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Les propriétés immunitaires des cellules souches de la pulpe dentaire dans un contexte infectieux

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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'École Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue publiée dans un journal scientifique afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Pr. Didier Raoult

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« Wa handra à jamais »

AH

Sommaire

Résumé :

Les cellules souches de la pulpe dentaire humaine (DPSCs) sont des cellules souches mésenchymateuses (MSCs) isolées du tissu de la pulpe dentaire. Comme pour toute cellule souche, les DPSCs sont capables de s'auto-renouveler et ont la capacité de se différencier en plusieurs types de cellules, comme les odontoblastes, les ostéoblastes, les chondrocytes, les neuroblastes et les adipocytes. Grâce à ces propriétés, les DPSCs deviennent une cible attrayante pour les applications cliniques en dentisterie et dans d'autres domaines tels que la neurologie, l'immunologie et la réparation du tissu osseux. À ce jour, il existe de plus en plus d'études sur les propriétés immunitaires des DPSCs. Elles hébergent des récepteurs à la surface de la cellule, il est donc possible que des ligands microbiens tels que le peptidoglycane (PGN) et le lipopolysaccharide (LPS), composants majeurs de la paroi bactérienne, puissent influencer la biologie des DPSCs.

Il a été démontré que les DPSCs interagissent avec les cellules immunitaires via un contact cellulaire ou en produisant une cytokine en tant que signal immunitaire. Cependant, les propriétés immunitaires sont peu investiguées. En effet, des propriétés immunitaires comme celles décrites dans les cellules immunitaires professionnelles telles que la phagocytose, la production de composés antimicrobiens et le nouveau concept d'«immunité entraînée» pourraient être étudiées. Suite à ces observations, nous avons décidé d'étudier les propriétés immunitaires des DPSCs.

Tout d'abord, une brève revue a été élaborée pour mettre en évidence l'ensemble des propriétés immunitaires des DPSCs décrites dans la littérature. Ensuite, expérimentalement, nous avons montré que les DPSCs pouvaient internaliser le pathogène bactérien *Bartonella quintana*. À notre connaissance, c'est la première fois qu'a été mise en évidence une

capacité phagocytaire des DPSCs. Cette internalisation est associée à une surexpression de gènes codant pour des facteurs inflammatoires tels que IL-6, IL-8, TNF-alpha et MCP-1, connus pour jouer un rôle important dans le recrutement des cellules immunitaires dans un site d'infection. Nous avons également montré une expression de la bêta-défensine 2, un peptide antibactérien. Cependant, cette activité antimicrobienne n'inhibe pas l'internalisation de *Bartonella quintana*. Au contraire, *Bartonella quintana* semble être internalisée dans un compartiment vacuolaire et se multiplier dans les cellules. Nous suggérons que les DPSCs pourraient être un sanctuaire pour *Bartonella quintana* expliquant l'infection chronique à *Bartonella quintana* chez l'Homme.

En outre, nous avons décrit la capacité des DPSCs à développer une immunité entrainée "trained immunity". Il s'agit d'un nouveau concept qui décrit la capacité d'un organisme à réagir plus rapidement contre une seconde infection en ne mettant en jeux que des composants issus de l'immunité innée. Dans notre étude, il s'agit d'une mémoire inflammatoire concernant deux cytokines IL-6 et MCP-1. L'amorçage des DPSCs avec le ligand bactérien LPS ou PGN induit une augmentation de l'expression et de la production de l'IL-6 et du PGN après un second stimulus.

Dans l'ensemble, l'étude des propriétés immunitaires des DPSCs montre que ces dernières peuvent agir comme des cellules immunitaires. De plus, le préconditionnement des DPSCs peut être utilisé pour améliorer la thérapie des cellules souches de la pulpe dentaire pour la dentisterie, la cicatrisation des plaies et d'autres types de tissu.

Abstract

Human Dental Pulp Stem Cells (DPSCs) are mesenchymal stem cells (MSCs) isolated from dental pulp tissue. As with any stem cell, DPSC are cell that undergo self-renewal and have the ability to differentiate into-multiple cell types such as odontoblasts, osteoblasts, chondrocytes, neuroblasts and adipocyts. Thanks to these properties DPSCs become an attractive target for clinical applications in dentistry and other field such as neurology, osteoblaty and immunology. To date, there is more and more studies relating the immune properties of DPSC. They harbour toll like receptor in the cell surface, so it's possible that microbial ligand such as Peptidoglycan (PGN) and Lipopolysacharide (LPS) the major component of wall bacteria might influence the biology of DPSCs.

It has been shown that DPSCs interact with immune cell via cell-to-cell contact or by producing cytokine as immune signal. Nevertheless, the immune properties have been poorly investigated. Indeed, immune properties like those found in professional immune cells, such as phagocytosis, production of antimicrobial compounds and the new concept « trained immunity » could be studied. Following these observations, we decided to investigate the immune properties of DPSCs.

Firstly, a brief review was elaborated to highlight the set of immune properties of DPSCs described in the literature. Then, experimentally, we have shown that DPSCs can internalize the bacterial pathogen *Bartonella quintana*. To our knowledge it is the first time that DPSCs is able to internalize bacteria. This internalization is associated with an over-expression of genes coding for inflammatory factors such as IL-6, IL-8, TNF-alpha and MCP-1 known to play an important role in the recruitment of immune cells at the site of infection. We have also shown an expression of Beta-defensin 2, an antibacterial peptide. However, this

antimicrobial activity does not inhibit the internalization of *Bartonella quintana*. In contrast *Bartonella quintana* seems to be internalized in vacuolar compartment and multiply into cells. We suggest that DPSCs could be a sanctuary for *Bartonella quintana* explaining the chronic infection in case of human infection with *Bartonella quintana*.

In addition, we have described, the capacity of DPSCs to develop a trained immunity. Trained immunity is a new concept which described the ability of an organism to respond faster against a second infection which only involved components from innate immunity. In our study, it is an inflammatory memory concerning two cytokines IL-6 and MCP-1. Priming DPSCs with bacterial ligands LPS or PGN induce an increase of the expression and production of IL-6 and PGN after a second challenge comparing to un-primed DPSCs.

Our investigations of the immune properties of DPSCs show they are acting like immune cells. Moreover, preconditioning DPSCs can be used to enhance the dental pulp stem cells therapy for dentistry and wound healing.

Introduction

Introduction :

Notre équipe a développé un modèle d'étude pour l'investigation des mécanismes d'interaction hôte pathogènes. Il s'agit de la planaire *Schmidtea mediteranea*, un ver plat d'eau douce amplement étudié du fait de sa capacité exceptionnelle de régénération (Sánchez Alvarado et al., 2002). En 1766, Peter Simon Pallasa a montré qu'un morceau de tissu de planaire peut régénérer une planaire entière (Odelberg, 2004). En 1898, Thomas Morgan confirme les premières découvertes en montrant qu'1/279 morceau de planaire régénère la planaire entière en 20 jours **Figure 1** (González-Estévez, 2009). Cette capacité régénérative est due au fait que la planaire est constituée de 30% de cellules souches pluripotentes (somatiques) qui après une lésion ou décapitation permettent de remplacer et de reconstruire des tissus et organes de la planaire à partir de structure préexistante (Reddien et al., 2005). *Schmidtea mediteranea* est donc essentiellement connue de la communauté scientifique par les études de développement et médecine régénérative alors que ses capacités immunitaires et de défense contre les agents pathogènes restent largement inexplorées.

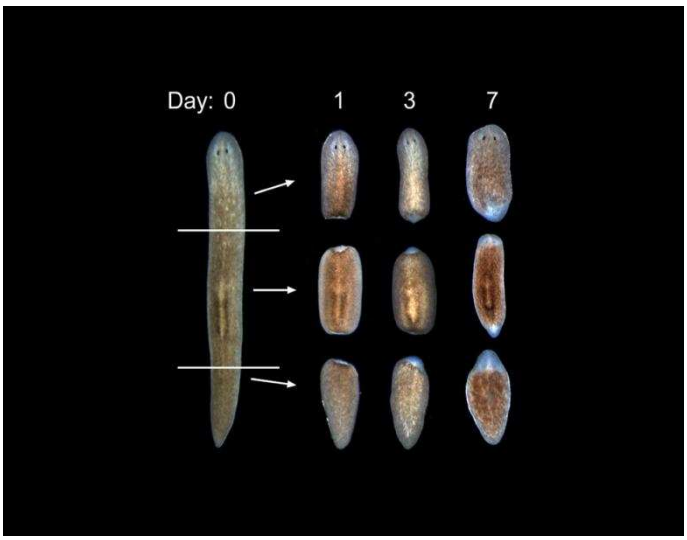

Figure 1 : Régénération de la planaire après amputation.

La première étude sur l'immunité des planaires a été effectuée dans les années 1990 par Morita, mettant en évidence des cellules, appelées réticulocytes, capables de reconnaitre des bactéries *Mycobacterium tuberculosis* H37RA tuées par la chaleur et de les éliminer dans l'intestin de la planaire *Dugesia dorotocephala* (Morita, 1991). Cependant dans ce travail princeps, les mycobactéries étaient inactivées par la chaleur. Au cours des cinq dernières années, notre équipe a mis en évidence les premières caractéristiques immunitaires de la planaire. La première étude a mis en évidence le fait que la planaire résistait à des bactéries pathogènes pour l'Homme, en utilisant un mécanisme de phagocytose **Figure 2** (Abnave et al., 2014). Ensuite, on a montré l'implication du rythme circadien dans le contrôle de l'infection à *Staphyloccocus aureus*. L'invalidation du gène Timeless, un gène de l'horloge moléculaire suivi d'une infection à *Staphyloccocus aureus* montre que la planaire n'arrive pas éliminer les bactéries par rapport aux vers non invalidés (Tsoumtsa et al., 2017).

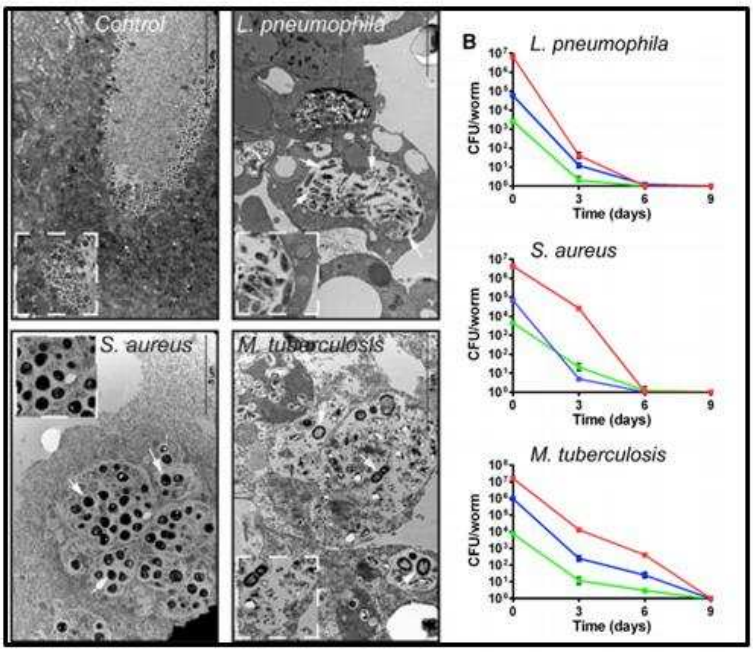

Figure 2 : Elimination bactérienne par la planaire D*ugesia japonica* (Abnave et al., 2014)

Ensuite, il a été mis en évidence un médiateur inflammatoire dans le contrôle de l'infection à *Staphylococcus aureus*. L'invalidation de LT4AH, un gène codant pour la production de l'enzyme leucotriène A4 hydrolase (LT4AH) impliquée dans la conversion de leucotriène A4 en B4, médiateurs de l'inflammation (Henderson, 1994), permettait d'augmenter la vitesse d'élimination de *Staphyloccous aureus* (Hamada et al., 2016).

Récemment, nous avons montré que la planaire avait la capacité de reconnaitre un agent pathogène et de l'éliminer rapidement lors d'un second contact 30 jours après la primo-infection. Cette mémoire immunologique est gouvernée par des néoblastes. L'invalidation des gènes impliqués dans le maintien des néoblastes au sein de la planaire, montre une perte de la mémoire immunologique (Torre et al., 2017a). Les néoblastes sont des cellules souches pluripotents, l'équivalent des cellules souches mésenchymateuses chez les vertébrés(Keating et al., 2017).

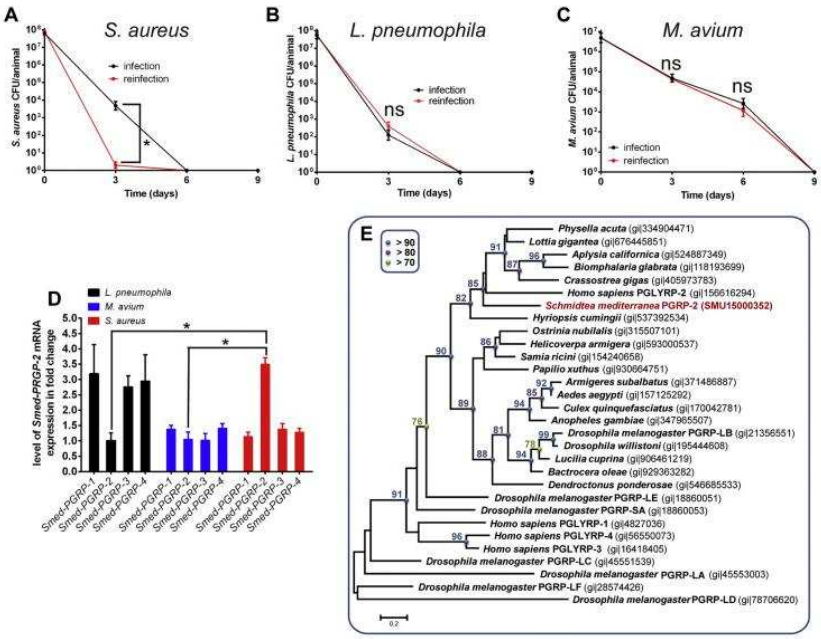

Figure 3 : Immunité entrainée dépendant du gène Smed-PGRP 2 contre *Staphylococcus aureus* chez la planaire *Schmidtea mediteranea* (Torre et al., 2017a)**.**

Au début de notre thèse, aucune étude n'avait mis en évidence la présence d'immunité entrainée dans les cellules souches. En effet, toutes les études portaient sur les cellules immunitaires innées telles que les monocytes, macrophages et cellules NK (Natural killer). Il s'agit de cellules différenciées de durée de vie limitée, plus courte que la durée de l'immunité entrainée. Partant de ce constat, Jorge Pereira à travers une revue intitulée « **A lasting impression : epigenetic memory of bacterial infections** ? (Pereira et al., 2016) avait suggéré d'investiguer la mémoire immunitaire des cellules pro-géniteurs de longue durée de vie. Sur la base de ces suggestions et les résultats issus de notre étude, nous avons décidé d'étudier l'immunité entrainée des cellules souches de l'Homme, plus particulièrement des cellules souches mésenchymateuses dérivées de la pulpe dentaire.

Les cellules souches de la pulpe dentaire que nous nommerons hDPSCs (human Dental Pulp Stem Cells) sont des cellules mésenchymateuses dérivées du tissu pulpaire de la dent. Les hDPSCs résident dans la zone apicale de la pulpe dentaire riche en cellules **Figure 4** (Galler et al., 2014; Martens et al., 2012). *In vitro*, les hDPSCs ont une forme similaire aux cellules fibroblastiques (fusiformes, coniques, présentant un cytoplasme volumineux avec un noyau central **Figure 5** (Varga et al., 2011). Comme toutes les cellules souches, les hDPSCs possèdent la propriété de s'auto-renouveler et de se différencier en plusieurs types de cellules telles que les ondotoblastes (Wang et al., 2011), les ostéoblastes (Liu et al., 2009), les chondrocytes (Iohara et al., 2006), les adipocytes (Iohara et al., 2006), les cellules nerveuses (Arthur et al., 2008) et les cellules vasculaires *in vitro* **Figure 6.**

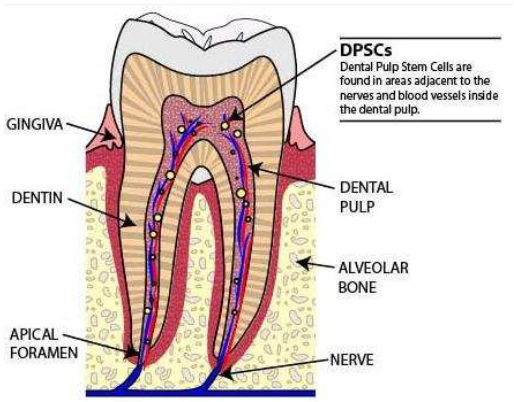

Figure 4 : Localisation des cellules souches dans la pulpe dentaire.

http://palmbeachdentistfl.com/dentistry/stem-cells-from-dental-pulp-used-to-make-corneal-cells/attachment/dental-pulp-stem-cells-dpscs/

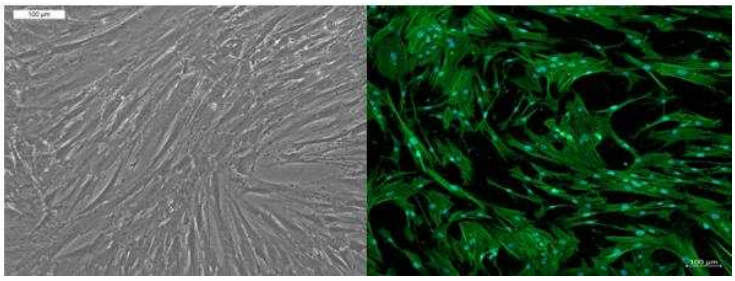

Figure 5 : Morphologie des cellules souches de la pulpe dentaire.

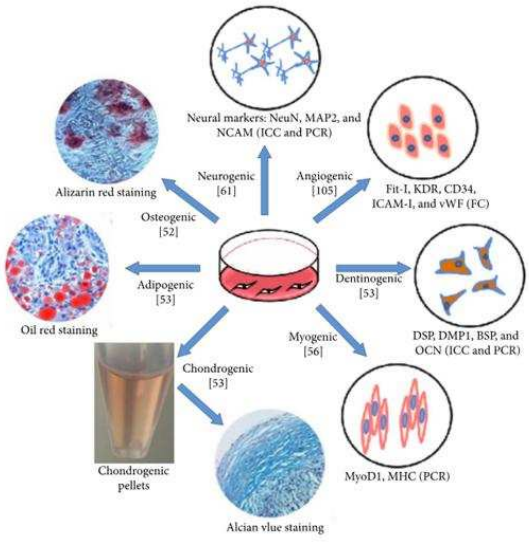

Figure 6 : Potentiel de multi différentiation des DPSC (Luo et al., 2018)**.**

Dans les conditions homéostatiques, les hDPSCs restent quiescentes et temporairement non prolifératives. Leur principale fonction est de produire des odontoblastes pour assurer la production de la dentine et le maintien de la vitalité biologique et physiologique de la dentine. En cas de lésion de la pulpe, causée par des facteurs mécaniques, chimiques ou thermiques, les hDPSCs sont activées et migrent vers le site de lésion où elles se différencient en odontoblastes afin de remplacer les cellules lésées et réparer la dentine (Rombouts et al., 2016; Téclès et al., 2005) **Figure 7.**

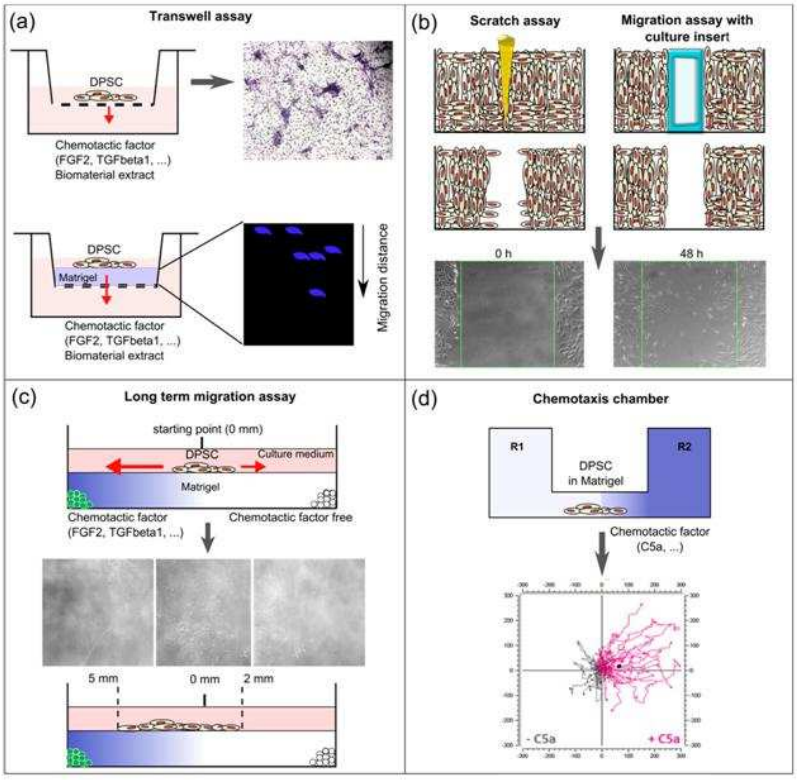

Figure 7 : Migration des hDPSC après lésions tissulaires (Rombouts et al., 2016)**.**

Ces fonctions naturelles de production d'odontoblastes et de différentiation multi-lignée sont utilisées pour des applications thérapeutiques telles que la réparation de la dentine et la régénération des structures dentaires. Des expériences récentes ont démontré le potentiel thérapeutique des cellules souches de pulpe dentaire pour la régénération

complète de la pulpe (Nakashima et al., 2017). Les hDPSCs sont également étudiées pour la réparation des tissus en dehors de la dent telle que la régénération neuronale des tissus œsophagiens (Chalisserry et al., 2017; Luo et al., 2018; Zhang et al., 2018) .

Outre les dommages chimiques et physiques, la pulpe dentaire peut subir des lésions causées par des bactéries ou par une maladie inflammatoire comme la parodontite. Les hDPSCs sont alors activées, prolifèrent et migrent vers le site de lésion. Ainsi, venant à proximité des bactéries et des composants bactériens où elles initient l'inflammation via leurs propriétés immunitaires, elles peuvent se différencier pour créer de nouvelles cellules et tissus différenciés comme la dentine afin d'assurer le maintien de la fondation de la structure dentaire (Li et al., 2014; Rombouts et al., 2016; Téclès et al., 2005).

Au cours des dix dernières années, de nouvelles connaissances ont été apportées sur la biologie des cellules souches qui prouvent que les hDPSCs contribuent également à la défense de l'hôte et à l'inflammation (He et al., 2017).

Des études ont montré que les hDPSC possèdent des propriétés immunitaires pour faire face aux agressions microbiennes. Fawzy El Sayd et ses collaborateurs ont montré qu'en réponse à un agent infectieux les hDPSCs sont activées et expriment des « pattern recognition receptors » (PRRs) plus particulièrement les Toll-like Receptors (TLRs) permettant ainsi de reconnaitre les « pathogen associated molecular patterns » (PAMPs) largement exprimés à la surface de différents types de microorganismes (Fawzy El-Sayed et al., 2016a) .

Une ensemble de mécanismes peuvent être activés suite à l'engagement des TLRs tel que la régulation à la hausse des effecteurs de l'immunité innée, y compris la production de peptides antimicrobiens contre les bactéries, de cytokines pro-inflammatoires et de chimiokines qui recrutent et activent les cellules immunitaires / inflammatoires résidant

dans les tissus (He et al., 2013, 2014; Tomic et al., 2011). Une étude a montré que des hDPSCs stimulées par le lipopolycasschariде (LPS), qui est un composé majeur de la paroi bactérienne des bactéries Gram-négatif, induit la production d'interleukine 8 (Il-8), par un processus dépendant de l'activation de TLR-4 et NF-KB, de manière similaire aux cellules immunitaires (He et al., 2013). Une étude semblable a montré également la production d'un ensemble de cytokines pro-inflammatoires IL-1b, IL-6, IL-8, TNF-α, MCP-1 en réponse au signal de danger LPS. Cette étude a montré en parallèle que les hDPSCs produisent aussi une cytokine anti-inflammatoire IL-10 comme les cellules immunitaires, permettant de moduler la réponse inflammatoire et de revenir dans une condition homéostatique (Bindal et al., 2018). Par ailleurs cet ensemble de réactions inflammatoires permet d'activer les cellules immunitaires et de les attirer sur le site de l'infection afin de développer une réponse immunitaire protectrice.

Grace à leurs propriétés régénératives et immunitaires, les cellules souches de la pulpe dentaires se présentent comme une source potentielle de cellules pour la médecine régénérative. Cependant, avant que les hDPSCs ne soient utilisées pour des applications cliniques, il est impératif de comprendre leurs propriétés biologiques en réponse aux bactéries. Les mécanismes immunitaires sous-jacents à la réponse des cellules de la pulpe dentaire humaine aux traumatismes ne sont pas complètement compris. D'autres propriétés immunitaires peuvent être investiguées. Effectivement les cellules souches situées dans la zone interne de la chambre de la pulpe dentaire en contact étroit avec la terminaison nerveuse, pourraient constituer des cellules sentinelles pour les blessures et l'invasion d'agents pathogènes circulant dans le sang.

Notre objectif principal au cours de notre thèse est d'apporter de nouvelles connaissances concernant les propriétés immunitaires des cellules souches de la pulpe dentaire en réponse aux bactéries. Pour répondre à l'objectif principal, différentes propriétés immunitaires ont été explorées.

En premier lieu, nous avons réalisé une revue bibliographique pour faire le point sur les nouvelles avancées biologiques concernant les propriétés immunitaires des cellules souches de la pulpe dentaire. Ensuite, nous avons poursuivi nos travaux de recherche bibliographique en rédigeant une deuxième revue bibliographique sur l'immunité entrainée, qui traite deux grands points : le paradoxe entre la durée de vie des cellules de l'immunité innée et la durée de l'immunité entrainée, le deuxième point étant l'immunité entrainée portée par des cellules souches et cellules non professionnellement immunitaires. Dans un troisième temps, nous avons réalisé des travaux de recherche expérimentale mettant en évidence une mémoire inflammatoire des hDPSCs aux ligands bactériens LPS et peptidoglycane (PGN). Enfin, nous avons étudié expérimentalement les propriétés phagocytaires des cellules souches de la pulpe dentaire en utilisant la bactérie pathogène intracellulaire facultative *Bartonella quintana* comme modèle.

