model, *in vivo* imaging of pulsed Tol-BMDCs generated with GM-CSF and VitD3 showed that these cells reached the liver and the spleen at 24 h after IV injection and remain stable for 7 days. A small amount of cells were also found in lymph nodes, thymus, and bone marrow (69). In order to support the importance of migration, DCs transduced with lentiviral vectors coding for CCR7 and IL-10 genes, prolonged cardiac allograft survival in mice, but this delay of rejection did not occur when DCs were transduced with IL-10 or CCR7 only. In this study, the authors also showed that DC transduced with CCR7 were able to migrate to LN and spleen (70). Additionally, in order to expose donor and recipient DC dynamics, a study was performed using intravital imaging in ear skin graft model in mice. In this work, authors showed that after transplantation donor dermal DCs migrate from allograft and are replaced by host DC. After donor antigen intake, these recipient DCs migrate to lymph nodes in order to present antigens to $CD8^+$T cells and prime anti-allograft response. This work suggested the dynamics of DC immunotherapy *in vivo* (71).

T Cell Inhibition

Even if tolerogenic DCs are able to migrate to lymphoid organs, the goal is to avoid the exacerbated proliferation of T cells in those organs and in the long term, the memory, and humoral responses. Conveniently, two common effects between the different works performed with tolerogenic DC therapy in transplantation have been observed: a decrease in the frequency of T cells in spleen, lymph nodes, and graft and an unresponsiveness of splenic T cells in contact to alloantigens (15, 58). This decrease of T-cell proliferation could be related to several tolerogenic DC molecules that lead to apoptosis, anergy, or hyporesponsivenes. There are many proposed mechanisms used by tolerogenic DCs to explain their tolerogenic activity, including contact-dependent and contact-independent mechanisms. Contact-dependent mechanisms include molecules, such as programmed-death-ligand 1 (PD-L1), Fas-Ligand (Fas-L), inducible T-cell costimulator-ligand (ICOS-L), but also other molecules, such as immunoglobulin-like transcript-2 (ILT-2), ILT-3, ILT4, HLA-G, and others. Contact-independent mechanisms could be classified into immunomodulatory cytokines, such as IL-10 and TGF-β, or enzymes that generate immunomodulatory molecules or related to nutrient deprivation, such as IDO, heme-oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS), and arginase 1 (Arg1) (**Figure 2**) (72).

Contact-Dependent Mechanisms

Contact-dependent mechanisms refer to those mechanisms that need contact between lymphocyte and DCs. The inhibition of proliferation through anergy, hyporesponsiveness, or apoptosis and the differentiation of regulatory cells depend in part of the combination of surface molecules and signal integration between both cells (73). As the different types of tolerogenic DCs have different combinations of inhibitory molecules, the following description is based uniquely on contact-dependent mechanisms observed in transplantation models no matter of tolerogenic DCs type.

Inducible T-cell costimulator-ligand, expressed in immature DC, could interact with ICOS expressed by T cells in order to induce a hyporesponse which is not recovered after restimulation (74). However, a recent study in NHP in kidney transplantation using a combinatorial therapy with belatacept and ICOS-Ig human Fc fusion protein, showed no improvement of allograft survival (75).

Another well-known immunomodulatory molecule related to allograft survival and present in DCs is PD-L1. The blockade of PD-L1 accelerates skin allograft rejection in a similar way to that of anti-CTLA4 treatment (76). Similarly, the use of anti-PD-L1 antibody accelerates heterotropic cardiac allograft rejection, abrogating the effect of cytotoxic T-lymphocyte associated protein-4 (CTLA-4)-Ig (77). Moreover, a recent study showed that DCs transfected with adenovirus coding for PD-L1 was able to induce an increase of kidney allograft survival in fully mismatched rats. This improvement was associated with impairment in $CD8^+$ T-cell proliferation and a decrease in pro-inflammatory cytokine production (78).

Interaction between ILT-2/ILT-4 and HLA-G in tolerogenic DC, has been shown to impair allogeneic T-cell proliferation. Nevertheless, ILT4-HLA-G pathway is more related to Treg generation (79).

Fas-ligand is another contact-dependent molecule that impairs T cell response *via* the induction of apoptosis. A study using BMDCs transfected with pBK-CMV coding for Fas-L demonstrated that these cells were able to improve cardiac allograft survival in a mouse model and to inhibit allogeneic MLR proliferation through apoptosis induction (80).

Immunomodulatory Cytokines

Cytokines related to tolerogenic DCs, such as IL-10, TGF-β, and others have been associated with several immunomodulatory functions, such as DCs impairment of maturation, inhibition of T-cell proliferation, and regulatory cell induction.

IL-10 is a well-known immunomodulatory cytokine that has been shown to be essential for the differentiation of several regulatory populations. IL-10 activates the tyrosine kinase IL-10 receptor leading to an activation of signal transducer and activator of transcription 3. This allows an activation of the suppressor of cytokine signaling 3 that inhibits NF-κB translocation leading to a hyporesponsiveness to pro-inflammatory stimuli (81). IL-10 is expressed by tolerogenic DCs under different dynamics depending on the type of tolerogenic DCs. For example, it has been shown that MoDCs generated with IL-10 spontaneously secrete IL-10 (82). However, other Tol-MoDCs require pro-inflammatory stimulation to produce IL-10, such as Dex- and VitD3- generated TolDCs (44). IL-10 leads to a state of anergy of human $CD4^+$T cells in allogeneic MLR and also after polyclonal stimulation with anti-CD3 antibody (83).

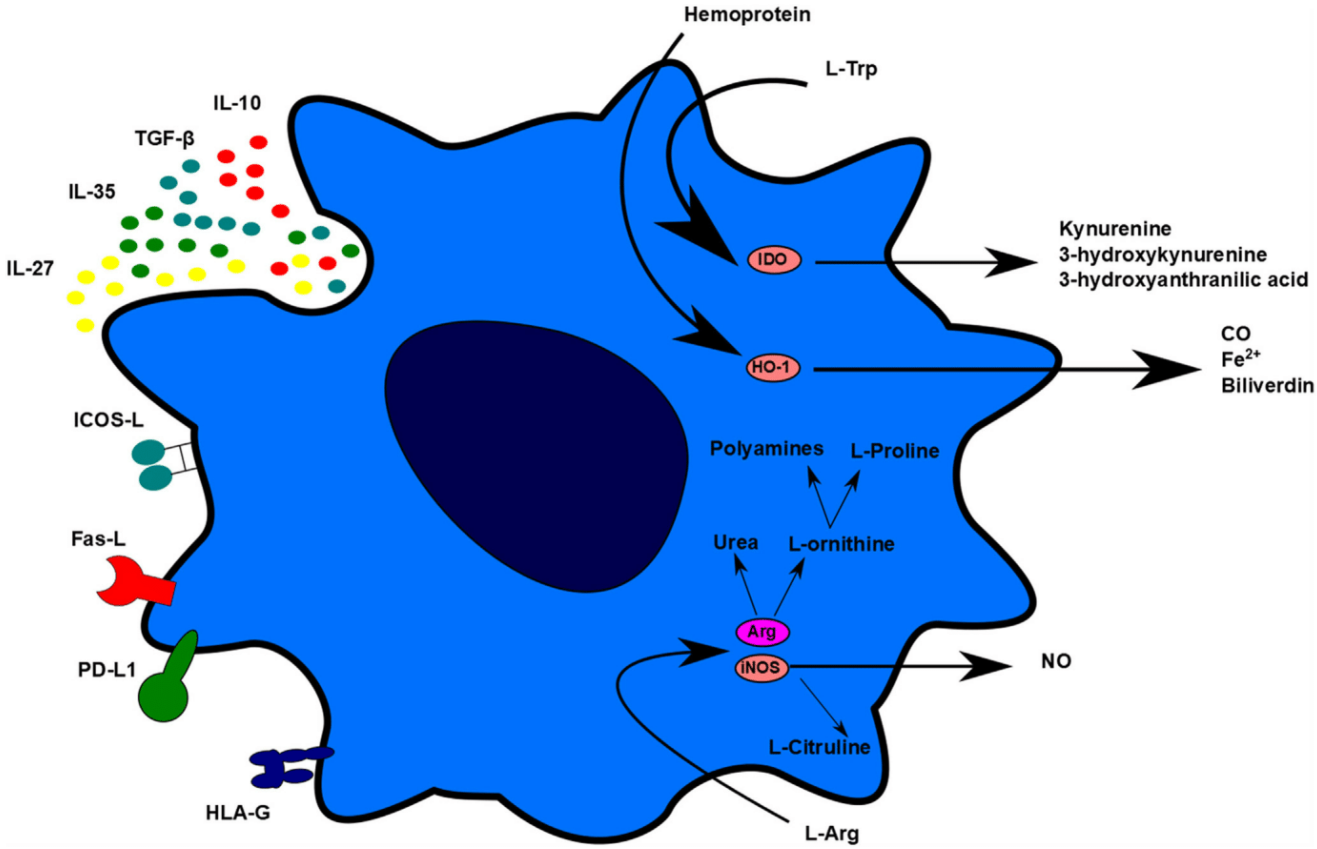


FIGURE 2 | Tolerogenic dendritic cells (DC) mechanisms. Tolerogenic DCs display different mechanisms of action to impair T-cell proliferation and stimulate regulatory cell induction. These mechanisms can be differentiated in contact-dependent and contact-independent mechanisms. Contact-dependent mechanisms include Inducible T-cell costimulator-ligand, Fas, programmed death-ligand 1, and human leukocyte antigen-G. Contact-independent mechanisms include the expression of immunomodulatory cytokines and the production of small molecules, such as carbon monoxide and nitric oxide. TolDC can also act indirectly through L-Arg and L-Trp deprivation.

TGF-β, for its part, is a pleiotropic cytokine related to immunosuppression. In one hand, TGF-β impairs both $CD4^+$ and $CD8^+$ T cell differentiation, activation, and proliferation, and in the other hand, it promotes Treg expansion. In fact, it has been shown that the lack of TGF-β signaling leads to the development of autoimmune inflammatory disease due to an uncontrolled $CD4^+$ activation (84). Moreover, it has been shown that Tol-BMDCs secrete TGF-β and this expression plays a crucial role in tolerance induction tolerance in several models (85). In cardiac allograft model in rat, the induction of tolerance by LF15-0195 is associated with an increase in *tgfb* expression in allograft of tolerant rats. Moreover, the adoptive transfer of splenocytes from tolerant rats to syngeneic rats receiving cardiac allograft and treated with Rapa in the presence or absence of anti-TGF-β blocking Ab showed that the tolerance was transferred and partially mediated through TGF-β (86).

Apart from classical immunomodulatory molecules, some other cytokines are potentially involved in tolerogenic DCs mechanisms. Among these cytokines, two of them share the Epstein–Barr virus-induced gene 3 (EBI3) monomer, IL-35, and IL-27. IL-35, a heterodimer of EBI3 and IL12p35, is related to immunosuppressive activity. Il-35 is mainly secreted by Treg although several studies demonstrated that APCs are also able to produce this cytokine. In fact, it has been shown that IL-35, but not other IL-12 members, is produced by Tol-BMDCs generated with Dex. In this study, the authors showed that the silencing of *Il12a* (IL-12p35) partially impaired the inhibitory effect of Tol-BMDC toward $CD4^+$ T cells (87). On the other hand, IL-27 is a heterodimer composed by EBI3 and IL27p28 that acts through IL-27R (gp130 and WSX1). IL-27 impaired several pro-inflammatory functions leading to a reduced effector T-cell response, a control of neutrophil migration, and an impairment of oxidative burst (88). Nevertheless, it has been suggested a dual role for IL-27 as it displayed a suppressive role in EAE model (89), but enhanced $CD8^+$ T cell anti-tumor activity in other models (90). In transplantation, IL-27 has an important relevance combined with TGF-β1. It has been demonstrated that the overexpression of IL-27 through injection of AAV-IL27 combined with Rapa improved cardiac allograft survival (86). However, monomeric function of EBI3 has been related also with tolerogenic potential in Tol-BMDCs. In fact, in heart allograft rodent model, our work highlighted that mice treated with autologous

Tol-BMDCs and low dose of IS displayed an increase of splenic TCRαβ$^+$CD3$^-$CD4$^-$NKRP1$^-$DN T cells expressing high amounts of IFNγ. The increase of this double-negative regulatory population and the allograft survival were related to the EBI3-expressing autologous Tol-DCs. We showed that *in vivo* blockade of either EBI3 or IFN-γ leads to allograft rejection, demonstrating that these molecules are playing a critical immunoregulatory role in this model of allograft tolerance (14).

Nutrient Deprivation and Other Mechanisms

On the other side, other mechanisms involving interaction between cells or nutrient competition have been observed in transplantation models for several years. These mechanisms open a new perspective on the understanding of graft microenvironment. Among these distinct mechanisms, IDO, iNOS, Arg1, and HO-1 have been related to the impairment of T-cell proliferation.

Inducible nitric oxide synthase and Arg1 are two enzymes commonly associated with macrophages. iNOS is an enzyme that metabolizes arginine and produce nitric oxide (NO) and citrulline, while arginase metabolizes arginine to ornithine and urea. Usually iNOS is known as a M1 macrophage marker and it is induced by pro-inflammatory stimuli, such as IFN-γ. The production of NO by macrophages is usually associated with pro-inflammatory response because this molecule belongs to the Reactive Nitrogen Species (RNS) family that is able to peroxidize membrane lipids in order to eliminate the inflammatory agent. On the other hand, the production of ornithine by M2 macrophages leads to the synthesis of L-Proline, which is essential for collagen production in the resolution of the inflammation (91, 92). However, it has been shown in DCs that these molecules are related to the inhibition of T-cell proliferation. To verify the implication of L-arginine in tolerance and transplantation, several studies were performed. In transplantation, a study demonstrated that the hypoproliferation of T cells isolated from grafted rats treated with Tol-BMDCs was induced by iNOS. Indeed, the use of an iNOS inhibitor (L-NMMA) allowed recovery of T-cell proliferation in treated mice (58). These results showed that iNOS was involved in allograft survival in this model. Similarly, another study demonstrated the relevance of L-arginine metabolism through iNOS and Arg1 in Tol-BMDCs. In this work, tolerogenic DCs were differentiated with retinoic acid (RA) and pulsed with OVA peptide in order to induce *in vivo* lymphoproliferation. The authors showed that Inos$^{-/-}$ RA do not display tolerogenic potential *in vivo* in the presence of OT-II cells. This study corroborated the results observed in transplantation models (93).

Indoleamine 2,3 dioxygenase and tryptophan metabolism, have been suggested as essential factors to inhibit T, B, and NK proliferation and to induce regulatory cells. Paradoxically, it has been shown that IDO is also essential for pro-inflammatory differentiation of DCs (94). A study performed by transfecting human DCs with adenovirus coding for IDO demonstrated that these cells were able to impair T-cell proliferation. Moreover, the study showed that this effect was led by the production of several metabolites of the Kynurenine pathway including kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, but not anthranilic acid nor quinolinic acid (95). Similarly, recent findings demonstrated that IDO$^+$BMDCs improved heart allograft survival in rodent models associated with an impairment of CD4$^+$ response and an increase of apoptosis (96).

Heme-oxygenase-1 is an enzyme that catalyzes the conversion of Fe-Protoporphyrin-IX (Heme group) to biliverdin, ferrous ion, and carbon monoxide (CO) (97). CO is usually associated with protective anti-apoptotic effect in a large range of cells, but in lymphocytes, it is usually associated with impaired proliferation and impaired production of inflammatory cytokines (98, 99). The use of a HO-1 inductor (cobalt protoporphyrin, CoPP) or HO-1 product CO, was already tested in pancreatic islet allograft in mice. Both, the pretreatment of allograft or the pretreatment of recipient with CO or CoPP result in an improvement of allograft survival. Moreover, the delay of graft rejection was even more significant when both recipient and allograft were treated (100). Like IDO, HO-1 expression is associated to DC maturation. Indeed, HO-1 is expressed in immature DCs, but not in mature DC. Our group demonstrated that immature DCs stimulated with the HO-1 inductor CoPP preserve an immature phenotype with a low production of IL-12p70, a high expression of IL-10, and were able to impair allogeneic T-cell proliferation in humans and rats (101). Based on these results and the observation that Tol-BMDCs expressed HO-1, we then investigated the role of HO-1 in the protective effect of Tol-BMDCs in our transplantation model of heart allograft in rats. Our results highlighted that the co-treatment of grafted rats with ATDC and an HO-1 inhibitor (tin protoporphyrin IX, SnPP), impaired the beneficial effect of autologous Tol-BMDC treatment. These results suggest that HO-1 is involved in the improvement of allograft tolerance mediated by autologous Tol-BMDCs in this model (102).