Chapitre 1 :

La planaire *Schmidtea mediterranea* : modèle d'étude pour des interactions hôte-pathogène

Article :

Inhibition of LTA4H expression promotes *Staphylococcus aureus* elimination by planarians

Attoumani Hamada, Cedric Torre, Catherine Lepolard, Eric Ghigo

Préambule de la revue

Malgré les dernières découvertes sur les caractéristiques immunitaires de la planaire, le système immunitaire des planaires reste encore largement inexploré. De ce fait, nous avons décidé de mettre en évidence chez la planaire, des agents antibactériens et médiateurs inflammatoires connus pour jouer un rôle sur la réponse immunitaire sur les vertébrés. Sur cette base nous avons étudié l'implication d'un ensemble de médiateurs de l'inflammation et des peptides antimicrobiens dans la réponse immunitaire antimicrobienne de la planaire *Schmidtea mediteranea*.

Par génétique comparative, en utilisant la base de donnés SMED GD (Robb et al., 2015), nous avons identifié chez la planaire des gènes homologues à des gènes de l'Homme incluant la cathelicidin (LL-37), un peptide antimicrobien (Krasnodembskaya et al., 2010), Indoleamine 2,3-dioxygenase IDO-1 un immuno-modulateur (Mbongue et al., 2015), le médiateur inflammatoire Macrophage mediator in resolving inflammation 1 (Mar1) (Serhan et al., 2012), l'enzyme leukotriene A4 hydrolase (LT4AH) impliquée dans la conversion de leukotriène A4 en B4, médiateurs de l'inflammation (Henderson, 1994), le peptide antimicrobien beta-defensin (Semple and Dorin, 2012) et le médiateur immunitaire Nitric Oxide (Madhu et al., 2016).

Des amorces spécifiques ont été désignées sur la base de ces gènes prédits et une PCR a été réalisée avec pour matrice l'ADNc des ARNm totaux de la planaire *Schmidtea mediterranea.* Les gènes *Smed-LL37, Smed-IDO-1,Smed- Maresin, Smed-LT4AH, Smed-Beta-defensin* et *Smed-Nitric Oxide*, ont été amplifiés avec succès en présence de témoins négatifs. Ces observations montrent l'existence, dans le génome de *Schmidtea mediterranea*, de régions capables de coder pour des protéines présentant une homologie significative avec les

peptides antimicrobiens et des médiateurs inflammatoires humains. Dans un deuxième temps, l'invalidation de ces gènes par RNA interference (RNAi), suivie d'une infection avec la bactérie *Staphylococcus aureus* a permis de montrer que seul le gène *Smed-LT4AH* avait un impact sur l'élimination des bactéries *Staphylococcus aureus*. L'invalidation de ce gène permettait aux vers d'éliminer rapidement les bactéries, passant de 6 jours chez les vers contrôles à 4 jours chez les vers invalidés. Cette observation est en total désaccord avec les observations publiées chez les vertébrés montrant plutôt une susceptibilité à l'infection bactérienne lors d'une déficience en LTA4H (Tobin et al., 2010). LT4AH est connu pour jouer un rôle dans la diminution de la douleur dans une inflammation induite dans un modèle de rat (Henderson, 1994).

Cette étude donne la voie pour étudier d'autres facteurs antimicrobiens connus chez les vertébrés.

Inhibition of LTA4H expression promotes *Staphylococcus aureus* elimination by planarians

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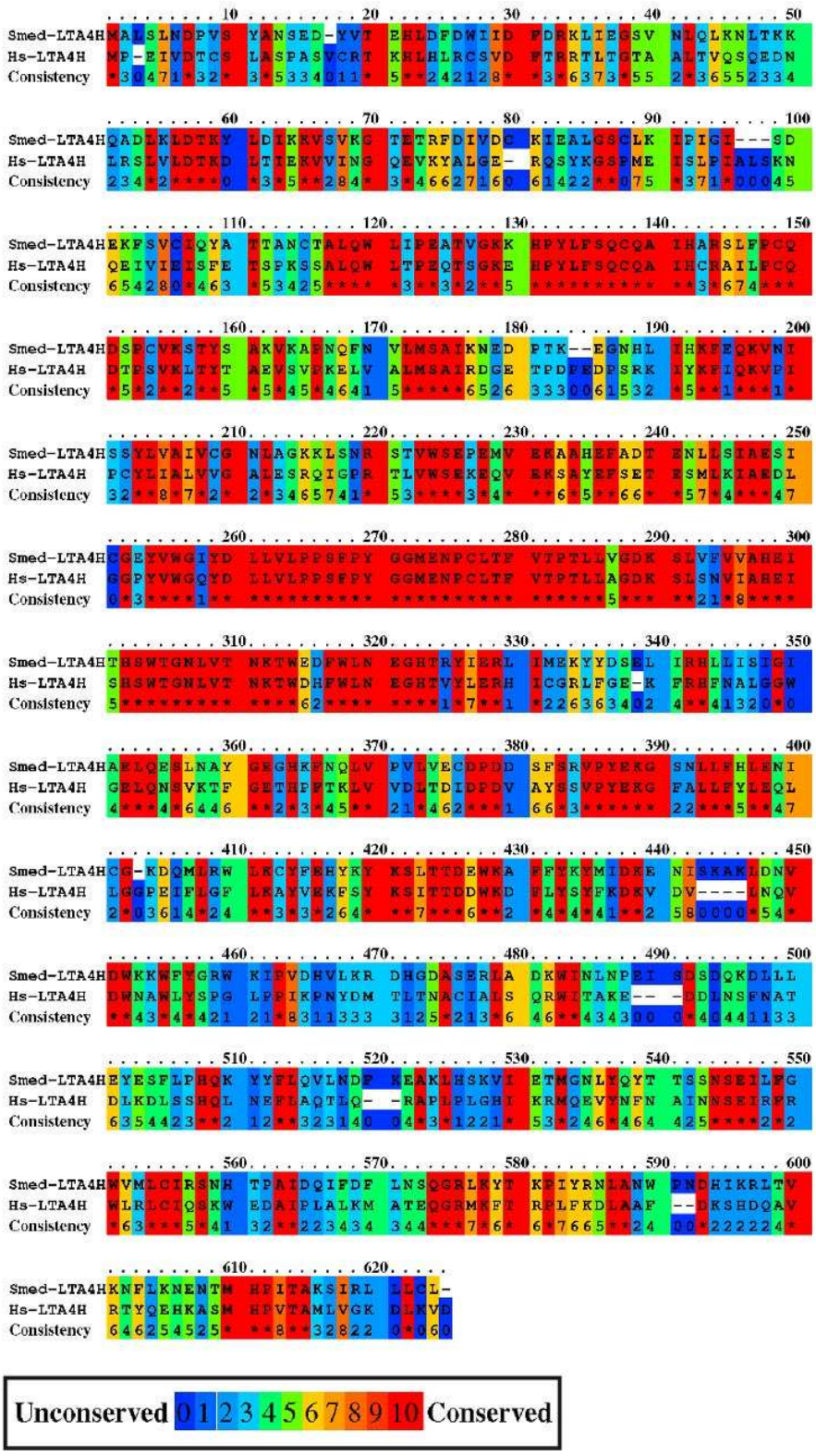
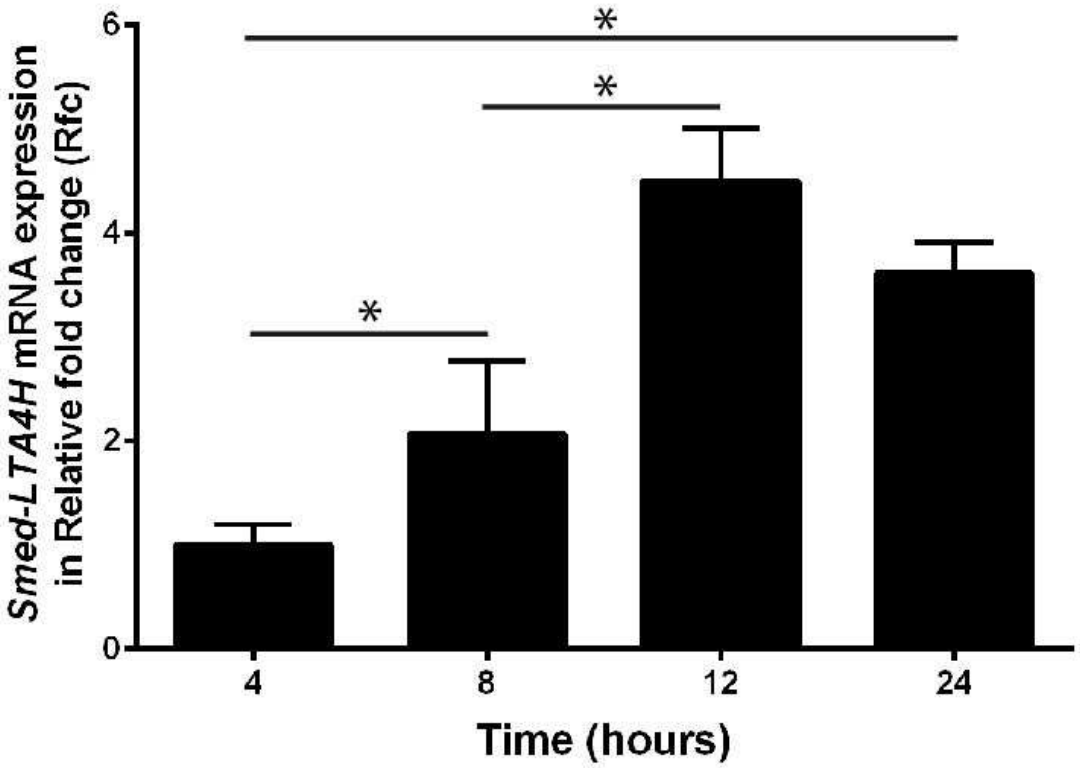
Abstract
The study of host-pathogen interactions in model organisms offers a means to characterize mechanisms and new critical innate immune genes that are conserved in higher eukaryotes. In vertebrates, leukotriene A4 hydrolase (LTA4H) is known to play a role in bacterial growth restriction. Planarian, a non-vertebrate organism, has the extraordinary ability to fight a wide spectrum of bacterial pathogens and have the faculty to regenerate an entire organism from a tissue fragment. However, the antibacterial response of the planarian remains poorly understood. We evaluated the contribution of LTA4H in the antibacterial response of the planarian, and we have observed that the silencing of the *Smed-LTA4H* gene by RNA interference promotes the *S. aureus* clearance, suggesting a role of LTA4H in the microbicidal activity of planarians.

Introduction
The planarian, a non-vertebrate, is actively used to investigate developmental and regeneration processes [1]. In addition, this platyhelminth is a model used to investigate the evolutionarily conserved mechanism of antibacterial response because of its resistance to infection [2]. Indeed, the planarian species *Schmidtea mediterranea* and *Dugesia japonica* are able to eliminate a large spectrum of human pathogens, including *Staphylococcus aureus* [2], a microbe responsible for nosocomial disease and for causing pneumonia, abscess, sepsis, toxic shock syndrome [3]. In vertebrates, Leukotriene A4 hydrolase (LTA4H) is a ubiquitously expressed enzyme that catalyzes the final step in the synthesis of leukotriene B4 (LTB4), a potent pro-inflammatory lipid mediator derived from arachidonic acid [4]. LTA4H controls the balance of pro-inflammatory and anti-inflammatory eicosanoids and determines the expression of tumor necrosis factor (TNF)-α. The expression of LTA4H induces the production of pro-inflammatory cytokines; in contrast, the inhibition of LTA4H reduces the LPS-induced production of pro-inflammatory cytokines, upregulates the production of the anti-inflammatory cytokine interleukin-10, and enhances bacterial invasion or bacterial susceptibility [5] [6] [7] [8] [9].

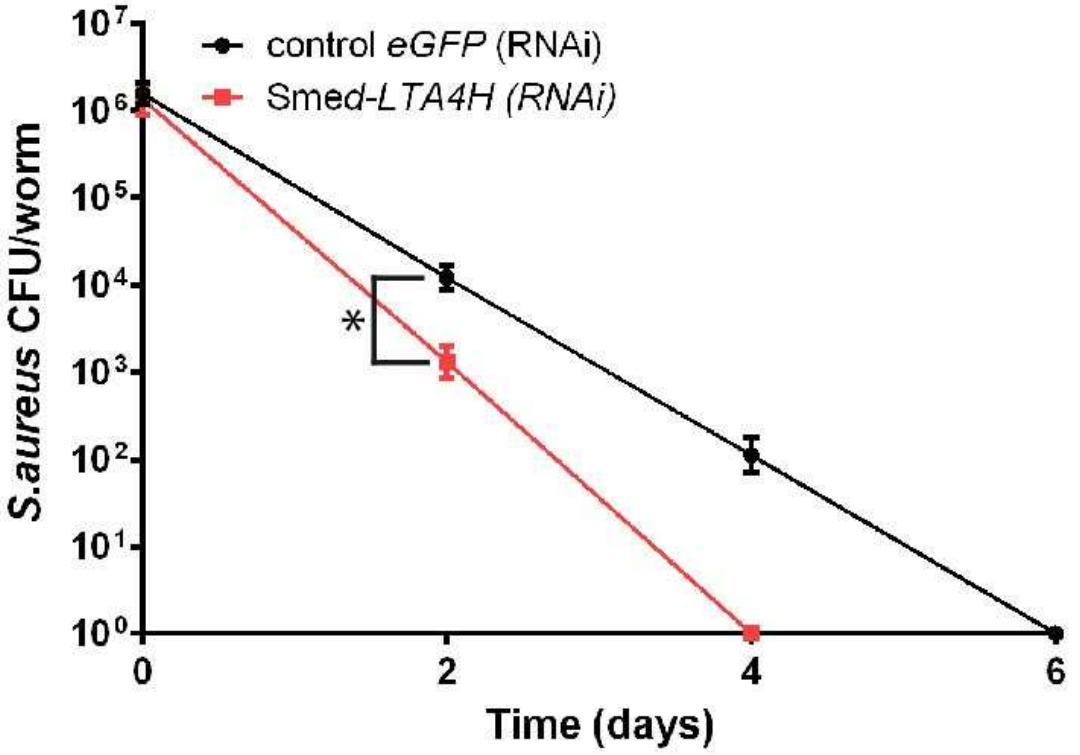
Objective
The role of LTA4H in resolving a bacterial infection in a model of resistance to bacterial infection, such as planarians, remains unknown. Therefore, we investigated the role of LTA4H in the planarian species *Schmidtea mediterranea*, infected with *S. aureus*.


Smed-LTA4H
Hs-LTA4H
Consistency
Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved

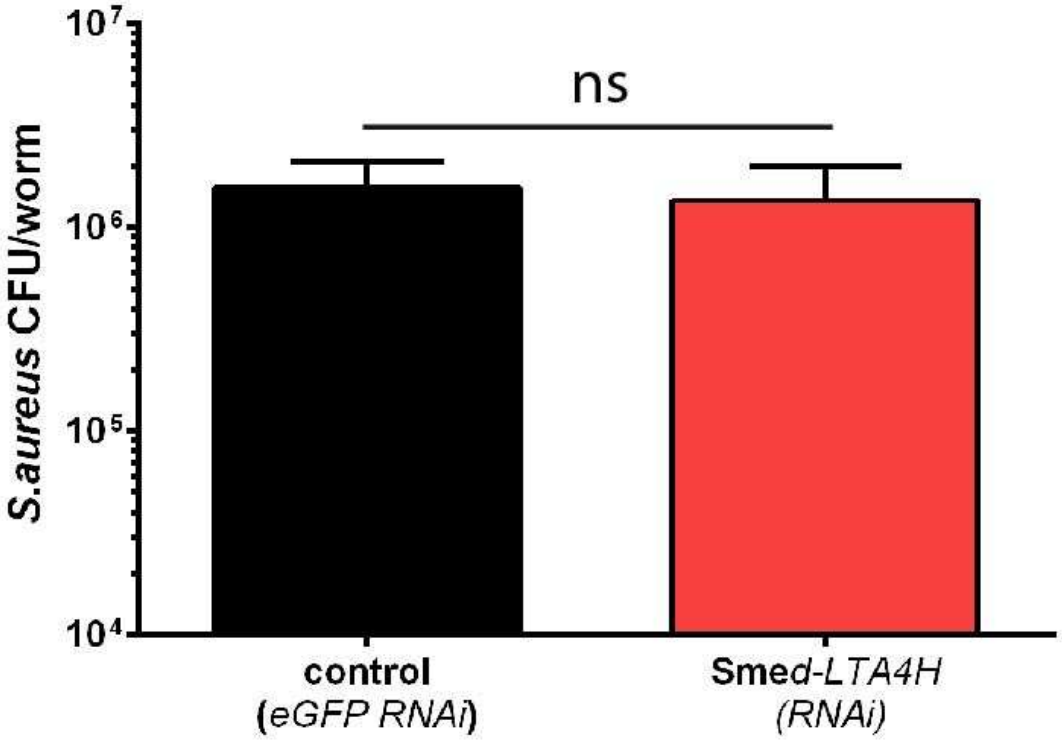
a


Smed-LTA4H mRNA expression in Relative fold change (Rfc)
6
4
2
0
*
*
*
4
8
12
24
Time (hours)

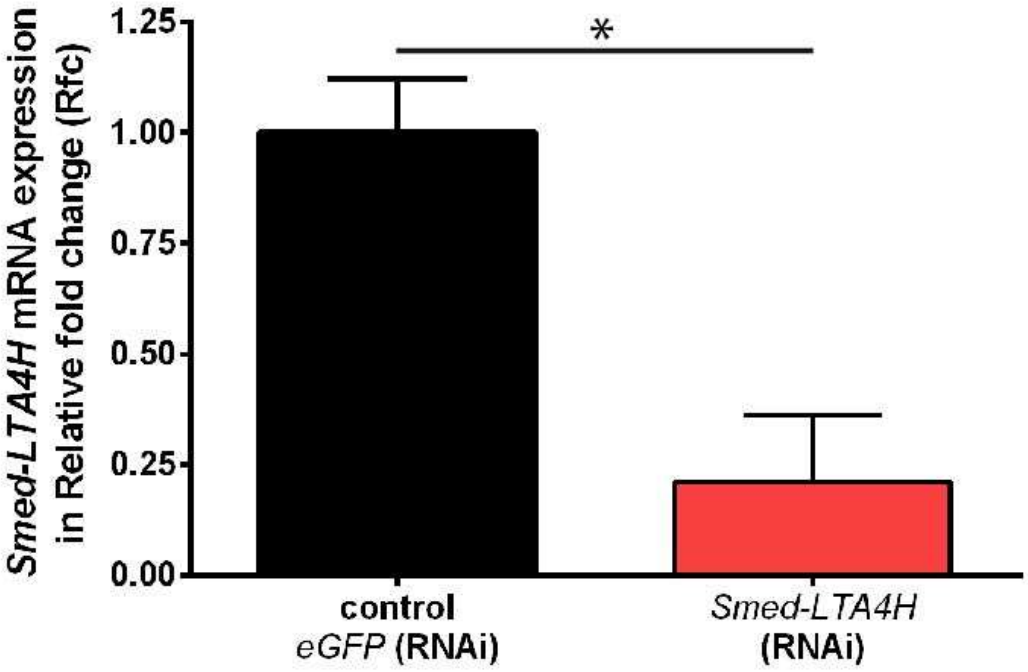
b


S.aureus CFU/worm
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
control eGFP (RNAi)
Smed-LTA4H (RNAi)
*
0
2
4
6
Time (days)

c



d



e

Figure Legend

Figure 1. The silencing of *Smed-LTA4H* enhanced bacterial elimination.

(A) Protein alignment between Hs-LTA4H (NP_000886.1) and predicted Smed-LTA4H. The results are color coded for amino acid conservation and the scoring scheme works from 0, for the least conserved alignment position, to 10, for the most conserved alignment position.
(B) Planarians were challenged with *S. aureus* (1×10e9 bacteria), and *Smed-LTA4H* expression was evaluated using RTqPCR. *S. aureus* induced a transient expression of *Smed-LTA4H*. The results are presented as the mean ± SD (3 worms per time point processed individually in triplicate, number of experiments= 3, *p <0.05).
(C) The *S. mediterranea eGFP (RNAi)* and the *S. mediterranea LTA4H* (RNAi) were fed with *S. aureus* (1×10e9 bacteria), and the CFUs per worm were counted over time. *S. aureus* were eliminated in 6 days in control and 4 days in planarians silenced for *Smed-LTA4H*. The results are expressed as the mean ± SD (10 worms per time point, the number of experiments= 3, *p <0.05).
(D) The amount of bacteria ingested by the *S. mediterranea eGFP (RNAi)* and the *S. mediterranea LTA4H (RNAi)* after feeding (Time 0 day) was determined by counting the CFUs. The *S. mediterranea eGFP (RNAi)* and the *S. mediterranea LTA4H (RNAi)* had ingested the same amount of bacteria. The results are expressed as the mean ± SD (10 worms per time point, number of experiments= 3).
(E) The efficiency of the *Smed-LTA4H* silencing by RNAi in the *S. mediterranea* was determined using RTqPCR. In *S. mediterranea LTA4H (RNAi)* animals, the level of expression of *Smed-LTA4H* is diminished of 79% compared to the control (*eGFP RNAi*). The results are expressed as the mean ± SD (3 worms per time point processed individually in triplicate, number of experiments= 3, *p <0.05).

Planarians

Used planarians belong to the species *Schmidtea mediterranea* (ClW4). Planarians were maintained as previously described [13] in autoclaved water at 20°C and fed once per week with calf liver. Animals were starved for at least 2 weeks prior to the experiments.

Bacteria

Staphylococcus aureus (ATCC25923) was grown on blood agar plates (BioMerieux SA) at 37°C.

Worm feeding with bacteria

S. mediterranea were fed with *S. aureus* (1×10e9 bacteria) using a protocol adapted from a dsRNA feeding method [14] as previously described [2]. Briefly, *S. aureus* were suspended in homogenized liver, mixed with ultra-low-gelling-temperature agar and red food coloring, and allowed to solidify on ice. Room temperature solidified food was fed to planarians. After 2 h (defined as day 0) of feeding, the planarians were extensively washed, and then collected or kept at 20°C in function of the experiments. Each experiment has been made in triplicate, for each experiment there were 10 worms per time point.

CFU Counting

As previously described [2], *S. mediterranea* were collected and homogenized in PBS. The lysate was passed 5 times through a sterile syringe with a 29G needle to disrupt planarian tissue clumps, and CFUs were counted after plating of 10 µl of a serial dilution of planarian lysates onto blood agar plates (BioMerieux SA) followed by an incubation of 24 h at 37°C.

Gene prediction

Smed-LTA4H has been identified using PlanMine transcriptomes database (http://planmine.mpi-cbg.de). Protein sequence of the Hs-LTA4H (NP_005082.1) has been blast via blastp against planmine transcriptomes dd_Smed_v6 using default parameters (e-value 1e-5). We identified the sequences producing a significant alignment with Hs-LTA4H. The top BLAST hit was used to predict *Smed-LTA4H* via FGENESH+ (http://www.softberry.com/) using default parameters. The homology at the protein level between predicted *Smed-LTA4H* and Hs-LTA4H (NP_005082.1) was analyzed using blastx. The conservation scoring was performed by PRALINE (http://www.ibi.vu.nl/ programs/pralinewww/) using default parameters. The results are color coded for amino acid conservation and the scoring scheme works from 0, for the least conserved alignment position, to 10, for the most conserved alignment position.

Cloning

To generate the *Smed-LTA4H* RNAi, cDNA from *S. mediterranea* was amplified via PCR designed with Primer3 (http://primer3.sourceforge.net/) and containing attB recombination sequences, and then the obtained PCR product was cloned as described elsewhere [14]. The dsRNA in silico accuracy prediction was defined as follows. Targeted transcript sequences were extracted between the 3' end of the 5' primer and the 5' end of the 3' primer used for cloning. The extracted sequences were then cut into 21 mers using a sliding window of 1 nucleotide. All possible RNAi sequences were then generated, and each putative RNAi sequence was aligned to the planarian transcriptome using BLAST with a word size of 21; only perfect matches were considered. For each transcript for which an RNAi was designed, a theoretical target accuracy was calculated based on the number of RNAi sequences matching the target divided by the total number of generated RNAi sequences. *Smed-LTA4H* primers (left primer CTCGTTCCGGTTCTTGTCGA, right primer GCAGGCGTGTGATTTGATCG), attB recombination sequences (CATTACCATCCCG).

Delivery of dsRNAs

dsRNAs were delivered to *S. mediterranea* as previously described [14]. The quality of *Smed-LTA4H* knock down was controlled via real-time RTqPCR as described elsewhere [15]. Primers used for real-time RTqPCR were for *Smed-LTA4H* (left primer TATGCCACTACCGCGAACTG, right primer CGGGCTATCTTGGCATGGAA). Results were normalized by the expression of the control housekeeping gene *Smed-ef2* [16].

RNA extraction

Total RNA (1 animal per sample, and 3 animals per experimental time point) were extracted from planarians using the Trizol method, as recommended by the manufacturer (Invitrogen). Experiments were made in triplicates.

Statistical Analysis

The results are expressed as means ± SD and were analyzed using the nonparametric Mann-Whitney U test. Differences were considered significant at $p < 0.05$.