Other molecules, such as thrombospondin-1 (TSP-1), PGE2, and adenosine could also influence the tolerogenic potential of tolerogenic DCs in transplantation. To test the role of these molecules in tolerance, a study was performed to compare human Tol-MoDCs differentiated with IL10, IL10/TGF-β, and IL10/IL-6. The results demonstrated that only Tol-MoDCs generated with IL10/TGF-β lost the suppressive potential *in vitro* in the presence of ARL67156 (CD39 inhibitor) or Indomethacin (PG inhibitor synthesis). However, IL-10 and TSP-1 inhibitors impaired tolerogenic potential in IL10 differentiated-DCs and IL10/IL6-DCs (103).

In conclusion, different types of tolerogenic DCs have different types of immunosuppressive mechanisms to elicit T-cell hypoproliferation.

REGULATORY CELL INDUCTION

Induction of CD4$^+$ Treg Cells

Nowadays, the main goal in post-transplantation therapy is to avoid chronic rejection. To be efficient in the long term, it is essential to induce regulatory cells. Different types of regulatory cells induced or expanded by tolerogenic DCs were described in several animal models and were also observed in the first clinical trials. Among them, the main ones are Tr1 cells, induced CD4$^+$CD25$^+$FoxP3^{hi} Treg, CD8$^+$Treg, CD3$^+$CD4$^-$CD8$^-$Treg (104) and Breg (**Figure 3**) (32).

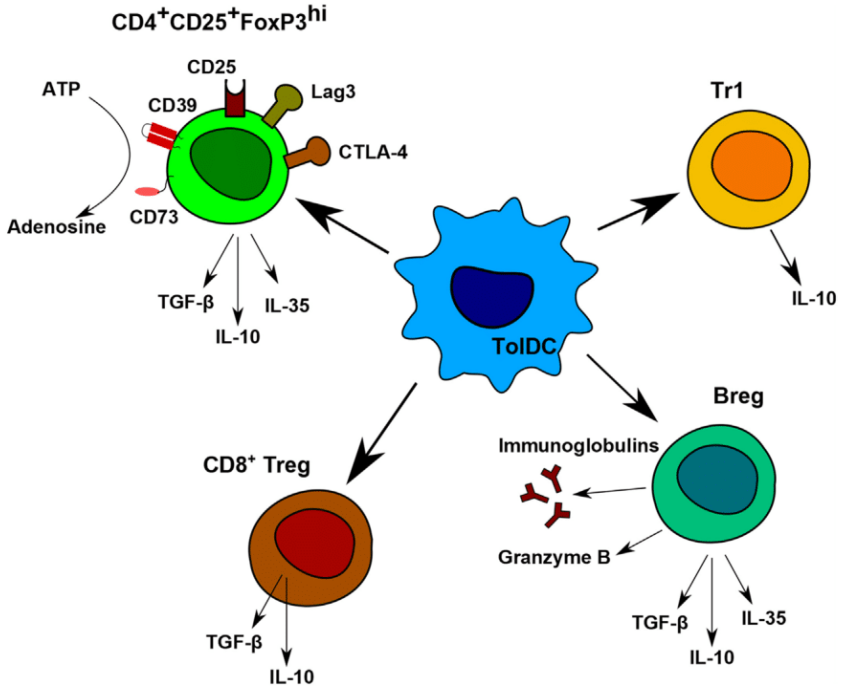


FIGURE 3 | Regulatory cells induced by tolerogenic dendritic cells (DC). Tolerogenic DCs are able to induce different populations of regulatory T and B cells. Each population has different immunomodulatory mechanisms to prevent allogeneic proliferation. $CD4^+CD25^+FoxP3^{hi}$ regulatory T (Treg) display contact-independent mechanisms, such as cytotoxic T-lymphocyte associated protein-4 and lymphocyte-activation gene 3 and contact-independent mechanisms by their secretion of IL-10, TGF-β, and IL-35. They are also able to produce adenosine by the degradation of ATP. Tr1 cells and $CD8^+$Treg are known to produce IL-10. Breg express anti-inflammatory molecules, Granzyme B, and immunoglobulins.

The important role of $CD4^+CD25^+FoxP3^{hi}$ T cells has already been demonstrated in transplantation. Indeed, it was highlighted that the transplantation of skin allografts from tolerant mice onto new recipients, receiving donor or third-party skin allografts leads to the transfer of tolerance. In this study, the authors demonstrated that the donor allograft was not rejected while the third-party one was, meaning that tolerance was led by specific mechanisms (105). $CD4^+CD25^+FoxP3^{hi}$ Treg are usually associated with several suppressive molecules, such as CTLA-4 and lymphocyte-activation gene 3 (Lag3) that trigger a signal to DCs in order to impair antigen presentation. $CD4^+CD25^+FoxP3^{hi}$ Treg are also associated with the production of granzyme B and immunomodulatory molecules such as IL-10, TGF-β, and IL-35. Apart from classical contact mechanisms, $CD4^+CD25^+FoxP3^{hi}$ Treg also compete with effector T cells for IL-2. The deprivation of IL-2 leads to an inhibition of proliferation and apoptosis in effector CD4 T cells (106, 107). Other mechanisms such as the production of adenosine through CD39 and CD73 have also been described (108). In transplantation models, several groups showed that tolerogenic DCs lead to the induction of $CD4^+CD25^+FoxP3^{hi}$ Treg. For example, a study using Tol-BMDCs generated with Rapa have been shown to favor $CD4^+CD25^+FoxP3^{hi}$ Treg population. In this study, the injection of syngeneic Rapa-DCs pulsed with donor antigens induced tolerance to heart allograft. The adoptive transfer of T cells from tolerant mice to syngeneic mice transplanted with heart allograft from the same source promote an increase in allograft survival due to the transfer of $CD4^+CD25^+FoxP3^{hi}$ Treg (47). Moreover, our recent studies in pancreatic islet allograft transplantation demonstrated that $CD4^+CD25^+FoxP3^{hi}$ Treg were increased in spleen, lymph nodes and graft of mice treated with autologous Tol-BMDCs and anti-CD3. As mentioned above, this Treg induction was essential for graft prolongation (15).

Other Treg-cell types commonly observed in tolerogenic DC therapy are Tr1 and Tr1-like cells (104). Tr1 are associated with a high expression of IL-10 after specific stimulation and the expression of Lag3 and CD49b markers (109). These Tr1 cells could be induced by Tol-MoDCs generated with IL-10 through the HLA-G/ILT4 pathway (41). Furthermore, it has been shown that Tol-MoDC generated with VitD3 stimulate the generation of Tr1-like cells with a high expression of IL-10 and are able to impair allogeneic T-cell proliferation (110). Interestingly, these Tr1-like cells are induced by contact with Tol-MoDCs notably by PDL-1/PD1 interaction (44). Tr1 have been shown to play an important role sustaining graft $CD4^+CD25^+FoxP3^{hi}$ Treg from the spleen through the expression of IL-10 in pancreatic islet allograft (111). These results indicated a network between different tolerogenic populations in order to prolong allograft survival. Another study demonstrated that Tr1-like cells ($IL\text{-}10^+FoxP3^-CTLA\text{-}4^+CD25^{hi}Egr2^+$ cells) could be differentiated from anergic $IL\text{-}10^-FoxP3^-CTLA\text{-}4^+CD25^+Egr2^+$T cells following their interaction with immature DCs (112). These Tr1-like cells were able to inhibit T-cell proliferation *in vivo* and *in vitro* in an antigen-specific manner (112).

Another CD4 T cell regulatory population potentially associated with tolerogenic DCs are the iTR35 cells. iTR35 are regulatory cells that suppress through IL-35 production but not through IL-10 nor TGF-β. Interestingly, these cells do not express FoxP3. iTR35 are generated *in vitro* with IL-10 and IL-35 but *in vivo* they are present in models such as intestine infection and cancer (113). IL-35 is highly expressed on human Dex induced-tolerogenic DCs after pro-inflammatory stimulation with IFN-γ, CD40-L, or LPS (87). However, the role of IL-35 secreting tolerogenic DCs and iTr35 differentiation *in vivo* remains a conjecture today.

Induction of Non-CD4$^+$ Regulatory Cells

Apart from CD4 regulatory cells, there are other regulatory populations involved in TolDC therapy in transplantation such as CD8 Treg and Breg. CD8 Treg cells are less characterized than CD4$^+$ regulatory cells but they are known to express IL-10 and TGF-β (114). In mice and humans, splenic CD8$^+$CD122$^+$PD-1$^+$ population is associated to an increased allograft survival (115) and also to an anti-inflammatory and suppressive function in other models (116). Moreover, there are several works that have demonstrated a link between tolerogenic DCs and CD8 Treg induction. In humans, a study performed in 2002 showed that antigen-specific CD8 T cells with suppressive activity are generated in healthy volunteers treated with immature DCs pulsed with influenza matrix peptide (117). Another study performed in NHP showed that animals treated with CTLA4-Ig and donor Tol-BMDCs prior to kidney transplantation developed an increased proportion of donor-specific Eomesodermin^{lo}CTLA4^{hi}CD8$^+$ T cells. This population is associated with an improvement in allograft survival (118). In our experiments, an increase of CD8$^+$CD11c$^+$ T cells was observed in a model of allograft skin transplantation in mice treated with autologous Tol-BMDCs and low doses of anti-CD3 antibody. The adoptive transfer of CD8$^+$ T cells purified from these animals was able to prolong allograft survival in new transplanted mice. These results suggest that CD8$^+$CD11c$^+$ T cells induced by autologous Tol-BMDCs could be regulatory cells (16).

Although B cells are well known to promote allograft responses, there is growing evidence that in some circumstances B cells also contribute to the maintenance of transplant tolerance (119). Different populations of regulatory B cells have been described from immature state to plasma cells. Breg effects were described to be mediated by immunomodulatory cytokines such as IL-10, IL-35, and TGF-β, contact-dependent mechanisms, cytotoxic activity mediated by Granzyme B and also by immunoglobulin secretion (120). In transplantation, the ability of B cells to delay graft rejection has already been demonstrated in different rodent transplantation models (121, 122) Furthermore, studies from our team and others demonstrated that the adoptive transfers of splenic B cells from tolerant animals (either total B cells or B cell subsets) were able to delay graft rejection both in heart transplantation in rats and in a mouse model of skin transplantation (123, 124). Other reports highlighted the induction of Breg following Tol-MoDC therapy. Interestingly, in the first phase I clinical trial with Tol-MoDC therapy in type 1 diabetic patients, an increase of B220$^+$CD11c$^+$ population was observed in the blood of patients treated with Tol-MoDCs modified with ODN anti-CD40/CD80/CD86 during the first 6 weeks. This phenotype coincides with a regulatory population (32). Additionally, the same authors demonstrated the contribution of suppressive B cells to control the development of T1D in NOD mice after Tol-BMDC treatment. In this study, the authors suggested that the expansion of pre-existing IL-10$^+$ B cells and the "*de novo*" generation from CD19$^+$ B cells could be mediated by the secretion of RA-DCs from Tol-BMDCs (125). However, the link between tolerogenic DCs, regulatory B cells, and allograft tolerance remains unclear.

Altogether these results show that tolerogenic DCs are able to induce regulatory cells leading to a regulatory network that could improve the allograft acceptance.

WHERE DO WE STAND?

From the first DCs vaccines back in 1995 (33) until today, the expectation on DCs therapy have increased due to the safety and potential demonstrated in animal models and in humans. Nowadays, four clinical trials using Tol-MoDCs in autoimmune diseases have already been completed (32, 48, 66, 126) (**Table 1**).

TABLE 1 | Completed clinical trials.

Differentiation protocol	Disease	Patients	Cohorts	Biological effect/safety
Tolerogenic monocyte-derived dendritic cells (Tol-MoDC) modified with oligonucleotides (ODN) anti-CD40/80/86	Type 1 diabetes	10	• Unmodified Tol-MoDC • ODN Tol-MoDC	• Increase in B220 B cells in blood • No adverse effects
Tol-MoDC treated with nuclear factor-κB inhibitor and pulsed with citrullinated peptides	Rheumatoid arthritis	18	• Low dose (1 million cells) • High dose (5 millions cells)	• Increase in Treg in blood • Decrease in T-cell response to vimentin 447–455 Cit450 • No adverse effects
Tol-MoDC differentiated with dexamethasone (Dex) and IL-6, TNF-α, IL-1β, and prostaglandin E2	Refractory Crohn's disease	12	• 2, 5 or 10 millions cells • Single dose or biweekly	• Increase in Treg in blood • Decrease in interferon-γ (IFN-γ) in blood • Three patients withdrew due to disease worsening
Tol-MoDC differentiated with Dex and loaded with autologous synovial fluid	Rheumatoid and inflammatory arthritis	13	• 1, 3, 10 millions cells	• No biological effect in blood • No adverse effects

First clinical trial was performed in insulin-requiring T1D patients. In this clinical trial, seven patients received Tol-MoDCs modified with ODN anti-CD40/80/86 and three were treated with unmodified Tol-MoDCs. An increase in B220 B cells and no adverse effects were observed (32). The second clinical trial using Tol-MoDCs was performed in rheumatoid arthritis patients. In this study, 18 HLA-positive RA patients were divided into two cohorts, patients from the first one received a low dose of Tol-MoDC (one million cells) and the others received high dose (five million cells). Tol-MoDCs used in this study were modified with an NF-κB inhibitor and pulsed with four citrullinated peptides. No adverse effects were observed. Additionally, the authors observed an increase in circulating Treg cells and a decrease in IL-6 expression in T cells in response to vimentin$_{447–455}$ Cit450 (66). The third clinical trial using Tol-MoDCs was performed in patients suffering from refractory Crohn's disease. In this clinical trial, 12 patients were divided in 6 cohorts, receiving 2, 5, or 10 million Tol-MoDCs in a single dose or biweekly. Despite that no adverse effects were observed in most patients, three of them withdrew the study due to worsening of the disease. Additionally, the authors found an increase in Treg cells and a decrease in IFN-γ in blood (48). Finally, the most recent clinical trial using Tol-MoDCs was performed in rheumatoid and inflammatory arthritis. In this study, 13 patients were divided in four cohorts, receiving 1, 3, or 10 millions cells and three patients receiving saline solution. Tol-MoDCs used in this clinical trial were differentiated using Dex and vitD3 and loaded with autologous synovial fluid. The outcome of this study showed that the treatment was safe and feasible. Moreover hypertrophy, vascularity and synovitis were stable in all cohorts and in placebo-treated patients. Nevertheless, two patients that have received 10 millions cells showed a decrease in synovitis score (126). Apart from these studies, there are many other ongoing clinical trials focused on other pathologies, such as allergy or multiple sclerosis. Among the ongoing clinical trials using Tol-MoDCs, we supervise a phase I/II clinical trial in kidney transplantation at Nantes university hospital (NCT02252055). This trial will evaluate the safety of autologous Tol-MoDCs in patients receiving living donor kidney transplantation and a minimized immunosuppression. In this trial, autologous Tol-MoDCs are generated in the presence of low-dose GM-CSF as the only cytokine used. These Tol-MoDCs are characterized by a weak capacity to stimulate allogenic T cells and a suppression of the proliferation of stimulated T cells. Furthermore, they are resistant to maturation stimuli. Patients receive their Tol-MoDCs the day before transplantation by intravenous route at a dose of one million/kg [for review (34)]. The team of Angus Thomson also evaluate the potential of Tol-MoDC in transplantation. In this trial, patients receive donor-derived Tol-MoDCs one-week prior to liver transplantation (NTC03164265) [for review (127)]. Due to the outcomes of these clinical trials, at least in terms of safety and biological effect, Tol-MoDC therapy appears more and more as an interesting strategy to treat several diseases. However, more clinical trials must be performed in order to find out the adequate dose, injection conditions, and associated drugs to efficiently treat patients.

CONCLUSION

Tolerogenic DCs have a solid background that corroborates their usefulness in transplantation, but also to treat autoimmunity and allergy diseases. Despite the different methods to generate them and the different models used, the common features of tolerogenic DCs converge in a low expression of costimulatory and presentation molecules, a maturation resistance, a high expression of immunomodulatory molecules, a low expression of pro-inflammatory molecules, and an impairment of T-cell proliferation. Moreover, tolerogenic DCs induce regulatory populations that are related to the protection of allograft in the long term. More importantly tolerogenic DCs have been proved to be safe supporting the feasibility of this cell therapy in humans. Finally, results confirming the efficacy and safety of autologous Tol-MoDC in humans in transplantation will be evaluated in the following years.