Nucleotide sequence and protein sequence of predicted Smed-LTA4H

>FGENESH:[mRNA] predicted Smed-LTA4H 1854 bp ATGGCCTTATCGC-
TAAACGATCCTGTGTCATATGCAAATTCAGAAGACTATGTTACTGAA-
CATTTGGATTTTGACTGGATAATAGATTTTGATAGAAAACTAATTGAAG-
GATCAGTAAATCTACAATTGAAAAACTTAACCAAAAAACAAGCTGACT-
TAAAATTAGATACGAAATATCTTGATATTAAAAAAGTGTCAGTGAAAG-
GAACGGAAACTAGATTTGATATTGTAGATTGTAAAATCGAAGCACTTG-
GATCGTGTTTAAAAATTCCAATTGGAATTTCCGATGAAAAATTTTCGGTTTG-
TATTCAATATGCCACTACCGCGAACTGTACAGCTCTTCAGTGGTTAATTCCG-
GAAGCAACTGTTGGAAAAAAACATCCCTATTTATTTAGTCAATGTCAAGC-
CATACACGCTAGAAGTCTTTTTCCATGCCAAGATAGCCCGTGTGTAAAATC-
GACTTATTCTGCAAAGGTAAAAGCTCCTAATCAATTCAATGTTTTAAT-
GAGTGCTATTAAAAATGAAGATCCTACTAAAGAAGGAAATCATTTGATACA-
CAAATTCGAACAGAAAGTTAATATTTCTAGCTATCTTGTTGCTATTGTATGTG-
GTAATTTGGCTGGAAAAAAGCTAAGTAACCGTTCAACTGTCTGGTCAGAAC-
CGGAAATGGTGGAAAAAGCCGCTCATGAGTTTGCAGACACTGAAAACT-
TATTATCTATTGCTGAAAGCATATGCGGCGAGTATGTTTGGGGCATATAT-
GACTTACTAGTATTGCCACCTTCGTTTCCATACGGGGGGATGGAAAATC-
CATGTCTTACATTTGTTACTCCAACGCTTTTGGTTGGGGATAAATCCCTTGT-
GTTTGTAGTTGCGCATGAAATCACTCATTCGTGGACTGGAAACCTAG-
TAACTAATAAAACATGGGAAGATTTTTGGTTAAATGAGGGTCACACTC-
GATATATCGAAAGATTGATAATGGAGAAGTATTATGATTCCGAACTGATAAG-
GCATCTGCTAATTTCTATCGGCATAGCAGAACTTCAAGAATCACTTAAT-
GCATACGGAGAAGGTCATAAATTTAATCAACTCGTTCCGGTTCTTGTC-
GAATGCGATCCAGACGATTCTTTCTCACGAGTGCCCTATGAGAAAG-
GCTCCAATTTATTATTCCATTTAGAAAATATTTGTGGCAAAGATCAAAT-
GTTGCGCTGGTTGAAATGCTATTTTGAGCACTACAAATATAAATCTCTTA-

CAACTGATGAGTGGAAGGCTTTCTTTTATAAATACATGATTGATAAGGAAAA-
CATTTCAAAAGCTAAACTTGATAATGTAGATTGGAAGAAATGGTTTTATGGAA-
GATGGAAAATTCCAGTTGACCACGTTCTCAAACGGGATCATGGTGATGCCAGT-
GAGAGACTTGCCGATAAATGGATTAATTTGAACCCAGAAATATCAGATTCC-
GATCAAAAGGATTTATTATTGGAATATGAATCGTTCTTACCGCATCAAAAATAT-
TACTTTTTGCAAGTGCTCAATGATTTTAAAGAGGCAAAGTTACATTCCAAAGT-
TATCGAGACAATGGGTAATTTGTACCAATACACCACTAGTTCTAATAGT-
GAAATACTATTCGGTTGGGTAATGCTGTGCATTCGATCAAATCACACGCCTG-
CAATTGATCAAATATTTGACTTTCTCAATTCACAAGGCCGATTGAAATATAC-
CAAGCCAATATATCGAAACCTTGCTAATTGGCCTAATGATCACATCAAACGAT-
TAACTGTTAAAAATTTCCTGAAAAACGAAAATACAATGCACCCAATAACTG-
CAAAAAGCATCAGACTTCTATTATGCCTCTGA

>FGENESH: predicted Smed-LTA4H 617 aa MALSLNDPVSYANSEDYVTEHLDFD-
WIIDFDRKLIEGSVNLQLKNLTKKQADLKLDTKYLDIKKVSVKGTETRFDIVDCKIEAL-
GSCLKIPIGISDEKFSVCIQYATTANCTALQWLIPEATVGKKHPYLFSQCQAIHARSLF-
PCQDSPCVKSTYSAKVKAPNQFNVLMSAIKNEDPTKEGNHLIHKFEQKVNISSYL-
VAIVCGNLAGKKLSNRSTVWSEPEMVEKAAHEFADTENLLSIAESICGEYVWGIY-
DLLVLPPSFPYGGMENPCLTFVTPTLLVGDKSLVFVVAHEITHSWTGNLVTNK-
TWEDFWLNEGHTRYIERLIMEKYYDSELIRHLLISIGIAELQESLNAYGEGHKFN-
QLVPVLVECDPDDSFSRVPYEKGSNLLFHLENICGKDQMLRWLKCYFEHYKYK-
SLTTDEWKAFFYKYMIDKENISKAKLDNVDWKKWFYGRWKIPVDHVLKRDHG-
DASERLADKWINLNPEISDSDQKDLLLEYESFLPHQKYYFLQVLNDFKEAKLHSKVIET-
MGNLYQYTTSSNSEILFGWVMLCIRSNHTPAIDQIFDFLNSQGRLKYTKPIYRNLANW-
PNDHIKRLTVKNFLKNENTMHPITAKSIRLLLCL

Results & Discussion

First, we searched for a homologue to *Homo sapiens (Hs)-LTA4H* (NM_000895.2) in *S. mediterranea* using the transcriptome database PlanMine (http://planmine.mpi-cbg.de/planmine/begin.do). Our TBLASTN analysis, using the Hs-LTA4H (NP_-000886.1) as a query and the planarian transcriptomic data (dd_Smed_v6), identified 10 sequences producing significant alignments with Hs-LTA4H (lcl|dd_Smed_v6_-733_0_2, e-value 1e-160; lcl|dd_Smed_v6_733_0_1, e-value 3e-160; lcl|dd_Smed_v6_-3027_0_1, e-value 8e-142; lcl|dd_Smed_v6_2224_0_1, e-value 2e-21; lcl|dd_Smed_v6_-15623_0_1, e-value 2e-20; lcl|dd_Smed_v6_6470_0_1, e-value 7e-20; lcl|dd_Smed_v6_-6958_0_1, e-value 7e-20; lcl|dd_Smed_v6_1249_0_1, e-value 2e-17; lcl|dd_Smed_v6_-8086_0_1, e-value 2e-13; lcl|dd_Smed_v6_7405_0_1, e-value 6e-06). Using FGENESH+ (http://www.softberry.com/), we predicted *Schmidtea mediterranea (Smed)-LTA4H*, as a homologue to *Hs-LTA4H*. Analysis with BLASTx showed a 44% homology at the protein level (97% of cover, e-value 2e-166) for predicted *Smed-LTA4H* with *Hs-LTA4H* (Fig. 1A). Second, using quantitative real-time PCR, we evaluated the expression level of the *Smed-LTA4H* in worms challenged by *S. aureus*. Our results demonstrated that *S. aureus* induced the mRNA expression of the *Smed-LTA4H*. We observed the level of mRNA expression of the *Smed-LTA4H* increase transiently from a relative fold change of 1 to a maximum relative fold change of 4.5, which was reached after 12 h of challenge with *S. aureus* (Fig. 1B). These data suggest that *Smed-LTA4H* is induced in response to *S. aureus* infection. Next, using RNA interference, we inhibited the expression of the *Smed-LTA4H* in planarians, and the *Smed-LTA4H (RNAi)* animals were fed *S. aureus*. We evaluated the clearance of *S. aureus* at 3, 6, and 9 days post-feeding using a direct measurement of the colony-forming units (CFUs) (Fig. 1C). 2 days after infection, we observed that the *S. aureus* CFU count was less important in the *Smed-LTA4H (RNAi)* worms (1.31×10e3 *S. aureus* CFU/worm) than in the control *Smed-eGFP (RNAi)* worms (1.20×10e4 *S. aureus* CFU/worm). 4 days after infection, the *Smed-LTA4H (RNAi)* worms had fully eliminated the *S. aureus*, whereas in the control, *eGFP (RNAi)* worms, *S. aureus* was still detected (1.12×10e2 ± 6.44×10e1 CFU/worm). *S. aureus* was detected for 2 additional days in the control *eGFP (RNAi)* worms. The observed

difference between the *Smed-LTA4H (RNAi)* worms and the control *eGFP (RNAi)* worms in the rate of *S. aureus* elimination was not due to differences in the level of infection at To, as there was no significant difference in the number of *S. aureus* CFU/worm detected at To between the *Smed-LTA4H (RNAi)* worms and the control *eGFP (RNAi)* worms (1.56×10e6 ± 5.31×10e5 vs 1.35×10e6 ± 6.50×10e5, respectively) (Fig. 1D). The inhibition of *Smed-LTA4H* expression via RNAi increases the rate of elimination of *S. aureus* in planarians. It is interesting to note that *S. aureus* induces the expression of *Smed-LTA4H*, suggesting that a survival strategies of *S. aureus* is to induce the expression of *Smed-LTA4H* to decrease the rate of its elimination in planarians. However, this strategy fails, probably because of other antibacterial mechanisms engaged by planarian to fight microbes. The knock-down efficiency of *Smed-LTA4H* was confirmed using real time RT-qPCR. The expression of *Smed-LTA4H* was reduced by 80% compared to the control *Smed eGFP (RNAi)* worms (Fig. 1E). Taken together, these data shows that the silencing of *Smed-LTA4H* does not affect the capacity of planarians to ingest bacteria and promotes the capacity of planarians to eliminate *S. aureus*. The specificity of the RNAi against *LTA4H* has been controlled. Indeed, for *Smed-LTA4H* transcript for which the RNAi was designed, the theoretical target accuracy has been calculated. We find a number of theoretical off target equal to 0, thus a target accuracy of 100%, excluding that the observed effect was due to an off-target effect. The data from this study demonstrate that (1) *S. aureus* induces the expression of the *Smed-LTA4H* and survives up to 6 days in planarians and (2) the knock down of the *Smed-LTA4H* significantly increased the antimicrobial activity of planarians, and *S. aureus* were eliminated by 4 days after infection. To date, there is no published data on the role of the LTA4H in the antibacterial processes of non-vertebrates. Therefore, the only comparisons we can make to the results from our study on non-vertebrate are based on the findings in vertebrates. In vertebrates, the overexpression of the LTA4H is responsible for an increase in TNF-induced cell necrosis leading to bacterial death. In contrast, a reduction in the LTA4H expression leads to an increase in bacterial growth [5] [9]. In addition, other work in mammals suggest LTA4H deficiency is significantly associated with tuberculosis meningitis, lymph node tuberculosis, bone tuberculosis and other extra-pulmonary tuberculosis with the exception of pleural tuberculosis in humans [6] [4] [8].Therefore in vertebrates, LTA4H deficiency likely leads to bacterial proliferation and a failure to resolve the bacterial infection due to the resultant anti-inflammatory properties, whereas overexpression of the LTA4H is associated with pro-inflammatory properties and thus leads to bacterial death. Here, we observed that LTA4H deficiency in planarians has the opposite effect compared to observations in vertebrates because LTA4H deficiency promotes the clearance of bacteria in planarians. This observed difference might be linked to the extraordinary capacity of planarians to regenerate any part of their body. In fact, it has been suggested that inhibition of the LTA4H expression by maresin-1 (MaR1) induced a faster regeneration of the planarian head [10]. In planarians, the autophagy process is required for tissue regeneration [11] and bacterial clearance [2]. The invalidation of the LTA4H gene could result in an increase in autophagy and a disruption of the tissue homeostasis equilibrium (controlled via autophagy), thereby leading to an increase in the elimination of the bacteria.

Conclusions

We can speculate through a comparison with mammalians that *LTA4H* expression is associated with the anti-inflammatory profile in planarians, but this must be proven by further experiments. However, we can conclude that in this study we have shown that the silencing of the *LTA4H* gene in planarians enhances the capability of planarians to kill *S. aureus*. These results suggest that in planarians, *LTA4H* expression is taking part to the anti-bacterial mechanisms engaged by planarians to fight microbes.

Limitations

These experiments were conducted using a non-vertebrate model that is highly resistant to infection and has the capacity to continuously regenerate. The particular biological properties of the planarians could explain the unexpected role of the LTA4H compared

to other organisms. In addition, we have specifically worked with *S. aureus*, it could be interesting to analyze *LTA4H* deficient planarians challenged with other microorganisms, such as *Mycobacterium tuberculosis*, which are often used to study the function of the LTA4H in vertebrates.
As suggested above, it would be interesting to evaluate the contribution of autophagy in the microbicidal mechanisms mediated by LTA4H in planarians. For that, it will be important to analyze the level of the autophagy in the *LTA4H* knock-down planarians, the expression level of autophagy markers, such as MORN2 [2], and the planarian homologue of Hs-dap--1 [11], and the level of apoptosis by tunnel assay. It will be also interesting to determine the cell population expressing *LTA4H*, as well as the co-expression of *LTA4H* and autophagy makers in planarians. In addition, whether the regeneration process failed or is affected by the knock down of *LTA4H* should be examined to explain the role of LTA4H in bacterial elimination by planarians. However, we cannot exclude a contribution of the α-toxin produced by *S. aureus* [12] in our observation. It will be interesting to see if other strains of *S. aureus* which do not produce α-toxin, such as the *S. aureus RN4220* strain, have the same effect on *Smed-LTA4H* expression, and the effect of the silencing of *Smed-LTA4H* on *S. aureus* RN4220 behaviors.

Additional Information

Methods

Planarians

Used planarians belong to the species *Schmidtea mediterranea* (ClW4). Planarians were maintained as previously described [13] in autoclaved water at 20℃ and fed once per week with calf liver. Animals were starved for at least 2 weeks prior to the experiments.

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Staphylococcus aureus (ATCC25923) was grown on blood agar plates (BioMerieux SA) at 37℃.

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S. mediterranea were fed with *S. aureus* (1×10e9 bacteria) using a protocol adapted from a dsRNA feeding method [14] as previously described [2]. Briefly, *S. aureus* were suspended in homogenized liver, mixed with ultra-low-gelling-temperature agar and red food coloring, and allowed to solidify on ice. Room temperature solidified food was fed to planarians. After 2 h (defined as day 0) of feeding, the planarians were extensively washed, and then collected or kept at 20℃ in function of the experiments. Each experiment has been made in triplicate, for each experiment there were 10 worms per time point.

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As previously described [2], *S. mediterranea* were collected and homogenized in PBS. The lysate was passed 5 times through a sterile syringe with a 29G needle to disrupt planarian tissue clumps, and CFUs were counted after plating of 10 µl of a serial dilution of planarian lysates onto blood agar plates (BioMerieux SA) followed by an incubation of 24 h at 37℃.

Gene prediction

Smed-LTA4H has been identified using PlanMine transcriptomes database (http://planmine.mpi-cbg.de). Protein sequence of the Hs-LTA4H (NP_005082.1) has been blast via blastp against planmine transcriptomes dd_Smed_v6 using default parameters (e-value 1e-5). We identified the sequences producing a significant alignment with Hs-LTA4H. The top BLAST hit was used to predict *Smed-LTA4H* via FGENESH+ (http://www.softberry.com/) using default parameters. The homology at the protein level between predicted *Smed-LTA4H* and Hs-LTA4H (NP_005082.1) was analyzed using blastx. The conservation scoring was performed by PRALINE (http://www.ibi.vu.nl/ programs/pralinewww/) using default parameters. The results are color coded for amino acid conservation and the scoring scheme works from 0, for the least conserved alignment position, to 10, for the most conserved alignment position.

Cloning

To generate the *Smed-LTA4H* RNAi, cDNA from *S. mediterranea* was amplified via PCR designed with Primer3 (http://primer3.sourceforge.net/) and containing attB recombina-

tion sequences, and then the obtained PCR product was cloned as described elsewhere [14]. The dsRNA in silico accuracy prediction was defined as follows. Targeted transcript sequences were extracted between the 3' end of the 5' primer and the 5' end of the 3' primer used for cloning. The extracted sequences were then cut into 21 mers using a sliding window of 1 nucleotide. All possible RNAi sequences were then generated, and each putative RNAi sequence was aligned to the planarian transcriptome using BLAST with a word size of 21; only perfect matches were considered. For each transcript for which an RNAi was designed, a theoretical target accuracy was calculated based on the number of RNAi sequences matching the target divided by the total number of generated RNAi sequences. *Smed-LTA4H* primers (left primer CTCGTTCCGGTTCTTGTCGA, right primer GCAGGCGTGTGATTTGATCG), attB recombination sequences (CATTACCATCCCG).

Delivery of dsRNAs

dsRNAs were delivered to *S. mediterranea* as previously described [14]. The quality of *Smed-LTA4H* knock down was controlled via real-time RTqPCR as described elsewhere [15]. Primers used for real-time RTqPCR were for *Smed-LTA4H* (left primer TATGCCACTACCGCGAACTG, right primer CGGGCTATCTTGGCATGGAA). Results were normalized by the expression of the control housekeeping gene *Smed-ef2* [16].

RNA extraction

Total RNA (1 animal per sample, and 3 animals per experimental time point) were extracted from planarians using the Trizol method, as recommended by the manufacturer (Invitrogen). Experiments were made in triplicates.

Statistical Analysis

The results are expressed as means ± SD and were analyzed using the nonparametric Mann-Whitney U test. Differences were considered significant at $p < 0.05$.

Nucleotide sequence and protein sequence of predicted Smed-LTA4H

>FGENESH:[mRNA] predicted Smed-LTA4H 1854 bp ATGGCCTTATCGC-
TAAACGATCCTGTGTCATATGCAAATTCAGAAGACTATGTTACTGAA-
CATTTGGATTTTGACTGGATAATAGATTTTGATAGAAAACTAATTGAAG-
GATCAGTAAATCTACAATTGAAAAACTTAACCAAAAAACAAGCTGACT-
TAAAATTAGATACGAAATATCTTGATATTAAAAAAGTGTCAGTGAAAG-
GAACGGAAACTAGATTTGATATTGTAGATTGTAAAATCGAAGCACTTG-
GATCGTGTTTAAAAATTCCAATTGGAATTTCCGATGAAAAATTTTCGGTTTG-
TATTCAATATGCCACTACCGCGAACTGTACAGCTCTTCAGTGGTTAATTCCG-
GAAGCAACTGTTGGAAAAAAACATCCCTATTTATTTAGTCAATGTCAAGC-
CATACACGCTAGAAGTCTTTTTCCATGCCAAGATAGCCCGTGTGTAAAATC-
GACTTATTCTGCAAAGGTAAAAGCTCCTAATCAATTCAATGTTTTAAT-
GAGTGCTATTAAAAATGAAGATCCTACTAAAGAAGGAAATCATTTGATACA-
CAAATTCGAACAGAAAGTTAATATTTCTAGCTATCTTGTTGCTATTGTATGTG-
GTAATTTGGCTGGAAAAAAGCTAAGTAACCGTTCAACTGTCTGGTCAGAAC-
CGGAAATGGTGGAAAAAGCCGCTCATGAGTTTGCAGACACTGAAAACT-
TATTATCTATTGCTGAAAGCATATGCGGCGAGTATGTTTGGGGCATATAT-
GACTTACTAGTATTGCCACCTTCGTTTCCATACGGGGGGATGGAAAATC-
CATGTCTTACATTTGTTACTCCAACGCTTTTGGTTGGGGATAAATCCCTTGT-
GTTTGTAGTTGCGCATGAAATCACTCATTCGTGGACTGGAAACCTAG-
TAACTAATAAAACATGGGAAGATTTTTGGTTAAATGAGGGTCACACTC-
GATATATCGAAAGATTGATAATGGAGAAGTATTATGATTCCGAACTGATAAG-
GCATCTGCTAATTTCTATCGGCATAGCAGAACTTCAAGAATCACTTAAT-
GCATACGGAGAAGGTCATAAATTTAATCAACTCGTTCCGGTTCTTGTC-
GAATGCGATCCAGACGATTCTTTCTCACGAGTGCCCTATGAGAAAG-
GCTCCAATTTATTATTCCATTTAGAAAATATTTGTGGCAAAGATCAAAT-
GTTGCGCTGGTTGAAATGCTATTTTGAGCACTACAAATATAAATCTCTTA-
CAACTGATGAGTGGAAGGCTTTCTTTTATAAATACATGATTGATAAGGAAAA-
CATTTCAAAAGCTAAACTTGATAATGTAGATTGGAAGAAATGGTTTTATGGAA-
GATGGAAAATTCCAGTTGACCACGTTCTCAAACGGGATCATGGTGATGCCAGT-
GAGAGACTTGCCGATAAATGGATTAATTTGAACCCAGAAATATCAGATTCC-
GATCAAAAGGATTTATTATTGGAATATGAATCGTTCTTACCGCATCAAAAATAT-
TACTTTTTGCAAGTGCTCAATGATTTTAAAGAGGCAAAGTTACATTCCAAAGT-

TATCGAGACAATGGGTAATTTGTACCAATACACCACTAGTTCTAATAGT-
GAAATACTATTCGGTTGGGTAATGCTGTGCATTCGATCAAATCACACGCCTG-
CAATTGATCAAATATTTGACTTTCTCAATTCACAAGGCCGATTGAAATATAC-
CAAGCCAATATATCGAAACCTTGCTAATTGGCCTAATGATCACATCAAACGAT-
TAACTGTTAAAAATTTCCTGAAAAACGAAAATACAATGCACCCAATAACTG-
CAAAAAGCATCAGACTTCTATTATGCCTCTGA
>FGENESH: predicted Smed-LTA4H 617 aa MALSLNDPVSYANSEDYVTEHLDFD-
WIIDFDRKLIEGSVNLQLKNLTKKQADLKLDTKYLDIKKVSVKGTETRFDIVDCKIEAL-
GSCLKIPIGISDEKFSVCIQYATTANCTALQWLIPEATVGKKHPYLFSQCQAIHARSLF-
PCQDSPCVKSTYSAKVKAPNQFNVLMSAIKNEDPTKEGNHLIHKFEQKVNISSYL-
VAIVCGNLAGKKLSNRSTVWSEPEMVEKAAHEFADTENLLSIAESICGEYVWGIY-
DLLVLPPSFPYGGMENPCLTFVTPTLLVGDKSLVFVVAHEITHSWTGNLVTNK-
TWEDFWLNEGHTRYIERLIMEKYYDSELIRHLLISIGIAELQESLNAYGEGHKFN-
QLVPVLVECDPDDSFSRVPYEKGSNLLFHLENICGKDQMLRWLKCYFEHYKYK-
SLTTDEWKAFFYKYMIDKENISKAKLDNVDWKKWFYGRWKIPVDHVLKRDHG-
DASERLADKWINLNPEISDSDQKDLLLEYESFLPHQKYYFLQVLNDFKEAKLHSKVIET-
MGNLYQYTTSSNSEILFGWVMLCIRSNHTPAIDQIFDFLNSQGRLKYTKPIYRNLANW-
PNDHIKRLTVKNFLKNENTMHPITAKSIRLLLCL

Supplementary Material

Please see https://sciencematters.io/articles/201604000011.

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Ethics Statement

Not applicable.

Citations

[1] Sarah A. Elliott and Alejandro Sánchez Alvarado. "The history and enduring contributions of planarians to the study of animal regeneration". In: *Wiley Interdisciplinary Reviews: Developmental Biology* 2.3 (July 2012), pp. 301–326. DOI: 10.1002/wdev.82. URL: http://dx.doi.org/10.1002/wdev.82.

[2] Prasad Abnave et al. "Screening in Planarians Identifies MORN2 as a Key Component in LC3-Associated Phagocytosis and Resistance to Bacterial Infection". In: *Cell Host and Microbe* 16.3 (Sept. 2014), pp. 338–350. DOI: 10.1016/j.chom.2014.08.002. URL: http://dx.doi.org/10.1016/j.chom.2014.08.002.

[3] Adam G. Peres and Joaquín Madrenas. "The broad landscape of immune interactions with Staphylococcus aureus: From commensalism to lethal infections". In: *Burns* 39.3 (May 2013), pp. 380–388. DOI: 10.1016/j.burns.2012.12.008. URL: http://dx.doi.org/10.1016/j.burns.2012.12.008.

[4] Robert J Snelgrove. "Leukotriene A4 hydrolase: an anti-inflammatory role for a proinflammatory enzyme". In: *Thorax* 66.6 (Apr. 2011), pp. 550–551. DOI: 10.1136/thoraxjnl-2011-200234. URL: http://dx.doi.org/10.1136/thoraxjnl-2011-200234.

[5] David M. Tobin et al. "The lta4h Locus Modulates Susceptibility to Mycobacterial Infection in Zebrafish and Humans". In: *Cell* 140.5 (Mar. 2010), pp. 717–730. DOI: 10.1016/j.cell.2010.02.013. URL: http://dx.doi.org/10.1016/j.cell.2010.02.013.

[6] James Curtis et al. "Association analysis of the LTA4H gene polymorphisms and pulmonary tuberculosis in 9115 subjects". In: *Tuberculosis* 91.1 (Jan. 2011), pp. 22–25. DOI: 10.1016/j.tube.2010.11.001. URL: http://dx.doi.org/10.1016/j.tube.2010.11.001.

[7] Sarah J. Dunstan et al. "LTA4H Genotype Is Associated with Susceptibility to Bacterial Meningitis but Is Not a Critical Determinant of Outcome". In: *PLOS ONE* 10.3 (Mar. 2015), e0118789. DOI: 10.1371/journal.pone.0118789. URL: http://dx.doi.org/10.1371/journal.pone.0118789.

[8] Jinghui Yang et al. "Relationship between human LTA4H polymorphisms and extra-pulmonary tuberculosis in an ethnic Han Chinese population in Eastern China". In: *Tuberculosis* 94.6

(Dec. 2014), pp. 657–663. DOI: 10.1016/j.tube.2014.08.014. URL: http://dx.doi.org/10.1016/j.tube.2014.08.014.

[9] David M. Tobin et al. "Host Genotype-Specific Therapies Can Optimize the Inflammatory Response to Mycobacterial Infections". In: *Cell* 148.3 (Feb. 2012), pp. 434–446. DOI: 10.1016/j.cell.2011.12.023. URL: http://dx.doi.org/10.1016/j.cell.2011.12.023.

[10] Charles N. Serhan et al. "Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain". In: *The FASEB Journal* 26.4 (Jan. 2012), pp. 1755–1765. DOI: 10.1096/fj.11-201442. URL: http://dx.doi.org/10.1096/fj.11-201442.

[11] C. Gonzalez-Estevez et al. "Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation". In: *Proceedings of the National Academy of Sciences* 104.33 (Aug. 2007), pp. 13373–13378. DOI: 10.1073/pnas.0703588104. URL: http://dx.doi.org/10.1073/pnas.0703588104.

[12] Bryan J. Berube and Juliane Bubeck Wardenburg. "Staphylococcus aureus α-Toxin: Nearly a Century of Intrigue". In: *Toxins* 5.6 (June 2013), pp. 1140–1166. DOI: 10.3390/toxins5061140. URL: http://dx.doi.org/10.3390/toxins5061140.

[13] Francesc Cebrià and Phillip A. Newmark. "Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture". In: *Development* 132.16 (July 2005), pp. 3691–3703. DOI: 10.1242/dev.01941. URL: http://dx.doi.org/10.1242/dev.01941.

[14] Peter W. Reddien et al. "Identification of Genes Needed for Regeneration, Stem Cell Function, and Tissue Homeostasis by Systematic Gene Perturbation in Planaria". In: *Developmental Cell* 8.5 (May 2005), pp. 635–649. DOI: 10.1016/j.devcel.2005.02.014. URL: http://dx.doi.org/10.1016/j.devcel.2005.02.014.

[15] David J. Forsthoefel et al. "An RNAi Screen Reveals Intestinal Regulators of Branching Morphogenesis, Differentiation, and Stem Cell Proliferation in Planarians". In: *Developmental Cell* 23.4 (Oct. 2012), pp. 691–704. DOI: 10.1016/j.devcel.2012.09.008. URL: http://dx.doi.org/10.1016/j.devcel.2012.09.008.

[16] Jordi Solana et al. "Defining the molecular profile of planarian pluripotent stem cells using a combinatorial RNA-seq, RNA interference and irradiation approach". In: *Genome Biology* 13.3 (2012), R19. DOI: 10.1186/gb-2012-13-3-r19. URL: http://dx.doi.org/10.1186/gb-2012-13-3-r19.

Chapitre 2 :

Caractérisation des propriétés immunitaires des cellules souches de la pulpe dentaire hDPSCs

Revue:

Immune properties of Human Dental Pulp Stem cells and interactions with immune system

Attoumani Hamada, Michel Drancourt, Eric Ghigo

Préambule de la revue

En 2000, Gronthos *et al.* ont isolé les hDPSCs pour la première fois à partir de la pulpe dentaire (Gronthos et al., 2000). Suite à ce travail pionnier, les hDPSCs ont attiré beaucoup d'attention dans le domaine de la médecine régénérative, principalement en raison de leur capacité à s'auto-renouveler et à se différencier en plusieurs types cellulaires spécifiques (d'Aquino et al., 2008; Gronthos et al., 2000; Nuti et al., 2016; Potdar and Jethmalani, 2015). Au cours de la dernière décennie, leur capacité régénérative a été étudiée en étroite relation avec l'immunité, montrant le rôle majeur de leurs processus immunitaires dans la régénération et l'homéostasie tissulaire. En effet, les cellules souches de la pulpe dentaire possèdent également un arsenal immunitaire comportant l'expression de TLRs capables de reconnaitre un agent pathogène et de le combattre directement en produisant des peptides antimicrobiens tel que des beta-defensines ou de manière indirecte via la production de cytokines pro- et anti-inflammatoires qui recrutent les cellules immunitaires et promeuvent la clairance bactérienne (Farges et al., 2015; Fawzy El-Sayed et al., 2016a; Goldberg et al., 2008).

Avant d'investiguer les propriétés immunitaires des hDPSCs face à un agent infectieux, notre premier travail a été de rédiger une revue bibliographique dont le titre est «**: Immune properties of Human Dental Pulp Stem cells and interactions with immune system »** afin d'énumérer l'ensemble des capacités immunitaires des hDPSCs et leurs interactions avec les cellules immunitaires.

Chez les vertébrés, lorsque les cellules de l'immunité innée sont infectées, elles réagissent en produisant des chimiokines, chemotaxiques et par la sécrétion de cytokines pro-inflammatoires et anti-inflammatoires, facteurs de croissance, chimiokines pour initier une

réponse immunitaire protectrice. De manière intéressante, les hDPSCs adoptent le même comportement lorsqu'elles sont stimulées par du LPS et produisent des cytokines pro-inflammatoires pour recruter les cellules immunitaires mais également des cytokines anti-inflammatoires. Par conséquent, les hDPSCs devaient être ajoutées à la liste des cellules professionnellement non-immunitaires mais qui possèdent des propriétés immunitaires telles que les fibroblastes, les cellules épithéliales et cellules souches mésenchymateuses. En consultant la base de données PubMed de NCBI, nous avons observé qu'aucune étude n'avait été publiée sur la capacité phagocytaire des hDPSCs. Récemment il a été montré que des cellules mésenchymateuses du tissu adipeux avaient la capacité d'internaliser *Mycobacterium tuberculosis* et de contrôler sa réplication par autophagie (Khan et al., 2017). Sur ces bases bibliographiques, nous avons suggéré l'hypothèse que les hDPSCs pourraient internaliser un agent infectieux et initier une réponse immunitaire.

Annals of Stem Cell Research & Therapy

Review Article
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Immune Properties of Human Dental Pulp Stem Cells and Interactions with the Immune System

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Abstract

Dental Pulp Stem Cells (DPSCs) are mesenchymal stem cells well known by the scientific community thanks to the studies about their regenerative properties. This regenerative capacity is mainly due to their ability to differentiate into multi-lined cells and their self-renewal. Since their isolation by Gronthos in 2000, their regenerative capacity has been intensively studied in relation to immunity. Thus, several studies have reported about the immune properties of DPSCs. Nevertheless, little is known about the mechanism of action that DPSCs exert in their micro-environment and their interactions with immune cells. The purpose of this review is to summarize the set of knowledge on the immune properties of DPSCs and their interactions with immune cells.

Keywords: Dental pulp stem cells (DPSC); Immunity; Immunomodulatory; TLRs; NF-kb

General Presentation

Characterization

Dental Pulp Stem Cells (DPSCs) were first isolated by Gronthos et al. [1] in 2000 from a permanent tooth pulp of the third molar. Morphologically, they are fusiform, tapered with a central nucleus and a bulky cytoplasm. In comparison with Bone Marrow Stem Cells (BMSCs), they are highly proliferative and have a high potential for clonogenicity [2]. They represent a large and accessible stock of post-natal stem cells. The basic physiological properties of DPSCs are based on their multipotence. DPSCs are able to self-renew and to differentiate into multi-lineage cells, they are responsible for the maintenance and repair of periodontal tissues. They also have the ability to differentiate into different cell types such as odontonblasts, chondrocytes, adipocytes and neuronal cells according to appropriate induction conditions [1,3-5]. In homeostatic conditions, they are in a quiescent state, but after stimulation (i.e. mechanical or bacterial aggression, when tooth decay occurs), the DPSCs can be activated, resulting in a proliferation and migration into the damaged tissues where they differentiate into periodontal cells and form a repaired dentin. According to the criteria required by the International Society for Cellular Therapy ISCT, DPSCs are mesenchymal stromal cells [6]. They adhere to the plastic when they are maintained under standard culture conditions, proliferate by forming colonies and express mesenchymal antigen markers (CD13, CD29, CD44, CD73, CD90 and CD105) but lack hematopoietic markers (CD34 and CD45) [7]. Interestingly, this type of cell does not differentiate into immune cells.

Isolation of the dental pulp stem cells

Avulsion is performed for orthodontic reasons. Starting from the tooth, there are basically two methods for isolating DPSCs: an enzymatic method and an outgrowth method [8]. After extraction, the tooth surface is cleaned, disinfected and mechanically fractured around the cement-enamel junction. Then the pulp tissue is gently recovered in a standard medium and minced with a scalpel in cubes of 2 mm^2. This first part is common to both techniques. The enzymatic method consists in digesting the pulp by using an enzyme cocktail (dispase or collagenase) to obtain a monocellular suspension that is seeded in a medium with a plastic-adherent container. The outgrowth method is based on the direct culturing of pulp tissue fragments in a standard culture medium with a plastic-adherent container, with the stem cells budding around the explants. The proliferation medium is supplemented with penicillin or streptomycin antibiotics and antifungal amphotericin B. After cell proliferation, a washing and purification step by flow cytometry will follow which will eliminate contaminant cells such as monocytes, fibroblasts and hematopoietic stem cells.

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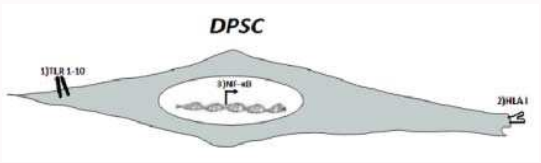

Figure 1: Immune characteristics of Dental Pulp Stem Cells (DPSC). DPSC are equipped with TLR 1-10, HLA-1 and produce NF-kB.

Immune Properties of Human Dental Pulp Stem Cells

TLR expression

The dental pulp is a richly vascularized and innervated tissue, containing different cell populations, such as fibroblasts, odontoblasts, immune cells (dendritic cells, macrophages and lymphocytes) and DPSCs [9-11]. This set constitutes a dynamic complex forming a functional unit conferring to the dental pulp its nutrition and repair functions as well as a primordial role in the response to aggressions. This activation is the consequence of the commitment of TLRs expressed on the cell surface by pathogens. Toll-like receptors are type I transmembrane glycoproteins with an extracellular domain Rich in Leucine Repeats (LRR) that is responsible for the recognition of PAMPs (Pathogens Associated Molecular Patterns) and a transmembrane and intracellular domain named Toll-Interleukin 1 cytoplasmic domain (TIR), that is required for downstream signaling [12]. TLRs are known to be expressed primarily in innate and adaptive immune cells, however, some studies have shown their expression on other cell types such as mesenchymal stromal cells [13]. Indeed, in 2012 Fawy El Sayed et al. [14] have shown for the first time that DPSCs express TLR 1-10 (Figure 1). In addition, the data showed that according to the inflammatory environment of the DPSCs, TLRs are expressed in different ways. Thus, in an inflammatory environment, TLR 2,3,4,5 and 8 are over-expressed while TLR 1,7, 9 and 10 are under-expressed, with an abolished TLR 6 expression [14].

Immunogenic profile

DPSCs are immuno-privileged cells also hypo immunogenic. As shown in Figure 1, they express on their surface the MHC I constitutively as all the nucleated cells and do not express MHC II [15]. Although DPSCs are not direct immune effectors and do not express MHC II, they may act indirectly in initiating the immune response by activating immune cells by a signalling cascade [16]. It has been shown that human Mesenchymal Stromal Cells (MSCs) have the ability to express MHC II in inflammatory conditions and present antigens, an important characteristic of immune cells [17-19]. It is therefore suggested that DPSCs under inflammatory or stress conditions could also express MHC II and become immunogenic.

NF-KB roles

Discovered in 1986 by Sen and Baltimore, NF-KB is a key transcription factor for the transcription of light chain kappa immunoglobulin genes. NF-KB's activity was first demonstrated in a murine model [20]. Soon after, it was found that its expression is ubiquitous, and present in almost all cells, including dental pulp stem cells (Figure 1). Numerous works have shown that NF-KB plays a central role in immunity through the production and the regulation of pro-inflammatory cytokines such as TNF-α, IL-8, chemokines (MCP-1), adhesion molecules (ICAM-1, VCAM-1, E-selectin), growth factors and antimicrobial peptides [21]. In 2005, Chang et al. [22] showed for the first time the expression of NF-KB in stem cells derived from the dental pulp and its implication in their immunomodulatory properties. DPSCs stimulated with both TNF and LPS induce the activation of NF-KB, leading to the production of pro-inflammatory cytokines such as IL-8. In addition, another study consolidates the idea that DPSCs possess an NF-KB dependent immune activity by reporting that *lipopolysaccharide* (LPS) induces IL-8 production via the TLR-4, MyD88 and NF-KB pathways [23]. NF-KB is also involved in the migration of DPSCs; indeed, the migration of DPSCs is important in the process of dentin repair during an attack of the tooth by an infectious agent during cariogenesis [24]. The pro-inflammatory cytokine IFN-γ has been shown to promote the proliferation as well as the migration of DPSCs depending on the activation of NF-KB [16]. Migration and immunity are indivisible processes, thus, the ability of cells to migrate is essential for tissue repair and immune response. In this same study, the authors also showed that NF-KB plays a role in the adhesion of DPSCs. The DPSCs stimulated by LPS activate the expression of NF-KB inducing an increase of the expression of IFN-γ, ICAM-1, integrin-β1 and VEGF and enhancing the adhesion of DPSCs [16]. In addition, NF-KB plays also a role in the process of cell differentiation. It has been shown that NF-KB is involved in the differentiation of mesenchymal stem cells. TNF-α promotes osteogenic differentiation of human mesenchymal stem cells via the NF-KB signaling pathway [25]. In agreement with mesenchymal stem cells, DPSCs stimulated by TNF-α also differentiate into osteogenic cells via NF-KB factor as shown in Figure 1 [26].

Immunomodulatory activity

In addition to multipotency and clonogenicity, DPSCs have been reported to regulate the immune response in many diseases and thus play an important role in immunity. Immunomodulation is the capacity of a substance to modify the immune responses by increasing (immuno-stimulator) or decreasing it (immuno-suppressor) to maintain homeostasis. Pierdomenico et al. [26] in 2005 have shown that DPSCs have immunoregulatory characteristics. Compared with BM-SCs, DPSCs are more immunosuppressive and the inhibition of Phyto-Hem -Agglutinin (PHA) stimulated T cell proliferation is more marked in the co-culture of DPSCs-T lymphocytes than of MSC-T lymphocytes [27]. The underlying mechanisms of these immunosuppressive properties of DPSCs have been discovered in 2011. This study shows that DPSCs suppressed the proliferation of Peripheral Blood Mononuclear Cells (PBMCs) by the production of Transforming Growth Factor (TGF), and this expression is correlated with the increased expression of TLR-4 and TLR-3 [28]. A recent study has shown that co-culture between naive T lymphocytes CD4+ and DPSCs induces an increase in Treg cells CD4+ and FOXP3+. This activation is the consequence of a strong TGF-β and IL-10 anti-inflammatory cytokines production [29]. Thus, this process confers immunotolerance to DPSCs. The molecular and cellular mechanisms underlying immunomodulatory properties rely heavily on the production of TGF-β, being the main soluble factor inhibiting hyper immune reactions. TGF-β is a pleiotropic cytokine known for its role in immune suppression. Several studies have shown its direct or indirect involvement in this process trough its link with the Treg, allowing the induction of the FOXP3 transcription factor, the master regulator of Treg naive cells. The master transcriptional factor of Treg cells is responsible for the suppression of pro-inflammatory T cells, inhibits the secretion of pro-inflammatory cytokines such as IL-2, IFN-γ, IL-4, IL-17 and enhances anti-inflammatory cytokine like IL -10 [30,31]. Others studies have also demonstrated the

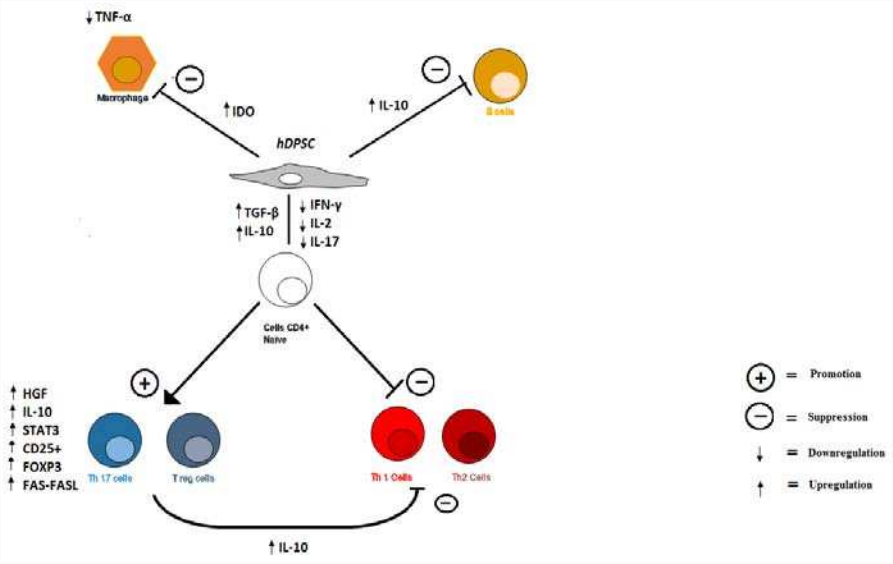

Figure 2: Immunosuppressive activity of Dental Pulp Stem Cells (DPSC). DPSC suppress Peripheral Blood Mononuclear Cell (PBMC) by producing immunosuppressive molecules such as Transforming Growth Factor beta (TGF- β) and interleukin 10. DPSC inhibit the function of macrophages and the production of B cells by producing respectively Indoleamine 2,3-Dioxygenase (IDO) and IL-10. DPSC induce a differentiation of CD4+ naïve cells to Treg cells and TH17 cells that activate STAT3, FOXP3, FAS-FASL, CD25+ produce also solubles factor IL-10 and Hepatocyte Growth Factor (HGF). Additionally DPSC inhibit the production of proinflammatory cytokine such as IL-2, IL-17 and IFN- to suppress Th1 and Th2 cells.

immunomodulatory properties of DPSCs in contact with immune cells, such as lymphocytes. Cell-cell contact of DPSCs and T lymphocytes induces the expression of FAS to induce their apoptosis [32]. FAS are a transmembrane cellular receptor inducing the death of cells expressing the Tumor Necrosis Factor (TNF6) receptor super family. The FAS / FASL interaction induces apoptotic cell death [33].

Interaction between Dpscs and Immune Cells

In teeth, dental pulp stem cells are found at the anatomical location of apical dental pulp. The microenvironment of this niche is composed of type I and III collagenic proteins, fibronectin, tenascin and other non-collagenous Extra Cellular Matrix (ECM) proteins. Cells such as fibroblasts, endothelial cells, pericytes and lymphatic vessels are also found, all of these elements constitute a functional unit. The main role of this complex is to regulate the homeostatic state of the dental pulp stem cells involved in the maintenance and repair of the pulp. In addition to their putative function, the DPSCs possess a role in immune defense based on a close relationship with defensive cells. Defensive cells such as inflammatory and immuno competent cells, namely dendritic cells, macrophages, lymphocytes and endothelial cells are also found, these cells are present in the dental pulp [34]. DPSCs interact with the cells of innate and adaptive immunity *via* direct cell-cell interactions or through the set of immunomodulatory molecules secreted (Figure 2).

Interaction between DPSCS and macrophages

Several researchers have already evidenced the presence of macrophages in healthy dental pulp, found in different morphological forms: round, oval, short-spindle, and dendritic [35]. Macrophages are the most predominant immune cells present in the dental pulp, with a sentinel role [10]. Macrophages play an important role in immunity and defense (acting mainly as trapping cells) thanks to their main function, the phagocytosis of foreign components, including infectious microorganisms. They also have the ability to communicate with other immune cells through the production of substances such as cytokines such as IL-1, IL-6, Tumor Necrosis Factor (TNF), antimicrobial peptides and Reactive Oxygen Species (ROS) as well as Growth Factors of Fibroblasts (FGF) and Endothelial Cells (EGF) that promote wound repair [36]. Recent studies have shown the interaction between dental pulp stem cells and macrophages. DPSCs are also able to modulate the immune system through their interaction with macrophages. A report has shown that DPSCs suppress macrophage activity via the TNF/IDO axis [37]. Moreover, DPSCs suppress the secretion of TNF produced by macrophages stimulated by LPS [37]. IDO is a cytosolic enzyme involved in the catabolism of tryptophan via the kynurenine degradation pathway. The products of tryptophan degradation are known to have immunoregulatory functions on immune cells, such as macrophages, they are also known for their strong immunosuppressive activity [38]. It was also reported that there are monocytes circulating in the dental pulp. Monocytes have a protective effect on DPSCs against cytotoxicity and cell-mediated NK cell lysis [39].

Interaction between DPSCs and T lymphocytes

In 1987, Jontel et al. [40] were the first to show the presence of CD8+ and CD4+ T lymphocytes in a healthy human dental pulp. This result was confirmed later by Hahn et al. [41]. Moreover, they demonstrate that the proportion of CD8+ is higher than that of CD4+ in normal teeth, establishing a CD4+/CD8+ ratio equal to 0.26 for normal pulp and 1.14 for pathological pulp. Gang Ding et al. [42] showed that DPSCs adopt an anti-inflammatory and immunosuppressive behavior when they are co-cultured with PBMC. DPSCs failed to stimulate the proliferation of allogene T cell and suppressed T cell proliferation and B cells by TGF-β secreting. In the same work, they also reported that DPSCs up-regulate IL-10, an anti-inflammatory cytokine and down-regulate the production of IL-2, IL-17 and IFN-gamma, pro-inflammatory cytokines, associated with an increase of Treg and Th 17 [43]. Moreover, according to the discovering of a perinatal murine model, DPSCs are capable of suppressing cell proliferation, inducing activated cell activation and ameliorating the inflammatory response. This process is associated with the expression

of FAS-FASL [32]. In addition, similar expressions of DPSC with other kinds of T cells have been reported. Recently Hong et al. [29] showed that co-cultured CD4+ T cells with DPSCs increase the number of CD4+, CD25+ and FOXP3+ regulatory T cell, trough the expression of TGF-β and IL-10. Thus, DPSCs can modulate immune tolerance by producing Treg. It has also been reported that DPSCs have an immunosuppressive activity on helper T cells, Th1 and Th2 subsets of CD4+ lymphocytes, and in parallel they stimulate Th17, a subset of Treg. A strong expression of Hepatocyte Growth Factor (HGF) is required to stimulate the production of Stat3 in Th17 cells, thus allowing for an inflammatory switch of pro-inflammatory Th17 in immunosuppressant Th17 [44].

Interaction between DPSC and B lymphocyte

The studies by Pulver et al. in 1977, Pekovic and Frillery in 1984 with immuno histochemistry or Jontel et al. in 1987 with monoclonal antibodies against B lymphocytes showed no evidence of the presence of B cells in a healthy human dental pulp. In 1989, thanks to the work done by Hahn et al., the presence of B lymphocytes in a healthy human dental pulp was evidenced. However, other teams have shown that there are a lot of antibodies in an inflamed pulp compared to a healthy pulp [10]. This presence of antibodies therefore suggests that there are B lymphocyte in the inflamed pulp [45,46]. This observation was confirmed by the work of Chin-Lo Hahn et al. [46] who they reported the presence of B lymphocytes in an infected pulp.

Conclusion

DPSCs possess immunological properties based on their hypo-immunogenicity and their secretome. These secretory activities enable them to interact with immune cells through the production of the identified soluble factors. Although there has been a major interest in their immune properties over the past decades, research has focused only on paracrine activity by analogy with mesenchymal cells in general. Other types of immune properties, like those found in professional immune cells, such as phagocytosis and the production of antimicrobial compounds could be studied in years to come.

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References

1. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA. 2000;97(25):13625-30.
2. Alge DL, Zhou D, Adams LL, Wyss BK, Shadday MD, Woods EJ, et al. Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. J Tissue Eng Regen Med. 2010;4(1):73-81.
3. Nemeth CL, Janebodin K, Yuan AE, Dennis JE, Reyes M, Kim DH. Enhanced chondrogenic differentiation of dental pulp stem cells using nanopatterned PEG-GelMA-HA hydrogels. Tissue Eng Part A. 2014;20(21-22):2817-29.
4. Jo YY, Lee HJ, Kook SY, Choung HW, Park JY, Chung JH, et al. Isolation and characterization of postnatal stem cells from human dental tissues. Tissue Eng. 2007;13(4):767-73.
5. El-Sayed KF, Dörfer C, Fändrich F, Gieseler F, Moustafa MH, Ungefroren H. Adult Mesenchymal Stem Cells Explored in the Dental Field. In Mesenchymal Stem Cells - Basics Clin Appl II. 2012;89-103. Berlin, Springer, Berlin, Heidelberg.
6. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.
7. Chalisserry EP, Nam SY, Park SH, Anil S. Therapeutic potential of dental stem cells. J Tissue Eng. 2017;8:2041731417702531.
8. Karamzadeh R, Eslaminejad MB, Aflatoonian R. Isolation, characterization and comparative differentiation of human dental pulp stem cells derived from permanent teeth by using two different methods. J Vis Exp. 2012;(69):4372.
9. Rombouts C, Giraud T, Jeanneau C, About I. Pulp Vascularization during Tooth Development, Regeneration, and Therapy. J Dent Res. 2017;96(2):137-44.
10. Jontell M, Okiji T, Dahlgren U, Bergenholtz G. Immune defense mechanisms of the dental pulp. Crit Rev Oral Biol Med. 1998;9(2):179-200.
11. Farges JC, Alliot-Licht B, Renard E, Ducret M, Gaudin A, Smith AJ, et al. Dental Pulp Defence and Repair Mechanisms in Dental Caries. Mediators Inflamm. 2015;2015:230251.
12. Leulier F, Lemaitre B. Toll-like receptors--taking an evolutionary approach. Nat Rev Genet. 2008;9(3):165-78.
13. Ledesma-Martínez E, Mendoza-Núñez VM, Santiago-Osorio E. Mesenchymal Stem Cells Derived from Dental Pulp: A Review. Stem Cells Int. 2016;2016:4709572.
14. Fawzy El-Sayed KM, Klingebiel P, Dörfer CE. Toll-like Receptor Expression Profile of Human Dental Pulp Stem/Progenitor Cells. J Endod. 2016;42(3):413-7.
15. Collart-Dutilleul P-Y, Chaubron F, De Vos J, Cuisinier FJ. Allogenic banking of dental pulp stem cells for innovative therapeutics. World J Stem Cells. 2015;7(7):1010-21.
16. He X, Jiang W, Luo Z, Qu T, Wang Z, Liu N, et al. IFN-γ regulates human dental pulp stem cells behavior via NF-κB and MAPK signaling. Sci Rep. 2017;7:40681.
17. Romieu-Mourez R, François M, Boivin M-N, Stagg J, Galipeau J. Regulation of MHC Class II Expression and Antigen Processing in Murine and Human Mesenchymal Stromal Cells by IFN-γ, TGF-β, and Cell Density. J Immunol. 2007;179(30):1549-58.
18. Chan JL, Tang KC, Patel AP, Bonilla LM, Pierobon N, Ponzio NM, et al. Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-γ. Blood. 2006;107(12):4817-24.
19. Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. Blood. 2006;107(6):2570-7.
20. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell. 1986;46(5):705-16.
21. Baldwin AS. Series Introduction: The transcription factor NF-κB and human disease. J Clin Invest. 2001;107(1):3-6.
22. Chang J, Zhang C, Tani-Ishii N, Shi S, Wang CY. NF-κB Activation in Human Dental Pulp Stem Cells by TNF and LPS. J Dent Res. 2005;84(11):994-8.
23. He W, Qu T, Yu Q, Wang Z, Lv H, Zhang J, et al. LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells. Int Endod J. 2013;46(2):128-36.
24. Gunzer M. Migration, cell-cell interaction and adhesion in the immune system. Ernst Schering Found Symp Proc. 2007;(3);97-137.
25. Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T. TNFα promotes

osteogenic differentiation of human mesenchymal stem cells by triggering the NF-κB signaling pathway. Bone. 2009;45(2):367-76.