AUTHOR CONTRIBUTIONS

All authors contributed in discussing the topic and wrote the manuscript.

FUNDING

The work performed in the INSERM U1064 and presented in this review was funded by IMBIO-DC, Fondation Progreffe, DHU Oncogreffe, The ONE Study (FP7-260687), and BIODRIM (FP7-305147) European Union seventh Framework Programs. The work of INSERM U1064 was also supported by funds from IHU-CESTI (Investissement d'Avenir ANR-10-IBHU-005, Région Pays de la Loire and Nantes Métropole) and the Labex IGO project (n° ANR-11-LABX-0016-01).

REFERENCES

1. Merrill JP, Murray JE, Harrison JH, Guild WR. Landmark article Jan 28, 1956: successful homotransplantation of the human kidney between identical twins. By John P. Merrill, Joseph E. Murray, J. Hartwell Harrison, and Warren R. Guild. *JAMA* (1984) 251:2566–71. doi:10.1001/jama.251.19.2566
2. Schwartz R, Eisner A, Dameshek W. The effect of 6-mercaptopurine on primary and secondary immune responses. *J Clin Invest* (1959) 38:1394–403. doi:10.1172/JCI103915
3. Cooper DKC. A brief history of cross-species organ transplantation. *Proc (Bayl Univ Med Cent)* (2012) 25:49–57. doi:10.1080/08998280.2012.11928783
4. Dalal P, Shah G, Chhabra D, Gallon L. Role of tacrolimus combination therapy with Mycophenolate mofetil in the prevention of organ rejection in kidney transplant patients. *Int J Nephrol Renovasc Dis* (2010) 3:107–15.
5. Salis P, Caccamo C, Verzaro R, Gruttadauria S, Artero M. The role of basiliximab in the evolving renal transplantation immunosuppression protocol. *Biologics* (2008) 2:175–88.
6. Stallone G, Infante B, Grandaliano G. Management and prevention of post-transplant malignancies in kidney transplant recipients. *Clin Kidney J* (2015) 8:637–44. doi:10.1093/ckj/sfv054
7. Libby P, Pober JS. Chronic rejection. *Immunity* (2001) 14:387–97. doi:10.1016/S1074-7613(01)00119-4

8. Argani H. Cell therapy in solid-organ transplant. *Exp Clin Transplant* (2016) 14:6–13.
9. Chabannes D, Hill M, Merieau E, Rossignol J, Brion R, Soulillou JP, et al. A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* (2007) 110:3691–4. doi:10.1182/blood-2007-02-075481
10. Franquesa M, Hoogduijn MJ, Reinders ME, Eggenhofer E, Engela AU, Mensah FK, et al. Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) Fourth Meeting: lessons learned from first clinical trials. *Transplantation* (2013) 96:234–8. doi:10.1097/TP.0b013e318298f9fa
11. Tan J, Wu W, Xu X, Liao L, Zheng F, Messinger S, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA* (2012) 307:1169–77. doi:10.1001/jama.2012.316
12. Riquelme P, Tomiuk S, Kammler A, Fandrich F, Schlitt HJ, Geissler EK, et al. IFN-gamma-induced iNOS expression in mouse regulatory macrophages prolongs allograft survival in fully immunocompetent recipients. *Mol Ther* (2013) 21:409–22. doi:10.1038/mt.2012.168
13. Hutchinson JA, Riquelme P, Sawitzki B, Tomiuk S, Miqueu P, Zuhayra M, et al. Cutting edge: immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients. *J Immunol* (2011) 187:2072–8. doi:10.4049/jimmunol.1100762
14. Hill M, Thebault P, Segovia M, Louvet C, Bériou G, Tilly G, et al. Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and Epstein-Barr virus-induced gene 3. *Am J Transplant* (2011) 11:2036–45. doi:10.1111/j.1600-6143.2011.03651.x
15. Baas MC, Kuhn C, Valette F, Mangez C, Duarte MS, Hill M, et al. Combining autologous dendritic cell therapy with CD3 antibodies promotes regulatory T cells and permanent islet allograft acceptance. *J Immunol* (2014) 193:4696–703. doi:10.4049/jimmunol.1401423
16. Segovia M, Louvet C, Charnet P, Savina A, Tilly G, Gautreau L, et al. Autologous dendritic cells prolong allograft survival through *Tmem176b*-dependent antigen cross-presentation. *Am J Transplant* (2014) 14:1021–31. doi:10.1111/ajt.12708
17. Todo S, Yamashita K, Goto R, Zaitsu M, Nagatsu A, Oura T, et al. A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. *Hepatology* (2016) 64:632–43. doi:10.1002/hep.28459
18. Geissler EK. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplant Res* (2012) 1:11. doi:10.1186/2047-1440-1-10
19. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* (1973) 137:1142–62. doi:10.1084/jem.137.5.1142
20. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J Exp Med* (1974) 139:380–97. doi:10.1084/jem.139.2.380
21. Mukherji B, Chakraborty NG, Yamasaki S, Okino T, Yamase H, Sporn JR, et al. Induction of antigen-specific cytolytic T cells in situ in human melanoma by immunization with synthetic peptide-pulsed autologous antigen presenting cells. *Proc Natl Acad Sci U S A* (1995) 92:8078–82. doi:10.1073/pnas.92.17.8078
22. Mellman I. Dendritic cells: master regulators of the immune response. *Cancer Immunol Res* (2013) 1:145–9. doi:10.1158/2326-6066.CIR-13-0102
23. Nussenzweig MC, Steinman RM, Gutchinov B, Cohn ZA. Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med* (1980) 152:1070–84. doi:10.1084/jem.152.4.1070
24. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol* (2012) 12:557–69. doi:10.1038/nri3254
25. Mildner A, Jung S. Review development and function of dendritic cell subsets. *Immunity* (2014) 40:642–56. doi:10.1016/j.immuni.2014.04.016
26. Ohnmacht C, Pullner A, King SBS, Drexler I, Meier S, Brocker T, et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med* (2009) 206:549–59. doi:10.1084/jem.20082394
27. Tisch R. Immunogenic versus tolerogenic dendritic cells: a matter of maturation. *Int Rev Immunol* (2010) 29:111–8. doi:10.3109/08830181003602515
28. Machen J, Harnaha J, Lakomy R, Styche A, Trucco M, Giannoukakis N. Antisense oligonucleotides down-regulating costimulation confer diabetes-preventive properties to nonobese diabetic mouse dendritic cells. *J Immunol* (2004) 173:4331–41. doi:10.4049/jimmunol.173.7.4331
29. Garcia F, Climent N, Assoumou L, Gil C, Gonzalez N, Alcami J, et al. A therapeutic dendritic cell-based vaccine for HIV-1 infection. *J Infect Dis* (2011) 203:473–8. doi:10.1093/infdis/jiq077
30. Butterfield LH. Dendritic cells in cancer immunotherapy clinical trials: are we making progress? *Front Immunol* (2013) 4:3–9. doi:10.3389/fimmu.2013.00454
31. Yang J, Bernier SM, Ichim TE, Li M, Xia X, Zhou D, et al. LF15-0195 generates tolerogenic dendritic cells by suppression of NF-κB signaling through inhibition of IKK activity that inhibited Th1 polarization and increased Th2. *J Leukoc Biol* (2003) (3):74.
32. Giannoukakis N. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. *Diabetes Care* (2011) 34:2026–32. doi:10.2337/dc11-0472
33. Dhodapkar MV, Steinman RM, Sapp M, Desai H, Fossella C, Krasovsky J, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J Clin Invest* (1999) 104:173–80. doi:10.1172/JCI6909
34. Moreau A, Varey E, Bériou G, Hill M, Bouchet-Delbos L, Segovia M, et al. Tolerogenic dendritic cells and negative vaccination in transplantation: from rodents to clinical trials. *Front Immunol* (2012) 3:218. doi:10.3389/fimmu.2012.00218
35. Arellano M, Lonial S. Clinical uses of GM-CSF, a critical appraisal and update. *Biologics* (2008) 2:13–27.
36. Shiomi A, Usui T, Mimori T. GM-CSF as a therapeutic target in autoimmune diseases. *Inflamm Regen* (2016) 36:8. doi:10.1186/s41232-016-0014-5
37. Yoo S, Ha S-J. Generation of tolerogenic dendritic cells and their therapeutic applications. *Immune Netw* (2016) 16:52–60. doi:10.4110/in.2016.16.1.52
38. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol* (2007) 7:610–21. doi:10.1038/nri2132
39. Corinti S, Albanesi C, la Sala A, Pastore S, Girolomoni G. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol* (2001) 166:4312–8. doi:10.4049/jimmunol.166.7.4312
40. Strobl H, Knapp W. TGF-β1 regulation of dendritic cells. *Microbes Infect* (1999) 1:1283–90. doi:10.1016/S1286-4579(99)00256-7
41. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood* (2010) 116:935–44. doi:10.1182/blood-2009-07-234872
42. Torres-Aguilar H, Sánchez-Torres C, Jara LJ, Blank M, Shoenfeld Y. IL-10/TGF-β-treated dendritic cells, pulsed with insulin, specifically reduce the response to insulin of CD4+ effector/memory T cells from type 1 diabetic individuals. *J Clin Immunol* (2010) 30:659–68. doi:10.1007/s10875-010-9430-5
43. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol* (2000) 164:2405–11. doi:10.4049/jimmunol.164.5.2405
44. Unger WWJ, Laban S, Kleijwegt FS, van der Slik AR, Roep BO. Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur J Immunol* (2009) 39:3147–59. doi:10.1002/eji.200839103
45. Trabanelli S, Lecciso M, Salvestrini V, Cavo M, Ocadlikova D, Lemoli RM, et al. PGE2-induced IDO1 inhibits the capacity of fully mature DCs to elicit an in vitro antileukemic immune response. *J Immunol Res* (2015) 2015:253191. doi:10.1155/2015/253191
46. Naranjo-Gómez M, Raïch-Regué D, Oñate C, Grau-López L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. *J Transl Med* (2011) 9:89. doi:10.1186/1479-5876-9-89
47. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol* (2007) 178:7018–31. doi:10.4049/jimmunol.178.11.7018

48. Jauregui-Amezaga A, Cabezón R, Ramírez-Morros A, España C, Rimola J, Bru C, et al. Intraperitoneal administration of autologous tolerogenic dendritic cells for refractory Crohn's disease: a phase I study. *J Crohns Colitis* (2015) 9(12):1071–8. doi:10.1093/ecco-jcc/jjv144
49. Chitta S, Santambrogio L, Stern LJ. GMCSF in the absence of other cytokines sustains human dendritic cell precursors with T cell regulatory activity and capacity to differentiate into functional dendritic cells. *Immunol Lett* (2008) 116:41–54. doi:10.1016/j.imlet.2007.11.013
50. Moreau A, Varey E, Bouchet-Delbos L, Cuturi M-C. Cell therapy using tolerogenic dendritic cells in transplantation. *Transplant Res* (2012) 1:13. doi:10.1186/2047-1440-1-13
51. Ingulli E. Mechanism of cellular rejection in transplantation. *Pediatr Nephrol* (2010) 25:61–74. doi:10.1007/s00467-008-1020-x
52. Smyth LA, Herrera OB, Golshayan D, Lombardi G, Lechler RI. A novel pathway of antigen presentation by dendritic and endothelial cells: implications for allorecognition and infectious diseases. *Transplantation* (2006) 82:S15–8. doi:10.1097/01.tp.0000231347.06149.ca
53. Rainienė T, Izvolskaja N, Dainys B, Kučinskis G, Razukas V. Donor-specific transfusions as a way of tolerance induction to living donor kidney transplant. *Biologija* (2009) 55:99–104. doi:10.2478/v10054-009-0016-1
54. Yabu JM, Anderson MW, Kim D, Bradbury BD, Lou CD, Petersen J, et al. Sensitization from transfusion in patients awaiting primary kidney transplant. *Nephrol Dial Transplant* (2013) 28:2908–18. doi:10.1093/ndt/gft362
55. Lutz MB, Suri RM, Niimi M, Ogilvie AL, Kukutsch NA, Rossner S, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *Eur J Immunol* (2000) 30:1813–22. doi:10.1002/1521-4141(200007)30:7<1813::AID-IMMU1813>3.0.CO;2-8
56. DePaz HA, Oluwole OO, Adeyeri AO, Witkowski P, Jin M-X, Hardy MA, et al. Immature rat myeloid dendritic cells generated in low-dose granulocyte macrophage-colony stimulating factor prolong donor-specific rat cardiac allograft survival. *Transplantation* (2003) 75:521–8. doi:10.1097/01.TP.0000048380.84355.4A
57. Divito SJ, Wang Z, Shufesky WJ, Liu Q, Tkacheva OA, Montecalvo A, et al. Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. *Blood* (2010) 116:2694–705. doi:10.1182/blood-2009-10-251058
58. Pêche H, Trinité B, Martinet B, Cuturi MC. Prolongation of heart allograft survival by immature dendritic cells generated from recipient type bone marrow progenitors. *Am J Transplant* (2005) 5:255–67. doi:10.1111/j.1600-6143.2004.00683.x
59. Bériou G, Pêche H, Guillonneau C, Merieau E, Cuturi M-C. Donor-specific allograft tolerance by administration of recipient-derived immature dendritic cells and suboptimal immunosuppression. *Transplantation* (2005) 79:969–72. doi:10.1097/01.TP.0000158277.50073.35
60. Chiffoleau E, Beriou G, Dutartre P, Usal C, Soulillou J-P, Cuturi MC. Induction of donor-specific allograft tolerance by short-term treatment with LF15-0195 after transplantation. Evidence for a direct effect on T-cell differentiation. *Am J Transplant* (2002) 2:745–57. doi:10.1034/j.1600-6143.2002.20808.x
61. Chatenoud L. CD3-specific antibody-induced active tolerance: from bench to bedside. *Nat Rev Immunol* (2003) 3:123–32. doi:10.1038/nri1000
62. You S, Zuber J, Kuhn C, Baas M, Valette F, Sauvaget V, et al. Induction of allograft tolerance by monoclonal CD3 antibodies: a matter of timing. *Am J Transplant* (2012) 12:2909–19. doi:10.1111/j.1600-6143.2012.04213.x
63. Ezzelarab M, Zahorchak AF, Lu L, Morelli AE, Chalasani G, Demetris AJ, et al. Regulatory dendritic cell infusion prolongs kidney allograft survival in non-human primates. *Am J Transplant* (2013) 13:1989–2005. doi:10.1111/ajt.12310
64. Ezzelarab MB, Raich-Regue D, Lu L, Zahorchak AF, Perez-Gutierrez A, Humar A, et al. Renal allograft survival in nonhuman primates infused with donor antigen-pulsed autologous regulatory dendritic cells. *Am J Transplant* (2017) 17:1476–89. doi:10.1111/ajt.14182
65. Moreau A, Vandamme C, Segovia M, Devaux M, Guilbaud M, Tilly G, et al. Generation and in vivo evaluation of IL10-treated dendritic cells in a nonhuman primate model of AAV-based gene transfer. *Mol Ther Methods Clin Dev* (2014) 1:14028. doi:10.1038/mtm.2014.28
66. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype-positive rheumatoid arthritis patients. *Sci Transl Med* (2015) 7:290ra87. doi:10.1126/scitranslmed.aaa9301
67. Moreau A, Varey E, Anegon I, Cuturi M-C. Effector mechanisms of rejection. *Cold Spring Harb Perspect Med* (2013) 3(11):a015461. doi:10.1101/cshperspect.a015461
68. Alvarez D, Vollmann EH, von Andrian UH. Mechanisms and consequences of dendritic cell migration. *Immunity* (2008) 29:325. doi:10.1016/j.immuni.2008.08.006
69. Mansilla MJ, Sellès-Moreno C, Fàbregas-Puig S, Amoedo J, Navarro-Barriuso J, Teniente-Serra A, et al. Beneficial effect of tolerogenic dendritic cells pulsed with MOG autoantigen in experimental autoimmune encephalomyelitis. *CNS Neurosci Ther* (2015) 21:222–30. doi:10.1111/cns.12342
70. Garrod KR, Chang CK, Liu F-C, Brennan TV, Foster RD, Kang S-M. Targeted lymphoid homing of dendritic cells is required for prolongation of allograft survival. *J Immunol* (2006) 177:863L–868. doi:10.4049/jimmunol.177.2.863
71. Celli S, Albert ML, Bousso P. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. *Nat Med* (2011) 17:744–9. doi:10.1038/nm.2376
72. Svajger U, Rozman P. Tolerogenic dendritic cells: molecular and cellular mechanisms in transplantation. *J Leukoc Biol* (2014) 95:53–69. doi:10.1189/jlb.0613336
73. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* (2013) 13:227–42. doi:10.1038/nri3405
74. Tuettenberg A, Huter E, Hubo M, Horn J, Knop J, Grimbacher B, et al. The role of ICOS in directing T cell responses: ICOS-dependent induction of T cell anergy by tolerogenic dendritic cells. *J Immunol* (2009) 182:3349–56. doi:10.4049/jimmunol.0802733
75. Lo DJ, Anderson DJ, Song M, Leopardi F, Farris AB, Strobert E, et al. A pilot trial targeting the ICOS-ICOS-L pathway in nonhuman primate kidney transplantation. *Am J Transplant* (2015) 15:984–92. doi:10.1111/ajt.13100
76. Sandner SE, Clarkson MR, Salama AD, Sanchez-Fueyo A, Domenig C, Habicht A, et al. Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo. *J Immunol* (2005) 174:3408–15. doi:10.4049/jimmunol.174.6.3408
77. Tanaka K, Albin MJ, Yuan X, Yamaura K, Habicht A, Murayama T, et al. PDL1 is required for peripheral transplantation tolerance and protection from chronic allograft rejection. *J Immunol* (2007) 179:5204–10. doi:10.4049/jimmunol.179.8.5204
78. Peng W, Ran B, Ma Y, Huang X, Chang Q, Wang X. Dendritic cells transfected with PD-L1 recombinant adenovirus induces T cell suppression and long-term acceptance of allograft transplantation. *Cell Immunol* (2011) 271:73–7. doi:10.1016/j.cellimm.2011.06.007
79. Wu J, Horuzsko A. Expression and function of ILTs on tolerogenic dendritic cells. *Hum Immunol* (2009) 70:353–6. doi:10.1016/j.humimm.2009.01.024
80. Min WP, Gorczynski R, Huang XY, Kushida M, Kim P, Obataki M, et al. Dendritic cells genetically engineered to express Fas ligand induce donor-specific hyporesponsiveness and prolong allograft survival. *J Immunol* (2000) 164:161–7. doi:10.4049/jimmunol.164.1.161
81. Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* (2008) 226:205–18. doi:10.1111/j.1600-065X.2008.00706.x
82. Amodio G, Gregori S. Human tolerogenic DC-10: perspectives for clinical applications. *Transplant Res* (2012) 1:14. doi:10.1186/2047-1440-1-14
83. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* (1996) 184:19–29. doi:10.1084/jem.184.1.19
84. Wan YY, Flavell RA. TGF-β and regulatory T cell in immunity and autoimmunity. *J Clin Immunol* (2008) 28:647–59. doi:10.1007/s10875-008-9251-y
85. Horton C, Shanmugarajah K, Fairchild PJ. ScienceDirect harnessing the properties of dendritic cells in the pursuit of immunological tolerance. *Biomed J* (2017) 40:80–93. doi:10.1016/j.bj.2017.01.002
86. Le Texier L, Thebault P, Carvalho-Gaspar M, Vignard V, Merieau E, Usal C, et al. Immunoregulatory function of IL-27 and TGF-beta1 in cardiac allograft transplantation. *Transplantation* (2012) 94:226–33. doi:10.1097/TP.0b013e31825b0c38
87. Dixon KO, van der Kooij SW, Vignali DAA, van Kooten C. Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. *Eur J Immunol* (2015) 45:1736–47. doi:10.1002/eji.201445217