26. Feng X, Feng G, Xing J, Shen B, Li L, Tan W, et al. TNF-α triggers osteogenic differentiation of human dental pulp stem cells via the NF-κB signalling pathway. Cell Biol Int. 2013;37(12):1267-75.

27. Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, et al. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation. 2005;80(6):836-42.

28. Tomic S, Djokic J, Vasilijic S, Vucevic D, Todorovic V, Supic G, et al. Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. Stem Cells Dev. 2011;20(4):695-708.

29. Hong JW, Lim JH, Chung CJ, Kang TJ, Kim TY, Kim YS, et al. Immune Tolerance of Human Dental Pulp-Derived Mesenchymal Stem Cells Mediated by CD4+CD25+FoxP3+ Regulatory T-Cells and Induced by TGF-β1 and IL-10. Yonsei Med J. 2017;58(5):1031-9.

30. Haiqi H, Yong Z, Yi L. Transcriptional regulation of Foxp3 in regulatory T cells. Immunobiology. 2011;216(6):678-85.

31. Mantel P-Y, Schmidt-Weber CB. Transforming growth factor-beta: recent advances on its role in immune tolerance. Methods Mol Biol Clifton NJ. 2011;677:303-38.

32. Zhao Y, Wang L, Jin Y, Shi S. Fas Ligand Regulates the Immunomodulatory Properties of Dental Pulp Stem Cells. J Dent Res. 2012;91(10):948-54.

33. Waring P, Müllbacher A. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol. 1999;77(4):312-7.

34. Goldberg M, Hirata A. The Dental Pulp: Composition, Properties and Functions. JSM. 2017;5(1):1079.

35. Orban BJ, Bhaskar SN. Orban's Oral Histology and Embryology. Mosby.1976.

36. Elhelu MA. The Role of Macrophages in Immunology. J Natl Med Assoc. 1983;75(3):314-7.

37. Lee S, Zhang QZ, Karabucak B, Le AD. DPSCs from Inflamed Pulp Modulate Macrophage Function via the TNF-α/IDO Axis. J Dent Res. 2016;95(11):1274-81.

38. Mbongue JC, Nicholas DA, Torrez TW, Kim N-S, Firek AF, Langridge WH. The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity. Vaccines (Basel). 2015;3(3):703-29.

39. Jewett A, Arasteh A, Tseng H-C, Behel A, Arasteh H, Yang W, et al. Strategies to Rescue Mesenchymal Stem Cells (MSCs) and Dental Pulp Stem Cells (DPSCs) from NK Cell Mediated Cytotoxicity. PLoS One. 2010;5(3):e9874.

40. Jontell M, Gunraj MN, Bergenholtz G. Immunocompetent Cells in the Normal Dental Pulp. J Dent Res. 1987;66(6):1149-53.

41. Hahn CL, Falkler WA, Siegel MA. A study of T and B cells in pulpal pathosis. J Endod. 1989;15(1):20-6.

42. Durutürk L, Sarı Ş, Şengül A. Immunocompetent cell level as a diagnostic reference for pulpal pathosis of primary teeth. Arch Oral Biol. 2013;58(10):1517-22.

43. Ding G, Niu J, Liu Y. Dental pulp stem cells suppress the proliferation of lymphocytes via transforming growth factor-β1. Hum Cell. 2015;28(2):81-90.

44. Özdemir AT, Özgül Özdemir RB, Kırmaz C, Sarıboyacı AE, Ünal Halbutoğlları ZS, Özel C, et al. The paracrine immunomodulatory interactions between the human dental pulp derived mesenchymal stem cells and CD4 T cell subsets. Cell Immunol. 2016;310:108-15.

45. Baumgartner JC, Falkler WA Jr, Bernie RS, Suzuki JB. Serum IgG reactive with oral anaerobic microorganisms associated with infections of endodontic origin. Oral Microbiol Immunol. 1992;7(2):106-10.

46. Hahn C-L, Falkler WA, Siegel MA. A study of T and B cells in pulpal pathosis. J Endod. 1989;15(1):20-6.

Article de recherche:

Internalization of the pathogen B*artonella quintana* into human dental pulp stem cells: new insights into *Bartonella quintana* relapsing infection

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(Unpublished data : subjecting to PLoS ONE)

Préambule de l'article

Au cours des dix dernières années, l'ensemble des articles sur les hDPSC portaient essentiellement sur la fonction chemotaxique, la sécrétion de cytokines pro-inflammatoires et anti-inflammatoires, de facteurs de croissance et de chimiokines (He et al., 2017; Özdemir et al., 2016; Zhao et al., 2012). Cependant, aucune étude sur la phagocytose par les cellules souches de la pulpe dentaire n'a été publiée. Pour mieux comprendre la biologie des hDPSCs lors d'une infection, il est important de savoir si les hDPSCs sont capables d'internaliser un agent pathogène par exemple par phagocytose. La phagocytose est l'un des mécanismes les plus importants de l'immunité innée chez l'homme, c'est un événement précoce et crucial dans le déclenchement des défenses de l'hôte contre les agents pathogènes(Gordon, 2016).

Classiquement la phagocytose est définie comme l'internalisation de particules de > 0,5 à 1 µm médiée par des types de cellules phagocytaires professionnelles et non professionnelles (Gordon, 2016; Lacy-Hulbert, 2009; Visan, 2013). Les phagocytes professionnels sont principalement des macrophages et des cellules dendritiques immatures (CD) résidant dans de multiples tissus et monocytes, neutrophiles et éosinophiles (Nagl et al., 2002; Shamri et al., 2011; Silva and Correia-Neves, 2012). Les phagocytes non-professionnels tels que les cellules épithéliales peuvent également capturer et internaliser des particules biologiques, y compris des cellules mourantes et bactéries (Monks et al., 2005; Rabinovitch, 1995; Tso et al., 2010; Visan, 2013). Récemment, une étude a montré que les cellules souches mésenchymateuses dérivées du tissu adipeux avaient la capacité d'internaliser *Mycobacterium tuberculosis* et de contrôler l'infection (Khan et al., 2017). Ce travail permet d'ajouter les cellules souches mésenchymateuses dans la liste des phagocytes non-professionnels. Néanmoins avant d'affirmer cela pour l'ensemble des cellules souches

mésenchymateuses, un travail d'investigation expérimentale doit être effectué sur toutes les Mesenchymal stem cells dérivées des différents tissus.

Sur la base de ces hypothèses et la conclusion du chapitre 1, suggérant une capacité phagocytique des hDPSCs, nous avons investigué si les hDPSCs avaient la capacité de reconnaitre et d'internaliser un agent infectieux. Nous avons choisi la bactérie *Bartonella quintana* comme agent infectieux modèle. En effet, *Bartonella quintana* est une bactérie intracellulaire Gram négative, responsable de la fièvre des tranchées (Foucault et al., 2006). Après une infection primaire, une bactériémie asymptomatique chronique se développe chez certains patients, suggérant l'existence d'un réservoir naturel de *Bartonella quintana* (Capo et al., 2003). Seule la lignée érythroblastique a été mise en évidence comme réservoir potentiel de *Bartonella quintana* (Rolain et al., 2002) alors que *Bartonella quintana* peut également infecter les cellules endothéliales (Brouqui and Raoult, 1996; Liberto et al., 2004). Ces deux types de cellules ont une durée de vie de respectivement 120 jours et 100 jour, plus courte que la persistance de *Bartonella quintana* dans l'organisme humain infecté (le record de persistance est de 7 ans). Des travaux au sein de l'équipe avaient mis en évidence la présence de *Bartonella quintana* dans la pulpe dentaire collectée à partir de squelettes d'individus inhumés depuis plusieurs siècles (Drancourt et al., 2005) mais aussi chez un individu vivant (Aboudharam et al., 2004) suggérant que la pulpe dentaire pourrait être un réservoir pour les infections chroniques à *Bartonella quintana*.

Notre travail expérimental a permis de montrer pour la première fois que les hDPSCs pouvaient internaliser un agent infectieux tel que *Bartonella quintana*. Cette internalisation est associée à un profil inflammatoire particulier: nous avons en effet observé une production significativement plus élevée de cytokines pro-inflammatoires MCP-1, TNF-α, IL-

6, IL-8 et de peptides antimicrobiens BD2 par les cellules infectées que par les cellules contrôles négatifs. Ce phénotype inflammatoire est connu pour activer le recrutement de cellules immunitaires sur le site infectieux afin d' initier une réponse immunitaire protectrice (Arango Duque and Descoteaux, 2014; Turner et al., 2014). Nous avons aussi observé la production d'IL-10 qui pourrait participer à la persistance *de Bartonella quintana*, IL-10 est une cytokine connue pour réduire l'inflammation. Cette production d'IL10 avait été mise en évidence pour jouer un rôle sur l'atténuation du syndrome inflammatoire chez des patients présentant une bactériémie chronique à *Bartonella quintana* (Capo et al., 2003).

Observation intéressante, les hDPSCs infectées par *Bartonella quintana* conservent leurs propriétés de cellules souches mésenchymateuses car nous n'avons pas observé de changement dans l'expression de marqueurs positifs et négatifs de MSC. Nous avons aussi observé une augmentation de l'expression du gène NANOG, ce qui renforce l'hypothèse que ces cellules conservent toujours leurs caractéristiques de cellules souches.

L'imagerie confocale nous a permis de constater que l'internalisation était accompagnée d'une co-localisation de *Bartonella quintana* au noyau cellulaire, suivie d'un réarrangement du réseau d'actine de la cellule infectée. Ces changements semblent induire la formation d'une vacuole dans laquelle est située *Bartonella quintana*.

L'internalisation de *Bartonella quintana* dans une vacuole suivie d'une production de l'IL-10 suggère que les cellules souches de la pulpe dentaire sont des sanctuaires possibles pour *Bartonella quintana* au cours des infections chroniques. Par ailleurs, ce modèle suppose que *Bartonella quintana* dispose de facteurs de virulence pour envahir les cellules hDPSCs et y persister pour une durée indéterminée. L'inhibition de l'apoptose des cellules infectées, est un mécanisme connu pour certaines espèces du genre *Bartonella* afin de persister chez leur

hôte (Kempf et al., 2005; Liberto et al., 2004). Cela nous a conduit à émettre l'hypothèse que *Bartonella quintana* induisait une inhibition de l'apoptose afin de persister dans les hDPSCs.

Internalization of the pathogen *Bartonella quintana* into human dental pulp stem cells: new insights into *Bartonella quintana* relapsing infection

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Abstract

Bartonella quintana is a facultative intracellular bacterium responsible for relapsing fever, an example of non-sterilizing immunity. The cellular sanctuary of *B*. *quintana* in-between febrile relapses remains unknown but repeated detection of *B*. *quintana* in dental pulp specimens suggests long-term half-life dental pulp stem cells (DPSCs) as candidates. As the capacity of DPSCs to internalize microscopic particles is unknown, we first observed that DPSCs internalized *B*. *quintana* bacteria. Confocal fluorescent microscopy localized *B*. *quintana* bacteria inside DPSC into a vacuolar compartment after 30 minutes of infection. The internalization of *B*. *quintana* correlated with modulated expression of genes involved in pro- and anti-inflammatory immune responses including interleukin (IL)-6, IL-8, monocytes chemoattractant protein-1, tumor necrosis factor-α and IL-10. Interestingly, we observed that DPSCs did not internalize heat-killed *B*. *quintana* bacteria, suggesting the role of heat-labile bacterial components interacting with DPSCs. Taken together, these data indicate for the first time that DPSCs have the capability to internalize bacteria, and that *B*. *quintana* induces a specific transcriptional modulation of genes involved in the immune response of DPSCs. Based on these observations, the possibility that DPSCs are sheltering *B*. *quintana* bacteria for their long-term persistence in the host warrants further experimental and clinical investigations.

Keywords: Human dental pulp stem cells, immunity, immunomodulatory, uptake, *Bartonella quintana*.

Introduction

Bartonella quintana is a facultative intracellular gram-negative bacterium which has been described in 1915 in World War I soldiers presenting with fever, headache, sore muscles, bones and joints and skin lesions on the chest and back [1,2]. This so-called trench fever is nowadays understood as one of the clinical forms of *B*. *quintana* bacteremia, also responsible for life-threatening endocarditis [3–6] and bacillary angiomatosis in immunocompromised populations [7]. *B*. *quintana* is also responsible of lymphadenopathy in the lymphatic territory of inoculation as *B*. *quintana* is an ectoparasite-borne pathogen transmitted from person to person by body lice [1,8], and probably from cat to persons by cat fleas [9].

Trench fever is a relapsing fever, and *B*. *quintana* has been consistently observed in circulating erythrocytes during febrile episodes[10]. However, the site where *B*. *quintana* resides in-between febrile episodes remains unknown even though the demonstration that bacillary angiomatosis results from the reactivation of quiescent *B*. *quintana* suggested that endothelial cells could be sanctuary cells[11,12]. Indeed, neither erythrocytes nor endothelial cells have been demonstrated to host *B*. *quintana* for a long time, in agreement with the fact that both cell types have a limited life span time of 120 and 100 days for erythrocytes and endothelial cells, respectively[13,14].

Interestingly, *B*. *quintana* has been consistently detected in the dental pulp, a highly vascularized organ with high erythrocyte trafficking[15]. Even in ancient past populations, *B*. *quintana* is detected with a prevalence of 2.5% to 21.4% in the dental pulp collected in buried populations[16,17]. In one particular burial site of Remiremont, the prevalence of *B*. *quintana* in 45 dental pulp specimens collected from these 5-10th century populations was as high as 53.3%[18]. Also, *B*. *quintana* has been detected in the dental pulp collected from one patient

who had been diagnosed with *B. quintana* bacteremia six months before tooth extraction and was free of bacteremia at the time of tooth extraction[19].

The dental pulp is composed of several cell types including dental pulp stem cells (DPSCs) which were investigated in the present study. DPSCs are mesenchymal stem cells isolated by Gronthos in 2000 and characterized by the expression of markers such as CD73, CD90 and CD105 but which are negative for CD34 (hematopoietic progenitor cell antigen) and CD45 (leukocyte common antigen)[20,21]. DPSCs are pluripotent cells which can differentiate into chondrocytes, adipocytes, neuronal cells and odontoblasts[21]. It has been previously shown that the lipopolysaccharide (LPS) induces the differentiation of DPSCs by modulating osteo/dentinogenic genes including alkaline phosphatase (ALP), osteocalcin (OCN) and dentin matrix protein 1 (DMP1)[22,23]. Self-renewal of DPSCs is characterized by the expression of NANOG, a gene involved in the maintenance and self-renewal in undifferentiated embryonic stem cells[24,25].

The DPSCs stemness capacity correlates with a long lifespan[25,26]. This is making DPSCs attractive cells to investigate the presence of *B. quintana* for extended periods of time In addition, DPSCs are located in the inner area of the dental pulp chamber in close contacts with nerve ending and could be sentinel cells for injury and blood-borne pathogen invasions. It has been found that DPSCs present immuno-privileged against immune responses[27]. Indeed, DPSCs possess an immunomodulatory activity following LPS stimulation. They produce pro-inflammatory cytokines such as interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α and monocytes chemoattractant protein (MCP)-1 to recruit immune cells in the site of inflammation, and anti-inflammatory cytokines including IL-10 to reduce the inflammation and maintain homeostasis[28–30].

Based on these findings, the aim of this present study was to investigate the role of DPSCs in host-pathogen interactions, using *B. quintana* as a paradigmal organism.

Materials and methods

Bacterial strain

B. quintana ATCC49793 was cultured on Columbia 5% sheep blood agar plates (bioMérieux, Craponne, France) at 37°C under 5% CO_2 atmosphere.The bacteria were alternatively heat-exposed at 96°C for two hours. To confirm that heat-exposed bacteria were killed, 10 µL of the suspension was incubated on Columbia 5% sheep blood agar plates at 37°C in atmosphere enriched with 5% CO_2 to assess the viability of *B. quintana*.

DPSC culture

DPSCs obtained from cell lines service (CLS, Eppelheim, Germany) were cultured in Dulbecco's Modified Eagle Medium F-12 (DMEM/F12, Invitrogen, Villebon-sur-Yvette, France) supplemented with 5% heat-inactivated fetal calf serum (FBS, qualified, EU-approved, South America origin, Gibco) at 37°C under a 5% CO_2 atmosphere.

FITC-labeled *B. quintana*

The above-mentioned *B. quintana* strain (both living and heat-killed bacteria) was labeled with fluorescein-5-isothiocyanate (FITC) according to the manufacturer's instructions (Sigma-Aldrich). Briefly, a bacterial suspension ($1x10^9$) was washed twice in phosphate buffered saline (PBS, Invitrogen). Bacteria were resuspended in 1M bicarbonate buffer (pH 9.5) containing 0.04 % FITC, agitated for two hours at room temperature and protected from light. The suspension was then centrifuged at 500 × *g* for 10 minutes at 4°C and the pellet was washed three times in PBS. Finally, the pellet was suspended in PBS and stored at −20°C in the dark.

Uptake assay and quantification

DPSCs were seeded onto glass coverslips at $5x10^4$ cells/mL in 24-well plates (Greiner Bio-One, United Kingdom) until 80% confluency. Cells were then co-incubated with FITC-labelled bacteria (either living or heat-killed bacteria) at different times at 37°C at a bacterium-to-cell ratio of 200:1. For each kinetic point, cells were washed with PBS to remove free bacteria and fixed with 4% paraformaldehyde for 15 minutes. Actin filaments were stained in red with phalloidin-fluor 555 (Invitrogen) and the nucleus was stained in blue using 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Finally, glass coverslips were mounted on object slide with Mowiol (Calbiochem, San Diego, United States of America). Imaging and evaluation of the number of internalized bacteria per cell was performed on a 63X oil objective using a Zeiss LSM 800 confocal inverted microscopy system. The uptake index was calculated as previously described[31]. Internalized bacteria were counted on a total of 100 DPSCs. The internalization index was calculated as follows: (% phagocytic cells containing ≥ 1 bacterium) × (mean number of bacteria/ DPSCs containing bacteria).

Transmission electron microscopy

DPSCs were infected with *Bartonella quintana* at a 200 :1 MOI during 24 hours, as described above. Cells were briefly incubated with trypsin/EDTA to unstick adherent cells. Cells were harvested, centrifuged at a speed of 200 g for 5 min and washed. The pellets were fixed with 4% glutaraldehyde in a 0.05 M phosphate-buffered solution containing 0.15 M NaCl at pH 7.3 at room temperature. We used the same protocols as described in this study [32].

Gene expression analysis

Total RNA was extracted from infected cells and non-infected cells (as negative control cells) using Trizol reagent according to the instructions of the manufacturer (Invitrogen) and quantified using a spectrophotometer at 260 nm (Nanodrop 1000 spectrophotometer, Thermo Scientific, Cambridge Scientific, Watertown, MA). All the samples were then normalized at

100 ng RNA before reverse-transcription to the first strand of cDNA by using oligo (dT) and Moloney murine leukemia virus–reverse transcriptase M-MLV Reverse Transcriptase, according to the manufacturer's instructions (Invitrogen). Gene expressions were evaluating using an ABI7900 Fast Real-Time PCR System, the SYBR Green Fast Master Mix (Roche Applied Science, Meylan, France), and primers listed in **Supplementary Table 1**. The data were obtained by calculating the fold change = $2^{-\Delta\Delta Ct}$, corresponding to $\Delta\Delta Ct = (Ct_{Target} - Ct_{AGAPDH})_{assay} - (Ct_{Target} - Ct_{GAPDH})_{control.}$ Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the relative expression of each gene under investigation.

Flow cytometry analysis

Mesenchymal stem cell markers of *B. quintana*-infected DPSCs were investigated using flow cytometry. Briefly, DPSCs were cultured to 80% confluence and infected with *B. quintana* for 24 hours (bacterium-to-cell ratio of 200:1). Thereafter, cells were washed two times in ice-cold PBS supplemented with 2% FBS and 2 mM EDTA. A total of 10^6 cells were incubated with antibodies against positive and negative mesenchymal stem cell markers listed in **Supplementary Table 2** in accordance with the manufacturer's protocol. Flow cytometry analyses were performed on a BD LSR Fortessa and data were analyzed using Flow Jo software.

Statistical analysis

Data were analyzed using GraphPad Prism 5.0 c software and using the unpaired *t*-test. $P < 0.05$ was considered statistically significant.

Results

Capacity of DPSCs to internalize *B. quintana* bacteria

We first investigated whether DPSCs could absorb and internalize *B. quintana* bacteria. Confocal cross-section analyses showed that *B. quintana* bacteria were absorbed and internalized into DPSCs after 30 minutes of co-incubation, 3-dimensional projection showing that *B. quintana* bacteria were localized in the same focal plane as the DPSC nucleus (**Supplemental Figure 1A**). Internalization was accompanied by an attachment of *B. quintana* to the cell nucleus and was followed by actin rearrangements. Of note, these changes seemed to induce the formation of a vacuole to internalize *B. quintana*. Indeed, as depicted in **Figure 1A**, the bacterium was found in great quantities in a vacuole delimited by arrows in the pictures. Uptake quantification indicated a time-dependent increase in the number of bacteria per cell (**Figure 1B**). Indeed, 11 ± 1% of DPSCs had internalized one bacterium, resulting in an internalization index of 15 after 30 minutes of co-incubation. The value of the internalization index exhibited a time-dependent increase of 217 ± 24 after a 24-hour co-incubation with 23% of DPSCs having internalized 10 bacteria per cell (**Figure 1B**). Interestingly, *B. quintana* internalization rate reached a plateau after 6 to 12 hours of incubation followed by a strong increase 12 hours later, suggesting a multiplication of the bacteria. Then, we confirmed that heat-exposed *B. quintana* bacteria were killed and observed that exposing DPSCs to heat-exposed *B. quintana* bacteria resulted in a significant decrease of DPSC infection compared to non-heat-treated *B. quintana* bacteria (*p=0.0096) (**Figure 1C and 1D**). Indeed, we found an uptake index of 217 ± 24 in live *B. quintana* phagocytized by DPSC, whereas only 18 ± 3 were observed for heat-exposed *B. quintana* (**Figure 1D**). Taken together, these results show for the first time that DPSCs do have the capability to absorb and internalize particles including bacteria. They also suggest that this activity depends on live bacteria, as evidenced for *B. quintana*.

Transmission electron microscopy observations of *Bartonella quintana*- infected DPSCs.

Transmission electron microscopy observations confirmed the internalization of *Bartonella quintana* in the presence of non-infected, negative control cells (**Figure 2).** Careful observations indicated different stages of internalization: first, attachement of bacteria as indicated by arrows in Figure 2 **(i),** then membrane ruffling forming cellular pseudopod-like structures hosting bacteria **(ii),** finally bacteria into a cytosolic compartment **(iii)** or into a vacuolic compartment **(iv).** Taken together, observations using transmission electron microscopy confirmed that DPSCs are able to internalize *Bartonella quintana* into a vacuolic compartment, 24 hours post-infection.

Immune response of DPSC to *B. quintana* infection.

We further investigated whether *B. quintana* internalization induced an immune pattern by DPSCs. *B. quintana* infection correlated with increased transcription of the pro-inflammatory gene TNF-α 0.5 to 18 hours post-infection, and peaked with a ~12.5-fold increase 2 hours post-infection (**Figure 3A**). Similar profiles were identified for MCP-1 and IL-6 during the first 6 hours, however a decrease of expression was observed at 8 and 18 hours, corresponding to the multiplication phase of *B. quintana* (**Figure 3A**). Concerning IL-8, we observed two peaks of up-modulation at 2 and 6 hours with respectively a 5.5 and a 2.4-fold increase (**Figure 3A**). Of note, a down-modulation was also observed during the multiplication phase of *B. quintana* at 8 and 18 hours post-infection. As depicted in **Figure 3B**, the anti-inflammatory gene IL-10 was globally not modulated, with only a peak at 2 hours post-infection and a fold change at 1.9 (**Figure 3B**). As we observed a pro-inflammatory response, we investigated the antimicrobial activity of *B. quintana*-infected DPSCs. The anti-bacterial gene BD2 was found up-modulated with a 2.2-fold change 30 minutes post-infection and a peak of 2.5-fold change at 2 hours post-infection (**Figure 3C**).

Stem and mesenchymal cell properties of *B. quintana*-infected-DPSCs.

Finally, we investigated whether *B. quintana* internalization could affect the mesenchymal stem phenotype of DPSCs. In a first step, we observed that *B. quintana*-internalization did not significantly impair the expression of CD90, CD105 and CD73 positive markers by DPSCs as measured using flow cytometry (**Figure 4A**). Furthermore, *B. quintana* internalization into DPSCs did not significantly increase the expression of the negative mesenchymal stem cell markers including CD34 and CD45 (**Figure 4A**). In a second step, we observed that the level of mRNA expression of ALP, OCN and DMP-1 genes was ≤ 1.5-fold change in DPSCs which had internalized *B. quintana* bacteria compared to control cells, using q-RT-PCR (**Figure 4B**). Finally, we observed an up-regulation of NANOG gene in DPSCs which had internalized *B. quintana* bacteria compared to control cells (**Figure 4C**). Altogether, these data showed that the internalization of *B. quintana* bacteria did not significantly change the stem cell phenotype of DPSCs.

Discussion

We observed that *B. quintana* was internalized by DPSCs. These findings support the emerging concept that mesenchymal stem cells are phagocytic cells able to absorb bacteria and cellular debris[31,33–35]. Although internalization was not firmly demonstrated to be phagocytosis in our study, the fact that the engulfment of *B. quintana* bacteria was accompanied by an increasing bacterial uptake over time and by actin rearrangements leading to intravacuolar *B. quintana* are suggestive of phagocytosis. Recently, human mesenchymal stem cells have been shown to phagocyte *Mycobacterium tuberculosis*[34]. Therefore, DPSCs could be added to the list of non-immune cells named « non-professional phagocytic cells » along with fibroblasts, astrocytes, Muller's glia and hepatocytes which were described to phagocyte particles, bacteria and cellular debris to maintain homeostasis[35–38].

Our observations made using both confocal and electron microscopy support the hypothesis that DPSCs could act as reservoir cells for *B*. *quintana*. This hypothesis is in accordance with a study reporting that intracellular *B*. *quintana* bacteria could be internalized into a vacuolic compartment (*B*. *quintana*-containing vacuoles) and multiply[39–41]. These observations correlate with the persistence of *B*. *quintana* in humans[42]. This is opening an exciting new role for DPSCs and further exploring their function could be done by additional observations including the co-localization of *B*. *quintana* and DPSCs in dental pulp specimens. If confirmed, the role of DPSCs as sanctuary cells for other pathogens will have to be investigated.