88. Goriely S, Goldman M. The interleukin-12 family: new players in transplantation immunity? *Am J Transplant* (2007) 7:278–84. doi:10.1111/j.1600-6143.2006.01651.x
89. Fitzgerald DC, Ciric B, Touil T, Harle H, Grammatikopolou J, Das Sarma J, et al. Suppressive effect of IL-27 on encephalitogenic Th17 cells and the effector phase of experimental autoimmune encephalomyelitis. *J Immunol* (2007) 179:3268–75. doi:10.4049/jimmunol.179.5.3268
90. Morishima N, Owaki T, Asakawa M, Kamiya S, Mizuguchi J, Yoshimoto T. Augmentation of effector CD8+ T cell generation with enhanced granzyme B expression by IL-27. *J Immunol* (2005) 175:1686–93. doi:10.4049/jimmunol.175.3.1686
91. Durante W. Role of arginase in vessel wall remodeling. *Front Immunol* (2013) 4:111. doi:10.3389/fimmu.2013.00111
92. Rath M, Muller I, Kropf P, Closs EI, Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. *Front Immunol* (2014) 5:532. doi:10.3389/fimmu.2014.00532
93. Bhatt S, Qin J, Bennett C, Qian S, Fung JJ, Hamilton TA, et al. All-trans retinoic acid induces Arg-1 and iNOS producing dendritic cells with T cell inhibitory function. *J Immunol* (2014) 192:5098–108. doi:10.4049/jimmunol.1303073
94. Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, et al. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. *Eur J Immunol* (2007) 37:3054–62. doi:10.1002/eji.200636704
95. Terness P, Bauer TM, Rose L, Dufter C, Watzlik A, Simon H, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J Exp Med* (2002) 196:447–57. doi:10.1084/jem.20020052
96. Li C, Liu T, Zhao N, Zhu L, Wang P, Dai X. Dendritic cells transfected with indoleamine 2,3-dioxygenase gene suppressed acute rejection of cardiac allograft. *Int Immunopharmacol* (2016) 36:31–8. doi:10.1016/j.intimp.2016.03.048
97. Araujo JA, Zhang M, Yin F. Heme oxygenase-1, oxidation, inflammation, and atherosclerosis. *Front Pharmacol* (2012) 3:119. doi:10.3389/fphar.2012.00119
98. Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol D-W, Flavell RA, et al. Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. *J Immunol* (2004) 172:1220–6. doi:10.4049/jimmunol.172.2.1220
99. Amano MT, Camara NOS. The immunomodulatory role of carbon monoxide during transplantation. *Med Gas Res* (2013) 3:1. doi:10.1186/2045-9912-3-1
100. Wang H, Lee SS, Gao W, Czismadia E, McDaid J, Ollinger R, et al. Donor treatment with carbon monoxide can yield islet allograft survival and tolerance. *Diabetes* (2005) 54:1400–6. doi:10.2337/diabetes.54.5.1400
101. Chauveau C, Remy S, Royer PJ, Hill M, Tanguy-Royer S, Hubert F-X, et al. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. *Blood* (2005) 106:1694–702. doi:10.1182/blood-2005-02-0494
102. Moreau A, Hill M, Thébault P, Deschamps JY, Chiffoleau E, Chauveau C, et al. Tolerogenic dendritic cells actively inhibit T cells through heme oxygenase-1 in rodents and in nonhuman primates. *FASEB J* (2009) 23:3070–7. doi:10.1096/fj.08-128173
103. Torres-Aguilar H, Aguilar-Ruiz SR, González-Pérez G, Munguía R, Bajaña S, Meraz-Ríos MA. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4+ T cells. *J Immunol* (2010) 184:1765–75. doi:10.4049/jimmunol.0902133
104. Raker VK, Domogalla MP, Steinbrink K. Tolerogenic dendritic cells for regulatory T cell induction in man. *Front Immunol* (2015) 6:569. doi:10.3389/fimmu.2015.00569
105. Graca L, Cobbold SP, Waldmann H. Identification of regulatory T cells in tolerated allografts. *J Exp Med* (2002) 195:1641–6. doi:10.1084/jem.20012097
106. de Goer de Herve M-G, Gonzales E, Hendel-Chavez H, Decline J-L, Mourier O, Abbed K, et al. CD25 appears non essential for human peripheral T(reg) maintenance in vivo. *PLoS One* (2010) 5:e11784. doi:10.1371/journal.pone.0011784
107. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol* (2007) 8:1353–62. doi:10.1038/ni1536
108. Schmitt EG, Williams CB. Generation and function of induced regulatory T cells. *Front Immunol* (2013) 4:152. doi:10.3389/fimmu.2013.00152
109. White AM, Wraith DC. Tr1-like T cells – an enigmatic regulatory T cell lineage. *Front Immunol* (2016) 7:355. doi:10.3389/fimmu.2016.00355
110. Garcia-Gonzalez P, Ubilla-Olguin G, Catalan D, Schinnerling K, Aguillon JC. Tolerogenic dendritic cells for reprogramming of lymphocyte responses in autoimmune diseases. *Autoimmun Rev* (2016) 15:1071–80. doi:10.1016/j.autrev.2016.07.032
111. Gagliani N, Jofra T, Valle A, Stabilini A, Morsiani C, Gregori S, et al. Transplant tolerance to pancreatic islets is initiated in the graft and sustained in the spleen. *Am J Transplant* (2013) 13:1963–75. doi:10.1111/ajt.12333
112. Pletinckx K, Vaeth M, Schneider T, Beyersdorf N, Hünig T, Berberich-Siebelt F, et al. Immature dendritic cells convert anergic nonregulatory T cells into Foxp3$^-$IL-10$^+$ regulatory T cells by engaging CD28 and CTLA-4. *Eur J Immunol* (2015) 45:480–91. doi:10.1002/eji.201444991
113. Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, et al. IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol* (2010) 11:1093–101. doi:10.1038/ni.1952
114. Lu L, Kim H-J, Werneck MBF, Cantor H. Regulation of CD8+ regulatory T cells: interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. *Proc Natl Acad Sci U S A* (2008) 105:19420–5. doi:10.1073/pnas.0810383105
115. Dai Z, Zhang S, Xie Q, Wu S, Su J, Li S, et al. Natural CD8+CD122+ T cells are more potent in suppression of allograft rejection than CD4+CD25+ regulatory T cells. *Am J Transplant* (2014) 14:39–48. doi:10.1111/ajt.12515
116. Dai H, Wan N, Zhang S, Moore Y, Wan F, Dai Z. Cutting edge: programmed death-1 defines CD8+CD122+ T cells as regulatory versus memory T cells. *J Immunol* (2010) 185:803–7. doi:10.4049/jimmunol.1000661
117. Dhodapkar MV, Steinman RM. Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans. *Blood* (2002) 100:174–7. doi:10.1182/blood.V100.1.174
118. Ezzelarab MB, Lu L, Guo H, Zahorchak AF, Shufesky WF, Cooper DKC, et al. Eomesoderminlo CTLA4hi alloreactive CD8+ memory T cells are associated with prolonged renal transplant survival induced by regulatory dendritic cell infusion in CTLA4 immunoglobulin-treated nonhuman primates. *Transplantation* (2016) 100:91–102. doi:10.1097/TP.0000000000000871
119. Redfield RR III, Rodriguez E, Parsons R, Vivek K, Mustafa MM, Noorchashm H, et al. Essential role for B cells in transplantation tolerance. *Curr Opin Immunol* (2011) 23:685–91. doi:10.1016/j.coi.2011.07.011
120. Durand J, Chiffoleau E. B cells with regulatory properties in transplantation tolerance. *World J Transplant* (2015) 5:196–208. doi:10.5500/wjt.v5.i4.196
121. Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, et al. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* (1995) 92:9560–4. doi:10.1073/pnas.92.21.9560
122. Niimi M, Pearson TC, Larsen CP, Alexander DZ, Hollenbaugh D, Aruffo A, et al. The role of the CD40 pathway in alloantigen-induced hyporesponsiveness in vivo. *J Immunol* (1998) 161:5331–7.
123. Durand J, Huchet V, Merieau E, Usal C, Chesneau M, Remy S, et al. Regulatory B cells with a partial defect in CD40 signaling and overexpressing granzyme B transfer allograft tolerance in rodents. *J Immunol* (2015) 195:5035L–5044. doi:10.4049/jimmunol.1500429
124. Moreau A, Blair PA, Chai J-G, Ratnasothy K, Stolarczyk E, Alhabbab R, et al. Transitional-2 B cells acquire regulatory function during tolerance induction and contribute to allograft survival. *Eur J Immunol* (2015) 45:843–53. doi:10.1002/eji.201445082
125. Di Caro V, Phillips B, Engman C, Harnaha J, Trucco M, Giannoukakis N. Involvement of suppressive B-lymphocytes in the mechanism of tolerogenic dendritic cell reversal of type 1 diabetes in NOD mice. *PLoS One* (2014) 9:e83575. doi:10.1371/journal.pone.0083575
126. Bell GM, Anderson AE, Diboll J, Reece R, Eltherington O, Harry RA, et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann Rheum Dis* (2017) 76:227–34. doi:10.1136/annrheumdis-2015-208456