DPSCs have the ability to differentiate in osteo/dentinogen cell lineages to contribute to the regeneration of dentin when dental pulp undergoes an agression[43]. Moreover, it has been shown that *Escherichia coli* LPS induced odontogenic differentiation of DPSCs by increasing the expression of ALP, OCN and DMP1[44]. In the present study, uptake and internalization of *B*. *quintana* did not induce such an odontogenic differentiation of DPSCs with the absence of modulation of the ALP, OCN and DMP1 genes. In addition, we observed that *B*. *quintana* infection did not alter the pattern of mesenchymal stem cell markers of DPSCs.

Internalization of *B*. *quintana* bacteria was associated with an expression of pro-inflammatory genes IL-6, IL-8, MCP-1 and TNF-α, as previously reported for DPSCs stimulated with LPS[30]. Acting together, these genes may induce a recruitment of inflammatory cells in the site of infection to fight *B*. *quintana* as well as a fever which could explain the typical trench fever[45]. Moreover, pro-inflammatory cytokines may recruit permissive cells that can support the growth and multiplication of *B*. *quintana* and protect infected cell by inhibition of apoptosis[46]. Here, we observed a moderate expression of the anti-inflammatory gene IL-10. IL-10 is known to resolve inflammation in order to avoid a

sepsis[47]. Moreover, it was reported in patient with bacteremia of *B*. *quintana* that Il-10 plays important role in the attenuation of inflammation and may participate to the persistency of *B*. *quintana* in blood[47].

In conclusion, this present study demonstrates for the first time that DPSCs, like phagocyte cells, can absorb and internalize *B*. *quintana* bacteria and that this engulfment is associated with an expression of pro-inflammatory and anti-inflammatory genes. This activity does not affect the stem and mesenchymal cell properties of DPSCs. Taken together, these findings support the hypothesis that the dental pulp and particularly the DPSCs could represent a sanctuary for chronic *B*. *quintana* infection, a hypothesis that will have to be confirmed in further experimental and clinical studies.

Figure legends

Figure 1. Phagocytic activity of DPSC. DPSC to 80% of confluence were stimulated with *B*. *quintana* at different times (ratio 200:1). (**A**) Confocal pictures and (**B**) phagocytosis index of DPSC infected by *B*. *quintana* were realized with bacteria in green, actin in red and nucleus in blue. White arrows show the delimitation of a vacuole in DPSC. (**C**) Confocal pictures and (**D**) uptake index were realized on DPSC infected by (left panel) activated *versus* (right panel) heat-exposed bacteria for 24 hours with bacteria in green, actin in red and nucleus in blue. The data were obtained in three independent experiments with mean ± SD.

Figure 2.Internalization of *Bartonella quintana* in DPSC by TEM

Bartonella quintana internalization were evaluated with TEM .(A) morphological characterization of *Bartonella quintana* (i) and DPSC (ii).(B) adhesion (i), Invasion with formation of cell pseudopods-like structure to keep bacteria **(ii),** *Bartonella quintana* in cytosolic compartment **(iii)** or in vacuolic compartment **(iv).**

Figure 3. Immune and anti-microbial response of DPSC infected by *B. quintana*. The level of mRNA expression in relative fold change was evaluated for (**A**) pro- (**B**), anti-inflammatory and (**C**) anti-microbial genes by q-RTPCR. All of these genes were normalized by using the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) between 0.5 and 18 hours. Data were obtained from three independently performed experiments with mean ± SEM.

Figure 4. Evaluation of stem and mesenchymal cell markers in DPSC infected by *B. quintana*. (**A**) The evaluation of mesenchymal cell markers was realized by flow cytometer

on (up-panel) unstimulated cells (as control) and (down-panel) *B. quintana* infected cells with positive (CD90, CD105 and CD73) and negative (CD34 and CD45) markers in red and appropriate isotype controls in blue. The level of mRNA expression in relative fold change was evaluated for (**B**) ALP, DMP1, OCN and (**C**) NANOG markers by q-RTPCR. All of these genes were normalized by using the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) between 0.5 and 18 hours. Data were obtained from three independently performed experiments with mean ± SEM.

Supplemental Figure 1A. Confocal sections of DPSC stimulated *B. quintana*. Confocal pictures were realized on DPSC stimulated by bacteria (**A**) during 24 hours with bacteria in green, actin in red and nucleus in blue. Bacteria found phagocytized are identified with white arrows.

Supplemental movie 1. Three dimensions movie of *B. quintana* phagocytosis by DPSC. Sequential spatial planes X, Y and Z-sections were acquired in order to show the internalization of bacteria in a 3D movie with *B. quintana* in green, actin in red and nucleus in blue.

Supplementary Table 1. Primers used for q-RTPCR analysis. The gene symbol and sequences are listed for the selected genes.

Supplementary Table 2. List of fluorescent reagents used for flow cytometry analysis. (AF, Alexa fluor; ECD, Phycoerythrin-Texas red; FITC, Fluorescein isothiocyanate and PE, Phycoerythrin).

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Declaration of interests

The authors have no conflict of interest to declare.

Author contributions

A.H, M.D. and E.G conceived and designed the experiments. A.H. performed experiments and analyzed the data. A.H, G.A, E.G and M.D wrote the paper.

Supplementary Table 1: Sequence of primers used for q-RTPCR analyses.

The gene symbol and sequences are listed for the selected genes.

Gene symbol	Forward	Reverse	References
GAPDH	CATCATCCCTGCCTCTACTG	GCCTGCTTCACCACCTTC	[48]
IL-6	CTAGAGTACCTCCAGAACAG	TGACCAGAAGAAGGAATGC	[49]
IL-8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT	[48]
IL-10	CTGTGAAAACAAGAGCAAGGC	GAAGCTTCTGTTGGCTCCC	[50]
MCP-1	AAGCAGAAGTGGGTTCAGGA	GCAATTTCCCCAAGTCTCTG	[51]
TNF-α	ATGAGCACTGAAAGCATGATC	AGAGAGGAGGTTGACCTTGGTCTGGTA	[52]
BD2	GAGGAGGCCAAGAAGCTGC	CGCACGTCTCTGATGAGGG	[53]
ALP	CCACGTCCTTCATTTGGTG	ATGGCAGTGAAGGGCTTCTT	[24]
OCN	GGCAGCGAGGTAGTGAAGAG	GCCGATAGGCCTCCTGAAAG	[24]
DMP 1	TGGGGATTATCCTGTGCTCT	TACTTCTGGGGTCACTGTCG	[54]
NANOG	ATTCAGGACAGCCCTGATTCTTC	TTTTGCGACACTCTTCTCTGC	[24]

Supplementary Table 2: Antibodies used for flow cytometry analysis.

Antibodies	**Conjugate**	**Clone**	**Manufacturer**
Positive mesenchymal stem cell markers			
CD73	FITC	AD2	Biolegend
CD90	AF-647	5E10	Biolegend
CD105	PE	43A3	Biolegend
Negative mesenchymal stem cell markers			
CD34	FITC	581	Beckman Coulter
CD45	ECD	J33	Beckman Coulter

References

1. Maurin M, Raoult D. Bartonella (Rochalimaea) quintana infections. Clin Microbiol Rev. 1996;9: 273–292.

2. Byam W, Carroll JH, Churchill JH, Dimond L, Sorapure VE, Wilson RM, et al. Trench Fever. A Louse-Borne Disease. Trench Fever A Louse-Borne Disease. 1919; Available: https://www.cabdirect.org/cabdirect/abstract/19192900231

3. Fournier PE, Lelievre H, Eykyn SJ, Mainardi JL, Marrie TJ, Bruneel F, et al. Epidemiologic and clinical characteristics of Bartonella quintana and Bartonella henselae endocarditis: a study of 48 patients. Medicine (Baltimore). 2001;80: 245–251.

4. Raoult D, Fournier PE, Drancourt M, Marrie TJ, Etienne J, Cosserat J, et al. Diagnosis of 22 new cases of Bartonella endocarditis. Ann Intern Med. 1996;125: 646–652.

5. Drancourt M, Mainardi JL, Brouqui P, Vandenesch F, Carta A, Lehnert F, et al. Bartonella (Rochalimaea) quintana endocarditis in three homeless men. N Engl J Med. 1995;332: 419–423. doi:10.1056/NEJM199502163320702

6. Drancourt M, Birtles R, Chaumentin G, Vandenesch F, Etienne J, Raoult D. New serotype of Bartonella henselae in endocarditis and cat-scratch disease. Lancet. 1996;347: 441–443.

7. Koehler JE, Sanchez MA, Garrido CS, Whitfeld MJ, Chen FM, Berger TG, et al. Molecular epidemiology of bartonella infections in patients with bacillary angiomatosis-peliosis. N Engl J Med. 1997;337: 1876–1883. doi:10.1056/NEJM199712253372603

8. Badiaga S, Brouqui P. Human louse-transmitted infectious diseases. Clinical Microbiology and Infection. 2012;18: 332–337. doi:10.1111/j.1469-0691.2012.03778.x

9. Chomel BB, Kasten RW, Floyd-Hawkins K, Chi B, Yamamoto K, Roberts-Wilson J, et al. Experimental transmission of Bartonella henselae by the cat flea. J Clin Microbiol. 1996;34: 1952–1956.

10. Rolain J-M, Foucault C, Guieu R, La Scola B, Brouqui P, Raoult D. Bartonella quintana in human erythrocytes. Lancet. 2002;360: 226–228.

11. Greub G, Raoult D. Bartonella: new explanations for old diseases. Journal of Medical Microbiology. 2002;51: 915–923. doi:10.1099/0022-1317-51-11-915

12. Mosepele M, Mazo D, Cohn J. Bartonella Infection in Immunocompromised Hosts: Immunology of Vascular Infection and Vasoproliferation. Clin Dev Immunol. 2012;2012. doi:10.1155/2012/612809

13. Kaestner L, Bogdanova A. Regulation of red cell life-span, erythropoiesis, senescence, and clearance. Front Physiol. 2014;5. doi:10.3389/fphys.2014.00269

14. Kliche K, Jeggle P, Pavenstädt H, Oberleithner H. Role of cellular mechanics in the function and life span of vascular endothelium. Pflügers Archiv - European Journal of Physiology. 2011;462: 209–217. doi:10.1007/s00424-011-0929-2

15. Jamal M. Dental Stem Cells and Their Potential Role in Regenerative Medicine. Journal of Medical Sciences. 2011;4: 53–61. doi:10.2174/1996327001104020053

16. Tran T-N-N, Forestier CL, Drancourt M, Raoult D, Aboudharam G. Brief communication: co-detection of Bartonella quintana and Yersinia pestis in an 11th-15th burial site in Bondy, France. Am J Phys Anthropol. 2011;145: 489–494. doi:10.1002/ajpa.21510

17. Nguyen-Hieu T, Aboudharam G, Signoli M, Rigeade C, Drancourt M, Raoult D. Evidence of a louse-borne outbreak involving typhus in Douai, 1710-1712 during the war of Spanish succession. PLoS ONE. 2010;5: e15405. doi:10.1371/journal.pone.0015405

18. Drancourt M, Tran-Hung L, Courtin J, Lumley H de, Raoult D. Bartonella quintana in a 4000-Year-Old Human Tooth. J Infect Dis. 2005;191: 607–611. doi:10.1086/427041

19. Aboudharam G, Fournier P-E, Drancourt M, Raoult D, Foucault C, Brouqui P. Molecular detection of Bartonella quintana DNA in the dental pulp of a homeless patient. Eur J Clin Microbiol Infect Dis. 2004;23: 920–922. doi:10.1007/s10096-004-1244-z

20. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8: 315–317. doi:10.1080/14653240600855905

21. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97: 13625–13630.

22. Huang Y, Jiang H, Gong Q, Li X, Ling J. Lipopolysaccharide stimulation improves the odontoblastic differentiation of human dental pulp cells. Mol Med Rep. 2015;11: 3547–3552. doi:10.3892/mmr.2014.3120

23. Kawashima N, Noda S, Yamamoto M, Okiji T. Properties of Dental Pulp–derived Mesenchymal Stem Cells and the Effects of Culture Conditions. Journal of Endodontics. 2017;43: S31–S34. doi:10.1016/j.joen.2017.06.004

24. Huang C-E, Hu F-W, Yu C-H, Tsai L-L, Lee T-H, Chou M-Y, et al. Concurrent Expression of Oct4 and Nanog Maintains Mesenchymal Stem-Like Property of Human Dental Pulp Cells. Int J Mol Sci. 2014;15: 18623–18639. doi:10.3390/ijms151018623

25. Allouba MH, ElGuindy AM, Krishnamoorthy N, Yacoub MH, Aguib YE. NaNog: A pluripotency homeobox (master) molecule. Glob Cardiol Sci Pract. 2015;2015. doi:10.5339/gcsp.2015.36

26. Graziano A, d'Aquino R, Laino G, Papaccio G. Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Rev. 2008;4: 21–26. doi:10.1007/s12015-008-9013-5

27. Collart-Dutilleul P-Y, Chaubron F, De Vos J, Cuisinier FJ. Allogenic banking of dental pulp stem cells for innovative therapeutics. World J Stem Cells. 2015;7: 1010–1021. doi:10.4252/wjsc.v7.i7.1010

28. Zhao Y, Wang L, Jin Y, Shi S. Fas Ligand Regulates the Immunomodulatory Properties of Dental Pulp Stem Cells. J Dent Res. 2012;91: 948–954. doi:10.1177/0022034512458690

29. Attoumani H, Drancourt M, Ghigo E. Immune Properties of Human Dental Pulp Stem Cells and Interactions with the Immune System. 2018;2: 5.

30. Bindal P, Ramasamy TS, Kasim NHA, Gnanasegaran N, Lin CW. Immune responses of human dental pulp stem cells in lipopolysaccharide induced microenvironment. Cell Biol Int. 2018; doi:10.1002/cbin.10938

31. Coulaud P-J, Lepolard C, Bechah Y, Berenger J-M, Raoult D, Ghigo E. Hemocytes from Pediculus humanus humanus are hosts for human bacterial pathogens. Front Cell Infect Microbiol. 2015;4. doi:10.3389/fcimb.2014.00183

32. Andreani J, Aherfi S, Bou Khalil JY, Di Pinto F, Bitam I, Raoult D, et al. Cedratvirus, a Double-Cork Structured Giant Virus, is a Distant Relative of Pithoviruses. Viruses. 2016;8. doi:10.3390/v8110300

33. Tso GHW, Law HKW, Tu W, Chan GCF, Lau YL. Phagocytosis of apoptotic cells modulates mesenchymal stem cells osteogenic differentiation to enhance IL-17 and RANKL expression on CD4+ T cells. Stem Cells. 2010;28: 939–954. doi:10.1002/stem.406

34. Khan A, Mann L, Papanna R, Lyu M-A, Singh CR, Olson S, et al. Mesenchymal stem cells internalize Mycobacterium tuberculosis through scavenger receptors and restrict bacterial growth through autophagy. Scientific Reports. 2017;7: 15010. doi:10.1038/s41598-017-15290-z

35. Arandjelovic S, Ravichandran KS. Phagocytosis of apoptotic cells in homeostasis. Nat Immunol. 2015;16: 907–917. doi:10.1038/ni.3253

36. Rasmussen JP, Sack GS, Martin SM, Sagasti A. Vertebrate Epidermal Cells Are Broad-Specificity Phagocytes That Clear Sensory Axon Debris. J Neurosci. 2015;35: 559–570. doi:10.1523/JNEUROSCI.3613-14.2015

37. Chang HY, Chi J-T, Dudoit S, Bondre C, van de Rijn M, Botstein D, et al. Diversity, topographic differentiation, and positional memory in human fibroblasts. Proc Natl Acad Sci U S A. 2002;99: 12877–12882. doi:10.1073/pnas.162488599

38. Brown GC, Neher JJ. Eaten alive! Cell death by primary phagocytosis: “phagoptosis.” Trends Biochem Sci. 2012;37: 325–332. doi:10.1016/j.tibs.2012.05.002

39. Brouqui P, Raoult D. Bartonella quintana invades and multiplies within endothelial cells in vitro and in vivo and forms intracellular blebs. Research in Microbiology. 1996;147: 719–731. doi:10.1016/S0923-2508(97)85119-4

40. Eicher SC, Dehio C. Bartonella entry mechanisms into mammalian host cells. Cellular Microbiology. 14: 1166–1173. doi:10.1111/j.1462-5822.2012.01806.x

41. Zhang P, Chomel BB, Schau MK, Goo JS, Droz S, Kelminson KL, et al. A family of variably expressed outer-membrane proteins (Vomp) mediates adhesion and autoaggregation in Bartonella quintana. Proc Natl Acad Sci U S A. 2004;101: 13630–13635. doi:10.1073/pnas.0405284101

42. Foucault C, Brouqui P, Raoult D. Bartonella quintana Characteristics and Clinical Management. Emerg Infect Dis. 2006;12: 217–223. doi:10.3201/eid1202.050874

43. Huang GT-J. Dental Pulp and Dentin Tissue Engineering and Regeneration – Advancement and Challenge. Front Biosci (Elite Ed). 2011;3: 788–800.

44. Nomiyama K, Kitamura C, Tsujisawa T, Nagayoshi M, Morotomi T, Terashita M, et al. Effects of lipopolysaccharide on newly established rat dental pulp-derived cell line with odontoblastic properties. J Endod. 2007;33: 1187–1191. doi:10.1016/j.joen.2007.05.015

45. Matera G, Liberto MC, Quirino A, Barreca GS, Lamberti AG, Iannone M, et al. Bartonella quintana lipopolysaccharide effects on leukocytes, CXC chemokines and apoptosis: a study on the human whole blood and a rat model. Int Immunopharmacol. 2003;3: 853–864. doi:10.1016/S1567-5769(03)00059-6

46. Liberto MC, Matera G, Lamberti AG, Barreca GS, Focà D, Quirino A, et al. Bartonella quintana-induced apoptosis inhibition of human endothelial cells is associated with p38

and SAPK/JNK modulation and with stimulation of mitosis. Diagn Microbiol Infect Dis. 2004;50: 159–166. doi:10.1016/j.diagmicrobio.2004.06.016

47. Capo C, Amirayan-Chevillard N, Brouqui P, Raoult D, Mege J-L. Bartonella quintana Bacteremia and Overproduction of Interleukin-10: Model of Bacterial Persistence in Homeless People. J Infect Dis. 2003;187: 837–844. doi:10.1086/368384

48. He W, Qu T, Yu Q, Wang Z, Lv H, Zhang J, et al. LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells. Int Endod J. 2013;46: 128–136. doi:10.1111/j.1365-2591.2012.02096.x

49. Liu G-Y, Liu Y, Lu Y, Qin Y-R, Di G-H, Lei Y-H, et al. Short-term memory of danger signals or environmental stimuli in mesenchymal stem cells: implications for therapeutic potential. Cell Mol Immunol. 2016;13: 369–378. doi:10.1038/cmi.2015.11

50. Chen P-M, Liu K-J, Hsu P-J, Wei C-F, Bai C-H, Ho L-J, et al. Induction of immunomodulatory monocytes by human mesenchymal stem cell-derived hepatocyte growth factor through ERK1/2. J Leukoc Biol. 2014;96: 295–303. doi:10.1189/jlb.3A0513-242R

51. Bronckaers A, Hilkens P, Fanton Y, Struys T, Gervois P, Politis C, et al. Angiogenic Properties of Human Dental Pulp Stem Cells. PLoS One. 2013;8. doi:10.1371/journal.pone.0071104

52. Capo C, Zugun F, Stein A, Tardei G, Lepidi H, Raoult D, et al. Upregulation of tumor necrosis factor alpha and interleukin-1 beta in Q fever endocarditis. Infect Immun. 1996;64: 1638–1642.

53. Sung DK, Chang YS, Sung SI, Yoo HS, Ahn SY, Park WS. Antibacterial effect of mesenchymal stem cells against Escherichia coli is mediated by secretion of beta-defensin- 2 via toll- like receptor 4 signalling. Cell Microbiol. 2016;18: 424–436. doi:10.1111/cmi.12522

54. Wang J, Liu X, Jin X, Ma H, Hu J, Ni L, et al. The odontogenic differentiation of human dental pulp stem cells on nanofibrous poly(L-lactic acid) scaffolds in vitro and in vivo. Acta Biomater. 2010;6. doi:10.1016/j.actbio.2010.04.009

Figure 1A.

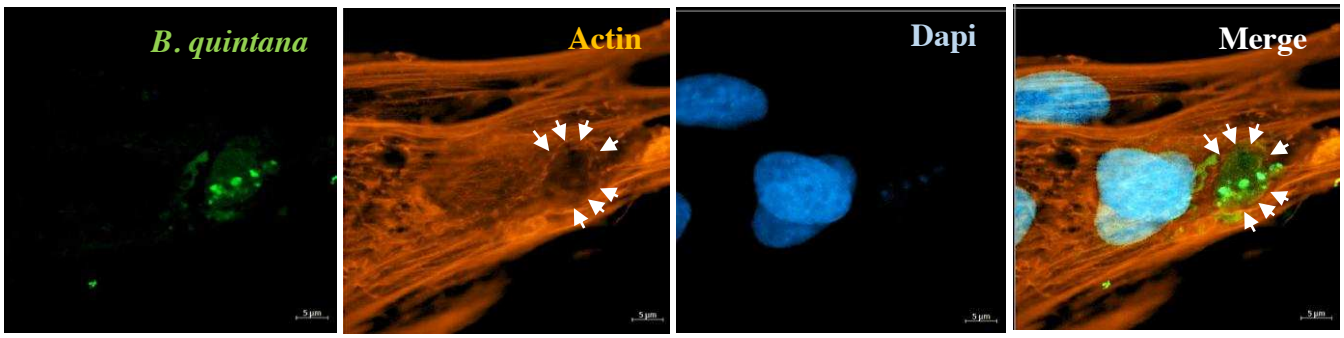


Figure 1B.

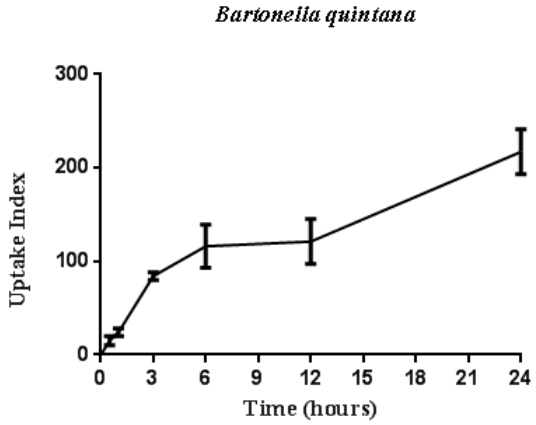


Figure 1C.

Activated *B. quintana* Inactivated *B. quintana*

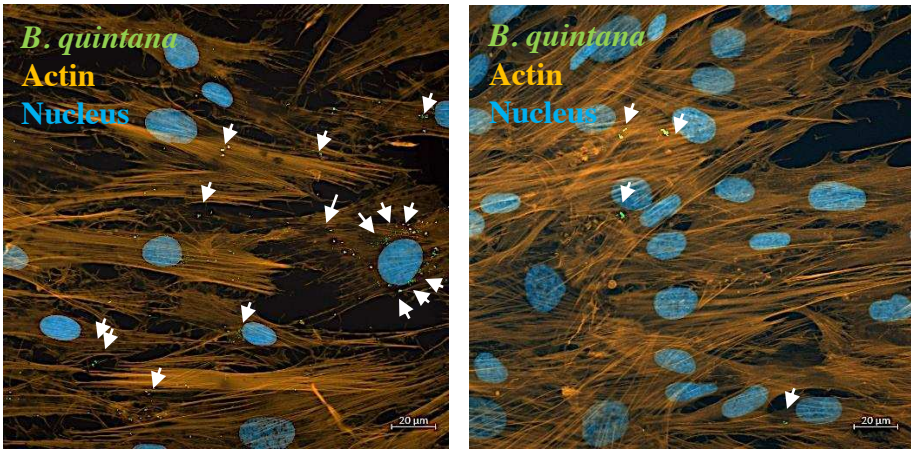


Figure 1D.

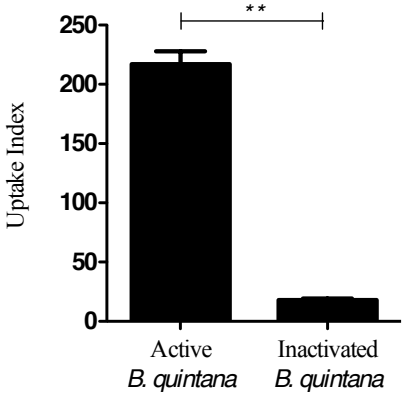
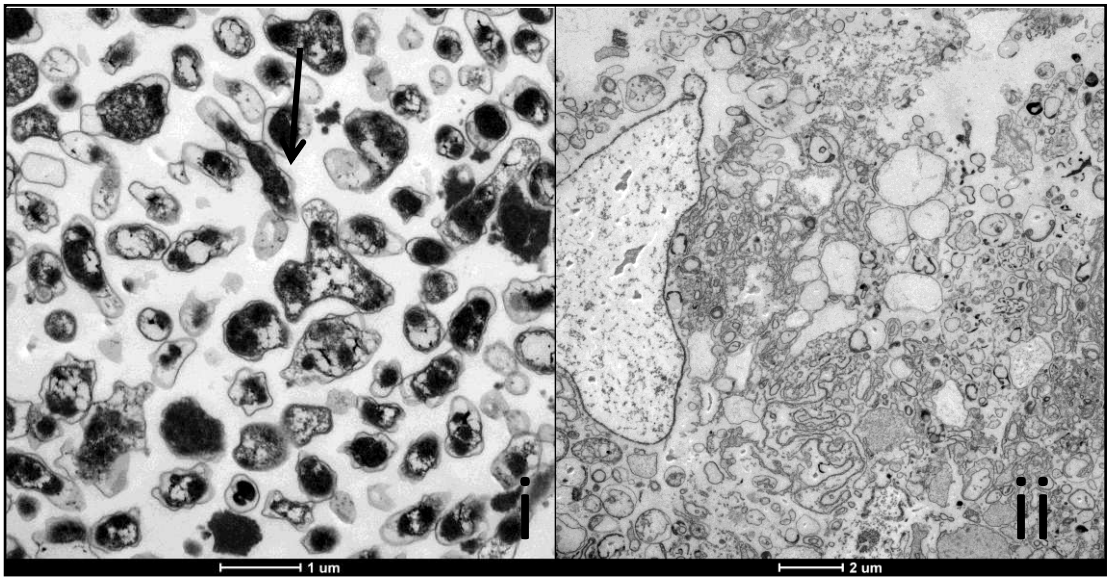


Figure 2A.



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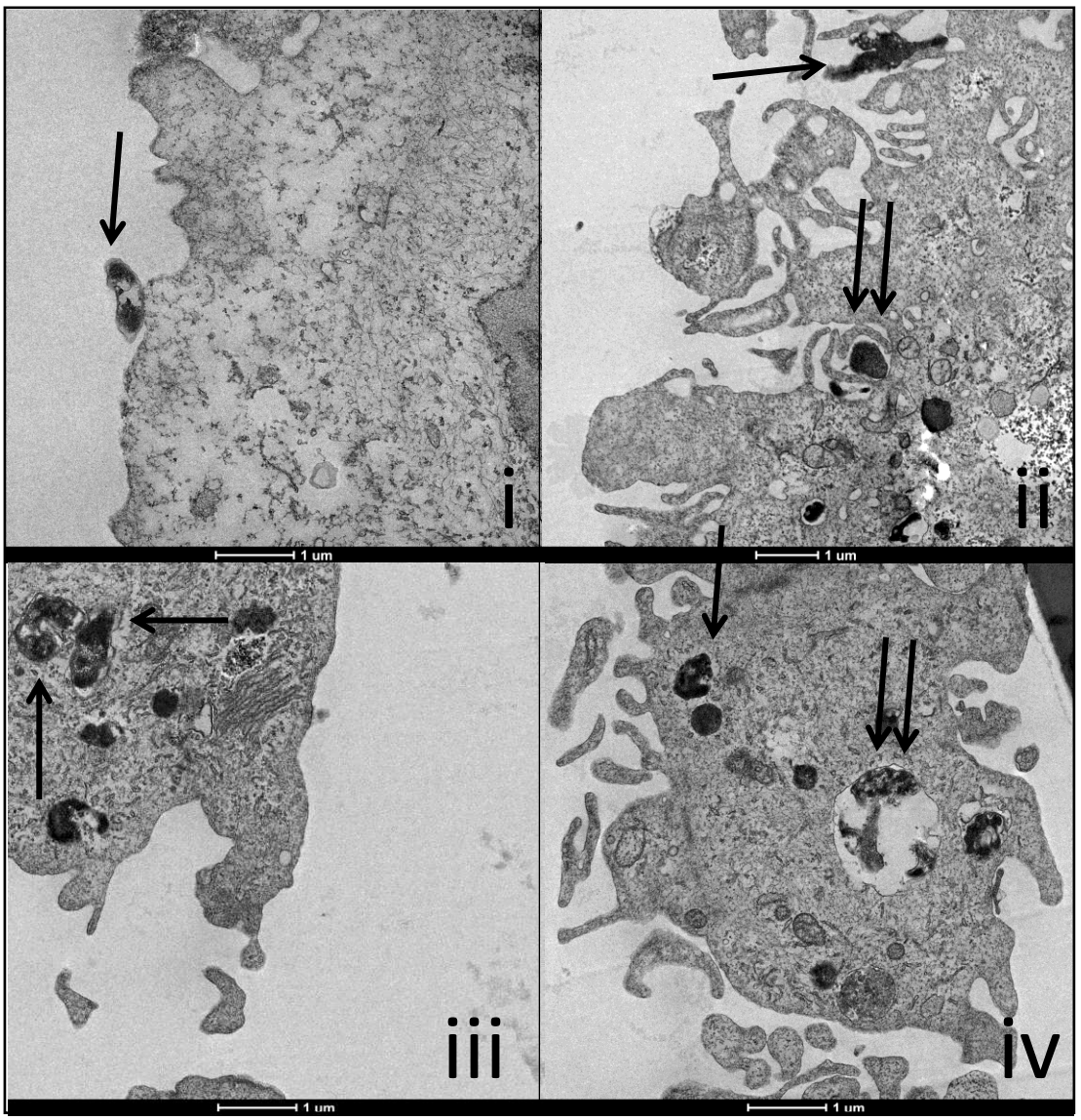
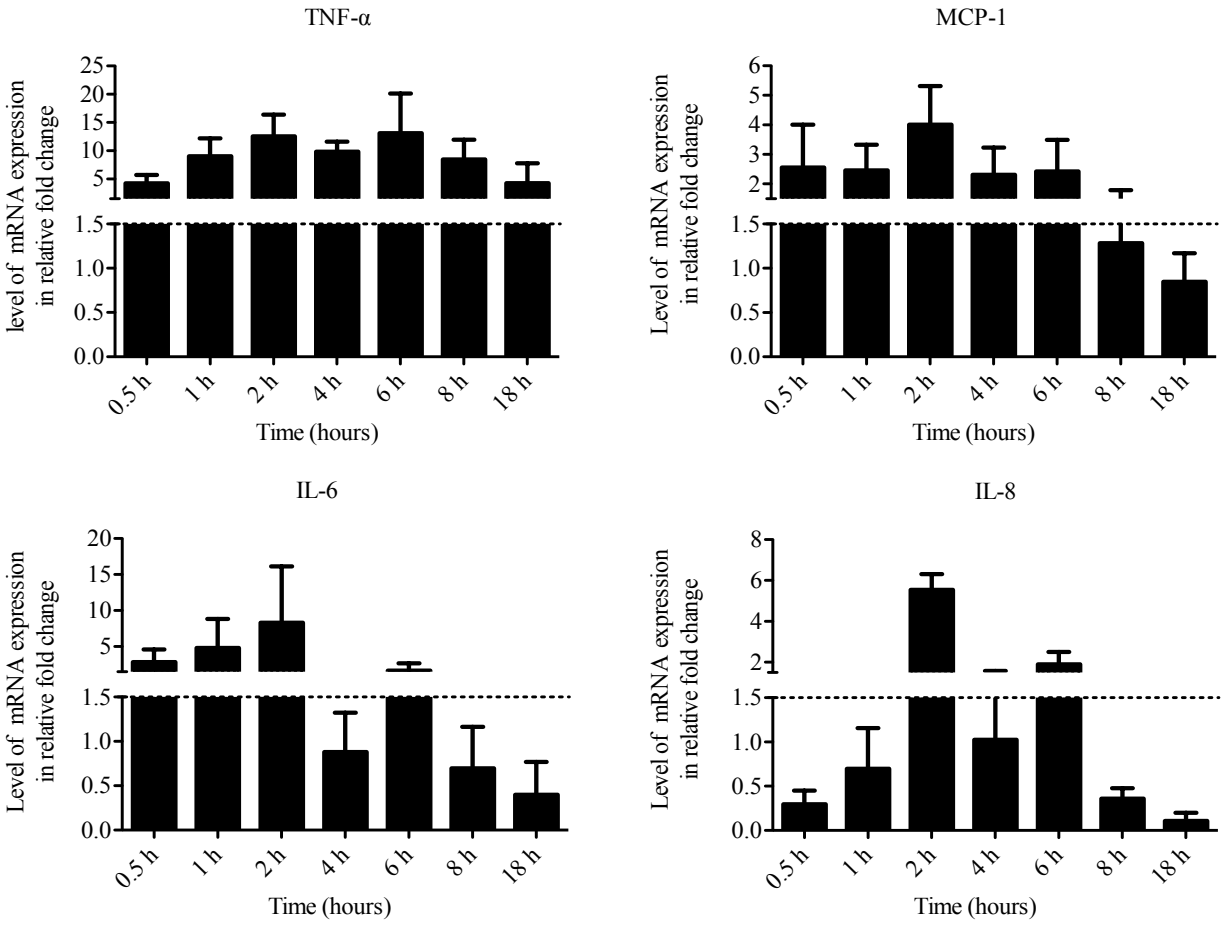


Figure 3A.



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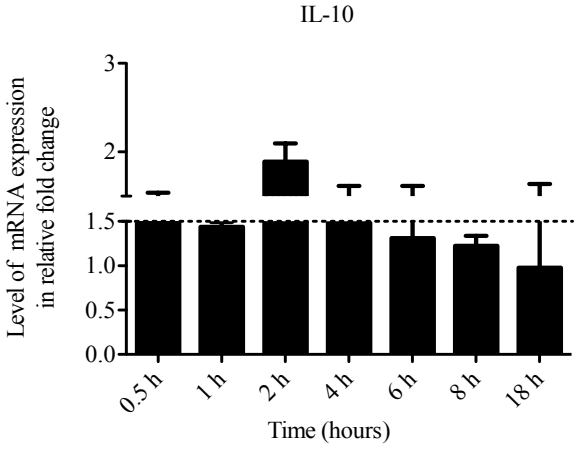


C.

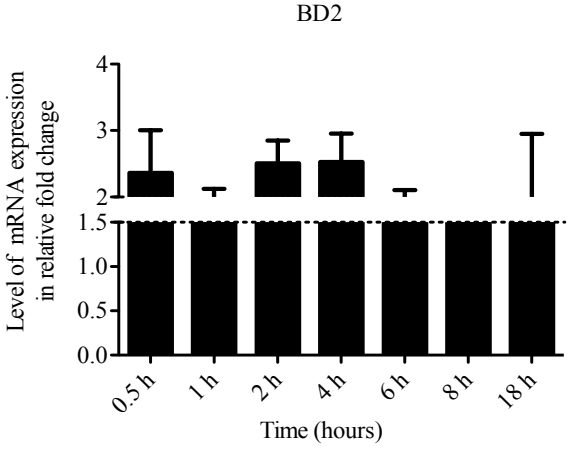
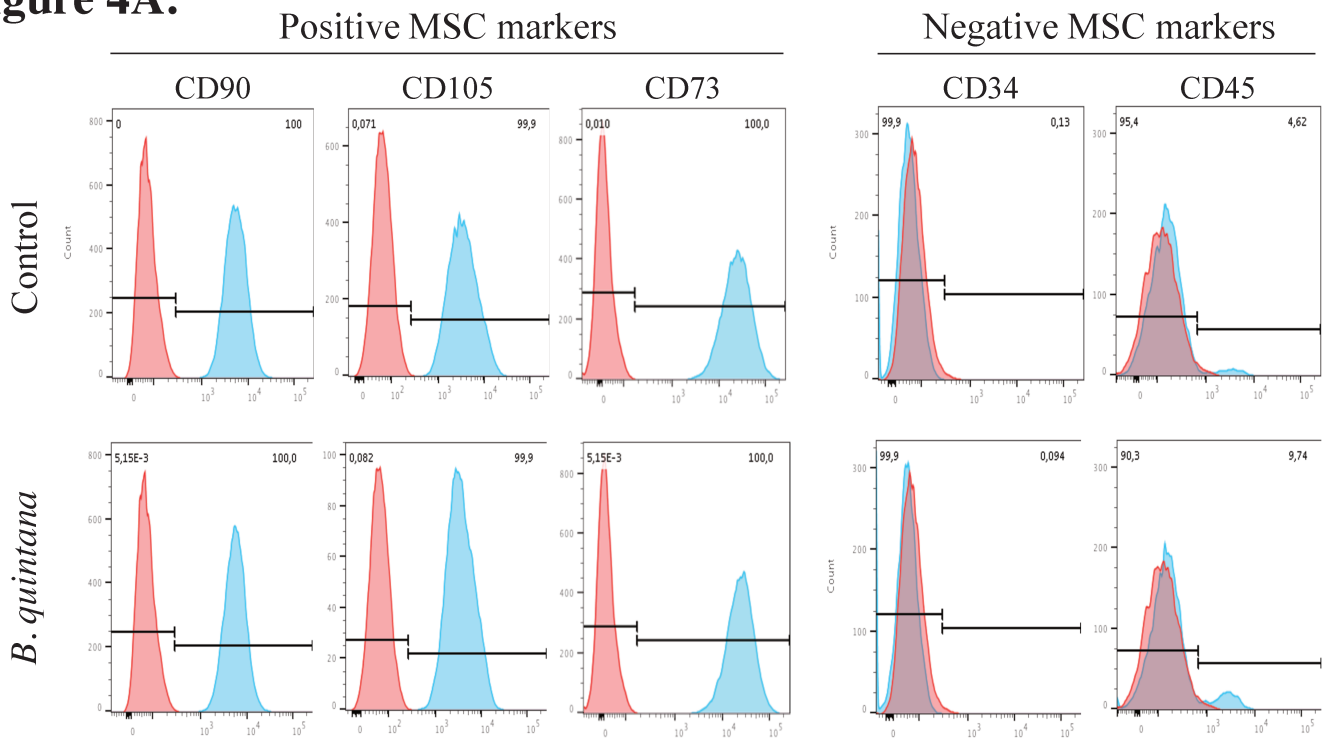


Figure 4A.



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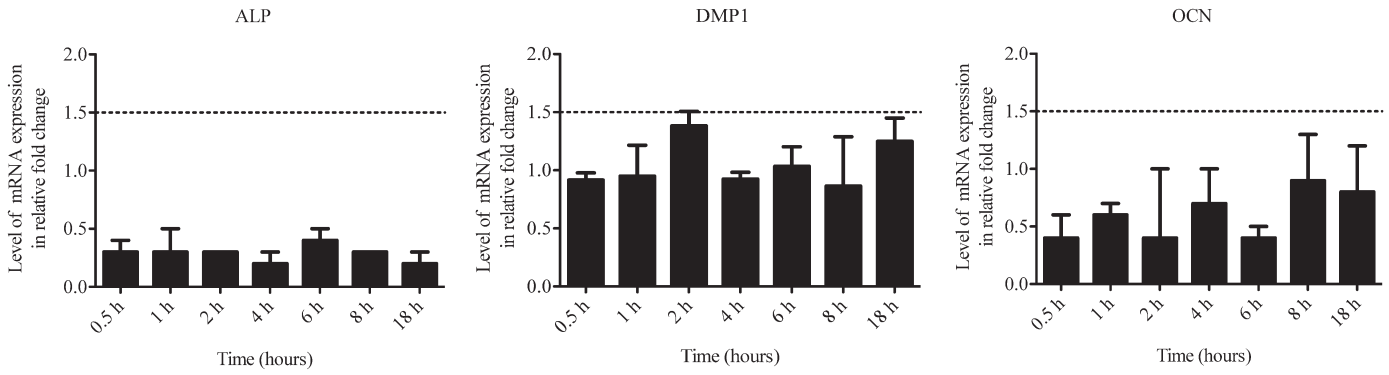

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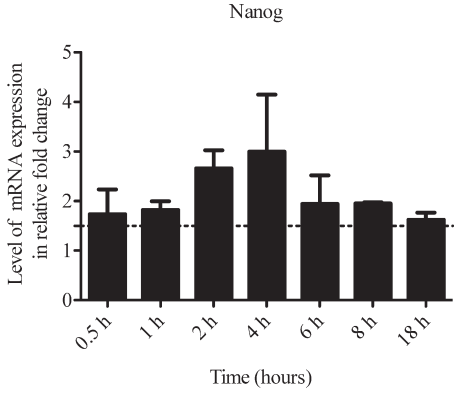
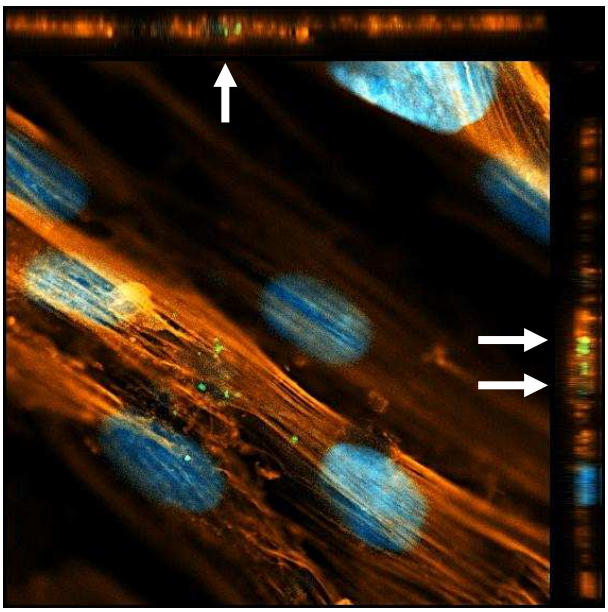


Supplemental figure 1A.



Revue :

Trained immunity carried by non-professional immune cells

Attoumani Hamada, Cedric Torre, Michel Drancourt, Eric Ghigo

(Submitted review to Frontiers in Microbiology)

Préambule de la revue

"Trained immunity" ou "innate immune memory" est un terme proposé par Netea en 2011 pour décrire le processus indépendant de l'immunité adaptative par lequel un organisme est capable de développer une réaction immunitaire exacerbée permettant de le protéger contre l'agent infectieux ou autre lors d' une seconde infection (Netea et al., 2011). Elle repose essentiellement sur la machinerie cellulaire et moléculaire de la réponse innée. En 1980, une équipe de chercheurs italiens de l'université de Perugia dirigée par le Pr Bistoni a observé que des souris infectées par une souche avirulente de *Candida albicans* étaient protégées au cours d'une infection par une souche virulente de *Candida albicans* mais étaient également protégées contre l'infection par des bactéries pathogènes telle que *Staphylococcus aureus* lors d'un second contact (Bistoni et al., 1986). Cette protection était particulièrement induite par les macrophages activés et la production de cytokines pro-inflammatoires et non pas par des lymphocytes T.

Les évolutions des techniques de manipulations génétiques ont permis de mettre en évidence sur des souris athymiques que la mémoire immunitaire dépendait essentiellement des monocytes, macrophages et des cellules natural killers (Quintin et al., 2012). Depuis ces travaux princeps, l'ensemble des études ont porté essentiellement sur les monocytes, macrophages et cellules natural killers, qui sont des cellules différenciées dont la durée de vie est courte compare à la durée de la "trained immunity". Pour résoudre ce paradoxe, il faut se tourner vers d'autres cellules possédant des propriétés immunitaires et une durée de vie très longue. John Pereira a suggéré d'investiguer la mémoire immunitaire des cellules souches de durée de vie illimitée du fait de leur auto-renouvellement (Pereira et al., 2016).

Au sein de notre équipe, Cédric Tore a montré que la planaire *Shmidtea mediterranea* possédait une mémoire immune innée portée par des néoblastes qui sont l'équivalent des cellules souches adultes chez les vertébrés (Torre et al., 2017b). Sur la base de ces observations, j'ai décidé d'écrire une revue dont le titre est **"Trained immunity carried by non-professional immune cells"**, soumise dans le journal Frontiers in Microbiology. Cette revue décrit l'ensemble des cellules de l'immunité innée possédant une mémoire immunologique et celles qui pourraient potentiellement la porter puis dresse une liste de cellules non-immunitaires possédant des propriétés immunitaires qui pourraient également porter une mémoire immunologique.

Trained immunity carried by non-immune cells

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ABSTRACT

"Trained immunity" is a term proposed by Netea to explain the ability of an organism to develop an exacerbated immunological response to protect against a second infection independant of the adaptative immunity. This immunological memory can last from one week to several months and is only described in innate immune cells such as monocytes, macrophages and natural killer cells. Paradoxically, the lifespan of these cells in the blood is shorter than the duration of trained immunity. This observation suggested that trained immunity could be carried by long lifespan cells such as stem cells and non-immune cells like fibroblasts. It is now evident that in addition to performing their putative function in the development and maintenance of tissue homeostasis, non-immune cells also play an important role in the response to pathogens by producing anti-microbial factors, with long-term inflammation suggesting that non-immune cells can be trained to confer long-lasting immunological memory. This review provides a summary of the current relevant knowledge about the cells which possess immunological memory and discusses the possibility that non-immune cells may carry immunological memory and mechanisms that might be involved.

Keywords: Trained immunity, Immunomodulatory, non-immune cells, stem cells, lifespan.

INTRODUCTION

Works regarding T cell and B cell-independent immune memory date back over half a century. A first report by Mackaness in 1969 showed that mice vaccinated against tuberculosis with the vaccine bacillus Calmette-Guérin (BCG) were effectively protected against a second infection by mycobacteria and other infectious agents such as *Salmonella typhimurium*, *Listeria monocytogenes, Staphylococcus aureus*, *Candida albicans* and *Schistosoma mansoni*(Blanden et al., 1969). These results were confirmed by Tribouley *et al.* showing the protective effect of BCG on athymic mice against *Schistosoma mansoni*(Tribouley et al., 1978).

In the *80-90s*, Bistoni and his colleagues showed that mice infected with attenuated *Candida albicans* exhibited protection against a lethal dose of *Candida albicans and other* pathogens such as *Staphylococcus aureus*(Bistoni et al., 1986). This protection was independent of acquired adaptative immune cells but depended on the innate immune cells as macrophages and a higher production of pro-inflamatory cytokines including interleukin (IL)-1, granulocyte macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α and interferon (IFN)-γ(Bistoni et al., 1986; Vecchiarelli et al., 1988). Then, O'leary *et al.* reported that natural killer (NK)-cells, another line of innate immune cells, possessed an immunological memory capacity. NK-cells primed with haptens developped an exacerbed immune response which protected against a second specific infection(O'Leary et al., 2006).

In 2012 Netea *et al.* highlighted the mechanism involved in the immune protection previously observed by Bistoni and others. Indeed, these authors showed that reprogramming cells and inflammatory response confered to monocytes/macrophages were associated with epigenetic modification mechanisms.

Following this work, the concept of “trained immunity or innate immune memory” has been proposed.

Recently, Cheng *et al.* showed a new mechanism to support “trained immunity” focusing especially on the metabolism of cells such as glycolysis. For example they showed that the cholesterol synthesis pathway was highly induced in β-glucan-trained macrophages(Cheng et al., 2014).

Trained immunity draws more and more attention and interest. A simple investigation of the number of publications based on the key words ”innate immune memory” between 1969 and 2018 shows a sharp increase in the number of papers, reaching 142 papers in July 2018 **(Figure 1).**

Despite new knowledge, fundamental questions need to be addressed about the paradoxe between the duration of trained immunity and the short lifespan of innate immune cells **(Table 1).** Until now all of the studies were performed in monocytes/macrophages and NK cells whereas trained immunity can last for one year and more.

Consequently, trained immunity is more and more investigated in non-immune cells such as stem cells which possess immune caracteristics (expression of TLRs, inflammatory response, production of antimicrobial peptides) with long life span **(Figure 2).**

BOX 1: Innate immunity

Innate immune system is the second line of defense against microbial infection in vertebrates and non-vertabrates. It helps containing most infectious agents behind the first line anatomical and physiological defenses(Turvey and Broide, 2010). The innate immune system comprises hematopoietic cells including mast cells, macrophages, dendritic cells, neutrophils and eosinophils derived from *myeloid lineage* and natural killer (NK) cells. When a microorganism succeeds in crossing the anatomical and physiological barriers, the innate immune system takes over to efficiently remove it. Innate immunity develops in several steps including recognition of the microorganism via surface pattern recognition receptors shared by all the innate immune cells, binding to common pathogen-associated molecular patterns (PAMPs), uptake of the microorganism and induction of a production of inflammatory cytokines, chemokines and chemotractant molecules to recrute additional immune cells. The processus of microbial clearance continues and immature dendritic cells which uptook the microorganism migrate to lymph nodes to initiate adaptative immunity(Turvey and Broide, 2010; Zak and Aderem, 2009).

BOX 2: Adaptative immunity

Adaptative immunity is the third line of defense composed by hematopeitic T cells and B cells derived from the *lymphoid lineage*. Dendritic cells recognize pathogen, uptake it, process microbial antigens and migrate in the secondary lymphoid organs to encounter naive T cells. Dendritic cells present the antigens by the human major histocompatibility (HMC)-II molecules to naive T cells (Th0). T-lymphocytes specific to the antigen are activated, leading to their clonal expansion and differentiation into effector T cells such as Th1, Th2 and Th17 cells. Functionnaly, effector T cells prime different types of immunity such as cellular immunity, humoral immunity and

tolerance immunity and secrete pro-inflammatory cytokines such as IFN-γ, IL-2, IL-4, IL-6, IL-17 and TNF-α to activate other immune cells, contributing to pathogen clearance. Some long half-life T cells become memory T cell and have the capability to quickly respond to subsequent exposure to the same pathogen (specific-protection)(Clark and Kupper, 2005; Pennock et al., 2013) .

BOX 3: Trained immunity

Trained immunity is a new concept designing the adaptative properties carried by innate immune cells such as macrophages and NK-cells. Innate immune cells including monocytes/macrophages and NK-cells are primed by recognition of PAMP such as lipopolysaccharides, bacterial DNA, mannans that bind to toll-like receptors (TLRs)(Janeway and Medzhitov, 2002). Priming induces a high protective inflammatory response via the release of cytokines such as IFN-γ confering a protection to a secondary presentation of PAMPs carried by the same or a different pathogen than the one which primed trained immunity (cross-protection). Epigenetic modifications and immunometabolism are underlying mechanisms for training immune cells to act efficiently during a second infection(Netea et al., 2015) .

BOX 4: Molecular and metabolic mechanims involved in trained immunity.

Epigenetic events are part of the mechanisms controlling the way innate immune cells maintain the immune response.

Epigenetic mechanism: Term composed by “epi” meaning “above” in Greek and “genetic” relating to genes. Basically, it is the set of chemical modifications occuring in the DNA and consequently modulating the expression of genes. The mechanism does not affect the sequence of the DNA but is transmissible to the offspring.

Epigenetic modifications include DNA methylation, histone methylation and acetylation(Hoeksema and de Winther, 2016; Saeed et al., 2014). In general, DNA methylation is an epigenetic mechanism which involves the addition of a methyl-CH_3 group on carbon predominantly to the CpG dinucleotides of the cytosine residues of DNA 5-methylcytosine (5mC). This process involving three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) is active in the regulation and maintenance of gene expression(Jaenisch and Bird, 2003). NK-cell memory trained by BCG is associated with DNA methylation(Schlums et al., 2015; Sun et al., 2012).

Immunometabolic mechanism: Contraction of "immune" and "metabolic", originally proposed to explain both the cellular metabolism of innate immune cells and the role of immune cells playing in metabolic diseases and/or organ metabolism in global. The first study on immunometabolic mechanism concerned the relation between immunity and metabolic diseases, diabetes and obesity(Ferrante, 2013). Recent studies showed that metabolic mechanisms are also highlighted in trained immunity(Arts et al., 2016; Hotamisligil, 2017). In monocytes trained by β-glucan, transcriptional and epigenetic analysis revealed an increase in the promoters of genes encoding enzymes involved in glycolysis (hexokinase and pyruvate kinase) and its master regulator mTOR (mammalian target of rapamycin)(Cheng et al., 2014).