127. Thomson AW, Zahorchak AF, Ezzelarab MB, Butterfield LH, Lakkis FG, Metes DM. Prospective clinical testing of regulatory dendritic cells in organ transplantation. *Front Immunol* (2016) 7:15. doi:10.3389/fimmu.2016.00015

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

UNIVERSITE BIOLOGIE BRETAGNE SANTE LOIRE

UNIVERSITÉ DE NANTES

Titre : Nouveaux mécanismes utilisés parles cellules dendritiques tolérogènes

Mots clés : Tol-DC, Lactate, Immunosuppression, Metabolisme, Terapie cellulaire

Résumé : Les recherches menées sur les cellules dendritiques tolérogènes au cours des 20 dernières années ont abouti à leur application thérapeutique dans plusieurs essais cliniques. Parmi ces essais, notre équipe est pionnière en transplantation rénale en réalisant de la thérapie cellulaire à l'aide des cellules dendritiques tolérogènes autologues générées avec une faible dose de GM-CSF (ATDC). Nos études précliniques ont démontré que ces cellules sont capables d'augmenter la survie de différentes allogreffes chez les rongeurs et n'induisent pas d'effets indésirables chez les primates. Lors de ma thèse, j'ai montré que les ATDC humaines présentent une faible expression de molécules co-stimulatrices, ne maturent pas et inhibent la prolifération des cellules T. De plus, les ATDC présentent un phénotype, un profil transcriptomique et un métabolisme particuliers qui les dissocient de d'autres cellules myéloïdes. Afin de déterminer les mécanismes suppressifs de ces cellules, j'ai réalisé différents tests démontrant que les ATDC sont capables d'inhiber la prolifération des cellules T $CD4^+$, d'altérer la production d'IFNγ et IL-17A et d'induire la différenciation $CD4^+CD25^+FoxP3^{hi}$Treg par des mécanismes indépendants du contact cellulaire. L'analyse du surnageant des ATDC (ATDC-SN) a révélé que ces cellules produisent une forte concentration de lactate,qui est en partie responsable de leur effet immunosuppresseur. Cette étude a permis de démontrer que la sécrétion d'acide lactique est un nouveau mécanisme des ATDCs et ouvre une nouvelle perspective de thérapie cellulaire associée à la production de petites molécules..

Title : Novel mechanisms in tolerogenic dendritic cells

Keywords : Tol-DC, Lactate, Immunosuppression, Metabolism, Cell Therapy

Abstract : The research focused on tolerogenic dendritic cells during the last 20 years has culminated on their therapeutic application in several clinical trials. Among these trials, our team is currently conducting the first trial in the context of kidney transplantation using tolerogenic dendritic cells generated with low dose of GM-CSF (ATDC). We previously reported that Tol-BMDC generated with this protocol prolong thesurvival of different allografts in rodent models and do not induce adverse effects in nonhuman primates. In this work I demonstrated that ATDC derived from human monocytes, similarly to their bone marrow equivalent, display a low expression of costimulatory molecules, do not maturate and impair T-cell proliferation. Interestingly, ATDC display a particular phenotype, transcriptomic profile and metabolism comparing to other myeloid cells. In order to determine the suppressive mechanisms of these cells, I performed different assays demonstrating that ATDC impaired $CD4^+$T-cells proliferation and IFNγ and IL-17A production, and induced de novo $CD4^+CD25^+FoxP3^{hi}$Treg by contact-independent mechanisms. The analysis of ATDC supernatant (ATDC-SN) revealed a high concentration of lactate. I demonstrated that this lactate production is in part responsible of ATDC immunosuppressive effects. This study allowed to demonstrate that lactic acid secretion is a novel mechanisms displayed by ATDC and opens a new perspective of cell therapy based on the production of small molecules.