TRAINED IMMUNITY IN INNATE IMMUNE CELLS

Monocytes/Macrophages:

Macrophages emerged as the gold standard cellular model to investigate the adaptative characteristics of innate immune cells. Several studies reported that monocytes trained with different stimuli such as microorganism ligands, endotoxin, LPS, β-glucan or attenuated *Candida albicans* and BCG, exhibited a high and long-term immune response confering them resistance against secondary infections(Bistoni et al., 1988; Kleinnijenhuis et al., 2012; Quintin et al., 2012)·. This protection is materialized by a strong production of pro-inflammotory molecules IL-1, GM-CSF, TNF-α and IFN-γ(Vecchiarelli et al., 1988), leading to the recruitment of innate immune cells, the activation of phagocytic cells and the activation of adaptative immunity(Muralidharan and Mandrekar, 2013; Newton and Dixit, 2012). Epigenetic reprogramming is one of the important mechanisms which sustain trained immunity in monocytes/macrophages.

Briefly, naive cells have a compacted DNA rendering it inaccessible to promoters/enhancers. After infection, the DNA decondensates making it accessible to the promoters/enhancers that could be mono-methylated and mono-acetylated in a way such that the DNA will be transcribed, allowing for the production of molecules involved in pathogen elimination(Mehta and Jeffrey, 2015). Once the infection is resolved, the acetylation is lost but methylation of lysine 4 of histone 3 (H3K4me) persists and keeps the promoters active so that during the second stimulation the DNA is rapidly transcribed allowing great production of genes involved in immunity(Ostuni et al., 2013; Quintin et al., 2014; Saeed et al., 2014). Metabolic induction is additional mechanisms underlying trained immunity(Bekkering et al., 2018).

The interplay between metabolite production and trained immunity has been recently shown (Arts et al., 2016). Cholestrol synthesis pathway requires an intermediate metabolite called mevalonate; which induces a trained immunity profile by promoting the expression of a set of genes required in β-glucan-trained immunity phenotype like fumarate(Arts et al., 2016; Bekkering et al., 2018) .

Trained immunity induces an aerobic glycolysis associated with an augmentation of glucose consumption, lactate production and elevated intracellular ratio of nicotinamide adenine dinucleotide (NAD+/NADH)(Cheng et al., 2014).

Natural killer cells

Natural killer cells (NK cells) are lymphocyte-like cells sharing common progenitor with T lymphocytes and B lymphocytes(Wickramasinghe et al., 2011). NK cells are distinguished from later cells by the absence of antigen-specific cell surface receptors and are therefore considered as components of innate immune defense(Bozzano et al., 2017; Paust et al., 2010). NK cells are well known for their cytotoxic activity against tumor cells and virus-infected cells(Shibuya, 2003; Vivier et al., 2008; Yokoyama, 2003). Several studies have shown that in the same way as monocytes, NK-cells exhibit immunological memory. O'Leary *et al.* showed that a hapten (small molecule triggering an immune response)(Erkes and Selvan, 2014) induced contact hypersensitivity in T and B cell-deficient mice during the second contact with same hapten(O'Leary et al., 2006). This activity was shown to be carried by a liver subpopulation of NK cells (Ly49C-I+)(O'Leary et al., 2006). An additional study showed that IL-12 and STAT4 are required to build immunological memory in a mouse infected with mouse cytomegalovirus (MCMV). They lead an expansion of MCMV-specific NK cell and generation of memory NK cells(Sun et al., 2012). The first infection leads to the robust expansion of a Ly49-I+ subpopulation of NK-cells surviving in both lymphoid and nonlymphoid organs for longtime. Moreover, NK

cells are cornerstones in the immune system, interacting with the adaptative and innate systems(Goldszmid et al., 2012; Hamerman et al., 2005). Effectively, Belkaid *et al.* showed that activated NK cells primed monocytes during an infection, leading to long-term immune response(Askenase et al., 2015; Marcus and Raulet, 2013). Consequently, in response to a second infection or another, the trained long-living NK-cells lead to highly efficient recognition and clearance of pathogens(Dokun et al., 2001; Sun et al., 2011). It is only recently that we have been interested in studying the memory capacity of NK cells, whose underlying mechanisms had not been explored. Epigenetic reprogramming, immunometabolic induction and cell reprogramming are among the mechanisms which could be explored by an analogy to what has already been discoverd in monocytes. Indeed, it has already been showed that NK cells exploit epigenetic regulatory mechanism through developpement, differentiation and effector functions. Perforin and granzyme were the factors related to the defence mechanisms of NK-cells(Salcedo et al., 1993). The production of these effectors are controled by promotor of gene, regulator sequence (enhancer–silencer) and transcription factors such as lymphotoxin α (*LTA*), tumor necrosis factor α (*TNFA*), lymphotoxin β (*LTB*) and interferon-γ (*IFNG*) genes wich may undergo epigenetic modifications(Cichocki et al., 2013; Wiencke et al., 2016). It needs to be investigated how the mechanisms of production of perforin and granzyme may be supported by an epigenetic modification.

Mast cells

Mast cells are innate immune cells derived from the hematopietic progenitor in the bone marrow. Once activated, mast cells leave the bone marrow and migrate to the mucosal and epithelial tissues(Krystel-Whittemore et al., 2016),(Hallgren and Gurish, 2011). They are commonly known to play a key role in allergy but also against

bacteria and parasites through inflammatory and phagocytic activity(Spicer et al., 1975; Urb and Sheppard, 2012). There is no study related to immunological memory of mast cells in the literrature. Nevertheless, Monticelli hypothesized that mast cells, like the other innate immune cells, could adopt a memory phenotype(Monticelli and Leoni, 2017). Effectivelly, as previously described, mast cells play an important role in the first line defence and possess a longer life than the other innate immune memory (monocyte/macrophage and NK-cells) cells which were well known to carry immune memory. It is now obvious that epigenetic modification mechanisms play a critical role in trained immunity(van der Heijden et al., 2017). Interestingly, mast cell biology can be regulated by epigenetic modifications as described in monocytes/macrophages and NK-cells. They play a critical role in the establishement and maintenance of mast cell identity, expansion, differentiation and regulation of mast cell response to a danger signal(Montagner et al., 2016; Monticelli and Leoni, 2017). A recent study reports that the DNMT3A DNA methyltransferase is important to modulate mast cell responses to chronic stimuli(Leoni et al., 2017). Therefore, mast cells could support an immunological memory which has yet to be investigated.

Dendritic cells

DCs are innate immune cells derived from hematopietic progenitors expressing the CD34 surface marker, offering these cells the possiblity to be committed to either lymphoid or myeloid lineages. There are mainly three types of DCs: *Myeloid DCs (mDCs), Plasmacytoid DCs (pDCs)* and interstitial DCs and *Langerhans cells (LCs)*(Collin et al., 2013),(Liu and Nussenzweig, 2010). Differentiation of various types of DC depends on the microenvironment and the stimuli present in primary lymphoid organs and peripheral tissues(Price and Tarbell, 2015),(Banchereau et al., 2000). DCs are able to sense and immediately respond against pathogen attack by

producing innate pro-inflammatory cytokines and priming naïve T cells. Their main function is to phagocyte microorganisms, to conserve intact antigens from the ingested microorganism and to present antigens to adaptive immune system cells(Savina and Amigorena, 2007). Exposure of monocytes to bacterial component promotes the differentiation of monocytes into DCs(Bullwinkel et al., 2011; Naik, 2008). This differentiation is supported by epigenetic modifications in the expression of CD14 and CD209 proper functionning of monocytes and DC, respectively. Basically, the downregulation of CD14 is associated with the acquisition of the repressive markers (H3K9me3, H4K20me3) at the promoter and the expression of CD209 is regulated by the acquision of histone marker (H3K9Ac) and the loss of repressive markers (H3K9me3, H4K20me3)(Bullwinkel et al., 2011). Another study showed that LPS-stimulated DCs are inducing a robust immune response lasting for several hours and involving epigenetic modifications of 25 transcription factors(Adams et al., 2012; Garber et al., 2012; Mildner et al., 2017). DCs possess the immunological arsenal against bacteria; have a longer lifespan than monocytes and epigenetic mechanisms to support trained immunity.

TRAINED IMMUNITY OF NON-IMMUNE CELLS

Stem cells

Mesenchymal stromal/stem cells

Mesenchmal stem cells possess immune properties such as immunosuppressive phenotype, inflamatory phenotype, anti-bacterial characteristics and are equipped with Pattern Recognition Receptors (*PRRs*), including Toll-Like Receptors (TLRs)(Coffelt et al., 2009; Krasnodembskaya et al., 2010; Liotta et al., 2008; Machado et al., 2013; Pevsner-Fischer et al., 2007). In 2006,Guang-yang Liu and his colleagues showed that adipose mesenchymal stem cells exhibit short-term memory when exposed a second time to bacterial ligand LPS or danger signal TNF-α. Preciselly, mesenchymal stem cells primed with LPS or TNF have the ability to produce more intensely pro-inflammatory cytokines IL-8, MCP-1 and IL-6 to the same stimulus upon a second encounter. Additionnaly, they showed that primed mesenchymal stem cells exhibited a better therapeutic effect on diabetic rat model than unprimed MSCs(Liu et al., 2016). Moreover, like in monocytes, this trained immunity is gouverned by epigenetic mechanisms. The authors showed the involvement of a set of microRNAs (miR146a, miR150 and miR155) and a modification of DNA by 5-hydroxymethylcytosine (5hmC)(Liu et al., 2016). This study was the first one to demonstrate that trained immunity could be carried by non-profesionnal immune cells, Nevertheless, it concerns a short-term memory only corresponding just to 7 days whereas the trained immunity can last from one week to months and several years(Nguipdop-Djomo et al., 2016).

Recently, it has been shown that the planarian *Schmidtea mediterranea* is exhibiting trained immunity function(Torre et al., 2017). Planarian previously infected with *Staphylococcus aureus* developped an improved immune response with increased bacterial clearance during a second infection. This immunological memory

is due to stem cells that support cell replacement during homeostasis and regeneration of any missing tissue(Eisenhoffer et al., 2008). RNAi-based experiments revealed the signaling Smed-PGRP-2/Smed-setd8-1 methyltransferases as key factors in instructed neoblastes (planarian stem cells)(Torre et al., 2017). This study provided additional information to explain the capacity of BCG to induce a long immune memory (30 days minimum) and to persist for months to years whereas the lifespan of many innate immune cells in circulation is limited on the order of hours or days(Kleinnijenhuis et al., 2012; Nayar et al., 2015; Sun et al., 2011). Therefore, it is important to study the immunological memory capacity of stem cells beyond one month and their ability to transmit information to innate immune cells.

Hematopoietic Stem Cells

Kauffman and her colleagues published an important study which brings additional information to explain how cells transmit their immunological memory to progenitors, providing long-lasting protection(Kaufmann et al., 2018). These authors showed that in mice, the inoculation of bone marrow with BCG induced protection against *Mycobacterium tuberculosis* during a second infection which was not the case after subcutaneous inoculation. Specifically, it was shown that bone marrow BCG inoculation initially increased the amount hematopoietic stem cells (HSCs) and myelopoiesis and trained HSCs which offspring had the ability to develop a memory response against virulent *Mycobacterium tuberculosis*. Moreover, BCG induces epigenetic modifications of 2,483 genes in the macrophages, inducing changes of histone H3K27ac in (BMDM) Bone-marrow-derived macrophage produced from BCG-vaccinated mice compared with PBS-control mice(Kaufmann et al., 2018).

Another recent study showed that trained immunity performed at the level of bone marrow precisely increased myelopoeisis(Mitroulis et al., 2018). A total of 1,383

differentially expressed genes in β-glucan-injected mice compared to PBS-treated control mice, were involved in some innate immune functions such as production of pro-inflammatory cytokine IL-1β, production of GM-CSF (granulocyte-macrophage colony-stimulating factor) and immunometabolism including the biosynthesis of cholesterol and glycolysis(Mitroulis et al., 2018).

Epithelial stem cells

Epithelial stem cells are progenitors of differentiated epithelial cells. Epithelial cells and fibroblasts are forming a physical and functionnal barrier against external agents(Sacco et al., 2004). They constitue the first line of defense in innate immunity(Ochiel et al., 2008). Like innate immune cell, epithelial cells can act as sentinel cells by expressing TLRs, producing immunomodulator factors and antimicrobial peptides when exposed to danger signals like bacteria, virus and noxious signals(Bautista-Hernández et al., 2017; Schaefer et al., 2004). In addition to these immune caracteristics, it was reported that skin epithelial stem cells exhibit trained immunity. By using an imiquimod (IMQ)-induced model of skin inflammation, the authors showed that the skin previously exposed to one inflammatory challenge responded faster to an unrelated secondary challenge, with faster wound healing in primed mice than in naïve mice(Naik et al., 2017). A recent review by Novakovic entitled ”I Remember You: Epigenetic Priming in Epithelial Stem Cells” speculated that this memory of inflammation could be due to epigenetic mechanisms(Novakovic and Stunnenberg, 2017). Additionnaly, epithelial cell possess a long lifespan averaging two years(Tunn et al., 1989).

Intestinal stromal cells

Intestinal stromal cells (iSCs) are part of non-professional immune cells including mesenchymal stem cells, fibroblasts, epithelial cells and endhotelial cells that exhibit immune properties and contribute to immunity processing. ISC express TLRs 1-9, act as sentinel cells and produce pro-inflammtory cytokines in response to a pathogen(Augenlicht et al., 2016)-(Owens et al., 2013). Recently, Owens assessed that iSCs possess the immune machinery to respond against bacterial pathogens and suggested that iSCs could exhibit an immunological memory (Owens, 2015). Indeed iSCs could develop a protective response to rapidly eliminate the pathogen or another microorganism during a second contact(Owens, 2015). ISCs produce prolonged pro-inflammatory cytokines (inflammatory memory) to recruit immune cells to the site of infection during a second contact(Owens, 2015; Owens et al., 2013; Owens and Simmons, 2013). Epigenetic modifications and immunometabolic mechanisms would be implicated in the persistence of immunological memory. Moreover, the lifespan of iSCs is longer than professional innate immune cells(Arts et al., 2016; Hotamisligil, 2017; Smith et al., 2017).

Fibroblasts

The main function of fibroblasts is to maintain the structural integrity of the connective tissue(Wong et al., 2007). Nevertheless, it has now been well established that fibroblasts play an important role in the immunity. Fibroblasts are equipped with 1 to 10 TLRs, produce proinflammatory and antimicrobial peptides, cytokines, chemokines and growth factors in response to pathogen invasion(Jordana et al., 1994; Van Linthout et al., 2014). Further studies reported that fibroblasts are sentinel cells responding to pathogens and interacting with other cells via the production of molecular signals (Kaufman et al., 2001). Recent studies

revealed that fibroblasts were involved in the persistence of inflammation. When activated with bacterial infection or danger signal (cytokines), tissue repair triggers a protective immune response by producing cytokines and interacting with immune cells(Flavell et al., 2008; Frank-Bertoncelj et al., 2017). The persistence of inflammation is controlled by epigenetic mechanisms. In response to injury or infection, fibroblasts first recruite immune cells in the site of infection to clear bacteria, then organize tissue repair and renewal(Flavell et al., 2008). Indeed, tissue repair and renewal are not random as fibroblasts possess a positional memory capacity enabling regenerating cells to recall spatial information from the uninjured tissue(Bustos-Arriaga et al., 2011; Van Linthout et al., 2014) .This process is gouverned by epigenetic regulation of the homeobox "HOX" genes also involved in regulating body formation during development(Coleman and Struhl, 2017; Francis and Kingston, 2001). Much remains to be investigated in the immune functions of the fibroblasts such as the immunological memory. Functionally fibroblasts possess the immunological arsenal as mentioned above: the ability to produce inflammatory cytokines, antimicrobial peptides, and possess a longer lifespan than the cells of innate immunity such as macrophgae once differentiated(Weissman-Shomer and Fry, 1975).

Microglial cells

Microglial cells are resident immune cells of the central nervous system (CNS) also know as resident macrophages of CNS(Wake and Fields, 2011). The main function of microglial cells is to ensure the homeostasis of synapses and the communication with the microenvironnement in the CNS. Once activated by virus or bacteria, microglial cells somal size increases while retraction and thickening of processes are faciliting their migration capacity ; they express TLRs, produce inflammatory cytokines and

acquire phagocytic ability like macrophages(Mariani and Kielian, 2009). After this priming, microglial cells become susceptible to a secondary danger signal leading an improve immune response(Haley et al., 2017; Perry and Holmes, 2014). Several reviews suggested a process of trained immunity in microglial cells(Haley et al., 2017; Lelios and Greter, 2018). Haley recently speculated the fact that microglial cells possess an innate immune memory caracterized by inflammatory pathways orchestrated with epigenetic mechanisms(Haley et al., 2017). A study comparing naive mice and mice primed with attenuated *Salmonella typhimurium* containing its LPS indicated that the second group exhibited increased microglial immunoreactivity in response to a second stimulation of LPS four weeks later. Intrestingly, there was no increased immunoreactivy in microglial for the naive mice(Püntener et al., 2012). However, the authors did not investigate a potential epigenetic mechanism to explain the long-term activation of microglia in this infectious model.

Conclusion

The established dogma that innate immunity system lacks memory and that only the adaptive immune sytem is able to recognize an infectious agent and to destroy it faster in a second encounter has been challenged. Current published data indicate that innate immune cells are able to build an immunological memory. Indeed, the new concept called « trained immunity » represents a paradigm change in the biology of immunity, emerging as a third way between the conventional dichotomy “innate immunity” and “adapatative immunity”.

We have reviewed that trained immunity is not restricted to innate immune cells such as monocytes/macrophages and NK-cells, as non-immune cells like stem cells and that stromal cells also recognize a danger and respond faster in a second contact. In order to avoid any confusion and to harmonize the concept of immunological memory, we propose to keep the term “trained immunity” because an increasing number of studies are showing that non-immune cells possess immunological memory which is therefore not restricted to innate immune cells.

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References

Adams, D., Altucci, L., Antonarakis, S. E., Ballesteros, J., Beck, S., Bird, A., et al. (2012). BLUEPRINT to decode the epigenetic signature written in blood. *Nat. Biotechnol.* 30, 224–226. doi:10.1038/nbt.2153.

Angiolilli, C., Grabiec, A. M., Ferguson, B. S., Ospelt, C., Fernandez, B. M., van Es, I. E., et al. (2016). Inflammatory cytokines epigenetically regulate rheumatoid arthritis fibroblast-like synoviocyte activation by suppressing HDAC5 expression. *Ann Rheum Dis* 75, 430–438. doi:10.1136/annrheumdis-2014-205635.

Arts, R. J. W., Joosten, L. A. B., and Netea, M. G. (2016). Immunometabolic circuits in trained immunity. *Seminars in Immunology* 28, 425–430. doi:10.1016/j.smim.2016.09.002.

Arts, R. J. W., Novakovic, B., ter Horst, R., Carvalho, A., Bekkering, S., Lachmandas, E., et al. (2016). Glutaminolysis and Fumarate Accumulation Integrate Immunometabolic and Epigenetic Programs in Trained Immunity. *Cell Metab* 24, 807–819. doi:10.1016/j.cmet.2016.10.008.

Askenase, M. H., Han, S.-J., Byrd, A. L., da Fonseca, D. M., Bouladoux, N., Wilhelm, C., et al. (2015). Bone marrow-resident NK cells prime monocytes for regulatory function during infection. *Immunity* 42, 1130–1142. doi:10.1016/j.immuni.2015.05.011.

Augenlicht, L. H., Li, W., Peregrina, K., and Houston, M. (2016). Abstract 1708: Intestinal stem cells are sentinel cells for nutritional exposure. *Cancer Res* 76, 1708–1708. doi:10.1158/1538-7445.AM2016-1708.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., et al. (2000). Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18, 767–811. doi:10.1146/annurev.immunol.18.1.767.

Bautista-Hernández, L. A., Gómez-Olivares, J. L., Buentello-Volante, B., and Bautista-de Lucio, V. M. (2017). Fibroblasts: The Unknown Sentinels Eliciting Immune Responses Against Microorganisms. *Eur J Microbiol Immunol (Bp)* 7, 151–157. doi:10.1556/1886.2017.00009.

Bekkering, S., Arts, R. J. W., Novakovic, B., Kourtzelis, I., van der Heijden, C. D. C. C., Li, Y., et al. (2018). Metabolic Induction of Trained Immunity through the Mevalonate Pathway. *Cell* 172, 135-146.e9. doi:10.1016/j.cell.2017.11.025.

Bistoni, F., Vecchiarelli, A., Cenci, E., Puccetti, P., Marconi, P., and Cassone, A. (1986). Evidence for macrophage-mediated protection against lethal Candida albicans infection. *Infect. Immun.* 51, 668–674.

Bistoni, F., Verducci, G., Perito, S., Vecchiarelli, A., Puccetti, P., Marconi, P., et al. (1988). Immunomodulation by a low-virulence, agerminative variant of Candida albicans. Further evidence for macrophage activation as one of the effector mechanisms of nonspecific anti-infectious protection. *J. Med. Vet. Mycol.* 26, 285–299.

Blanden, R. V., Lefford, M. J., and Mackaness, G. B. (1969). The host response to Calmette-Guérin bacillus infection in mice. *J. Exp. Med.* 129, 1079–1107.

Bozzano, F., Marras, F., and De Maria, A. (2017). Natural Killer Cell Development and Maturation Revisited: Possible Implications of a Novel Distinct Lin−CD34+DNAM-1brightCXCR4+ Cell Progenitor. *Front Immunol* 8. doi:10.3389/fimmu.2017.00268.

Bullwinkel, J., Lüdemann, A., Debarry, J., and Singh, P. B. (2011). Epigenotype switching at the CD14 and CD209 genes during differentiation of human monocytes to dendritic cells. *Epigenetics* 6, 45–51. doi:10.4161/epi.6.1.13314.

Bustos-Arriaga, J., García-Machorro, J., León-Juárez, M., García-Cordero, J., Santos-Argumedo, L., Flores-Romo, L., et al. (2011). Activation of the Innate Immune Response against DENV in Normal Non-Transformed Human Fibroblasts. *PLoS Negl Trop Dis* 5. doi:10.1371/journal.pntd.0001420.

Cheng, S.-C., Quintin, J., Cramer, R. A., Shepardson, K. M., Saeed, S., Kumar, V., et al. (2014). mTOR- and HIF-1α–mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* 345, 1250684. doi:10.1126/science.1250684.

Cichocki, F., Miller, J. S., Anderson, S. K., and Bryceson, Y. T. (2013). Epigenetic regulation of NK cell differentiation and effector functions. *Front Immunol* 4. doi:10.3389/fimmu.2013.00055.

Clark, R., and Kupper, T. (2005). Old Meets New: The Interaction Between Innate and Adaptive Immunity. *Journal of Investigative Dermatology* 125, 629–637. doi:10.1111/j.0022-202X.2005.23856.x.

Coffelt, S. B., Marini, F. C., Watson, K., Zwezdaryk, K. J., Dembinski, J. L., LaMarca, H. L., et al. (2009). The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3806–3811. doi:10.1073/pnas.0900244106.

Coleman, R. T., and Struhl, G. (2017). Causal role for inheritance of H3K27me3 in maintaining the OFF state of a Drosophila HOX gene. *Science* 356, eaai8236. doi:10.1126/science.aai8236.

Collin, M., McGovern, N., and Haniffa, M. (2013). Human dendritic cell subsets. *Immunology* 140, 22–30. doi:10.1111/imm.12117.

Dokun, A. O., Kim, S., Smith, H. R., Kang, H. S., Chu, D. T., and Yokoyama, W. M. (2001). Specific and nonspecific NK cell activation during virus infection. *Nat. Immunol.* 2, 951–956. doi:10.1038/ni714.

Eisenhoffer, G. T., Kang, H., and Alvarado, A. S. (2008). Molecular analysis of stem cells and their descendents during cell turnover and regeneration in the planarian Schmidtea mediterranea. *Cell Stem Cell* 3, 327–339. doi:10.1016/j.stem.2008.07.002.

Erkes, D. A., and Selvan, S. R. (2014). Hapten-Induced Contact Hypersensitivity, Autoimmune Reactions, and Tumor Regression: Plausibility of Mediating Antitumor Immunity. *Journal of Immunology Research*. doi:10.1155/2014/175265.

Ferrante, A. W. (2013). Macrophages, fat, and the emergence of immunometabolism. *J Clin Invest* 123, 4992–4993. doi:10.1172/JCI73658.

Flavell, S. J., Hou, T. Z., Lax, S., Filer, A. D., Salmon, M., and Buckley, C. D. (2008). Fibroblasts as novel therapeutic targets in chronic inflammation. *Br J Pharmacol* 153, S241–S246. doi:10.1038/sj.bjp.0707487.

Francis, N. J., and Kingston, R. E. (2001). Mechanisms of transcriptional memory. *Nat. Rev. Mol. Cell Biol.* 2, 409–421. doi:10.1038/35073039.

Frank-Bertoncelj, M., Trenkmann, M., Klein, K., Karouzakis, E., Rehrauer, H., Bratus, A., et al. (2017). Epigenetically-driven anatomical diversity of synovial fibroblasts guides joint-specific fibroblast functions. *Nat Commun* 8. doi:10.1038/ncomms14852.

Gabrielli, S., Ortolani, C., del Zotto, G., Luchetti, F., Canonico, B., Buccella, F., et al. (2016). The Memories of NK Cells: Innate-Adaptive Immune Intrinsic Crosstalk. *J Immunol Res* 2016. doi:10.1155/2016/1376595.

Garber, M., Yosef, N., Goren, A., Raychowdhury, R., Thielke, A., Guttman, M., et al. (2012). A high-throughput chromatin immunoprecipitation approach reveals principles of dynamic gene regulation in mammals. *Mol. Cell* 47, 810–822. doi:10.1016/j.molcel.2012.07.030.

Goldszmid, R. S., Caspar, P., Rivollier, A., White, S., Dzutsev, A., Hieny, S., et al. (2012). NK cell-derived interferon-γ orchestrates cellular dynamics and the differentiation of monocytes into dendritic cells at the site of infection. *Immunity* 36, 1047–1059. doi:10.1016/j.immuni.2012.03.026.

Haley, M. J., Brough, D., Quintin, J., and Allan, S. M. (2017). Microglial Priming as Trained Immunity in the Brain. *Neuroscience*. doi:10.1016/j.neuroscience.2017.12.039.

Hallgren, J., and Gurish, M. F. (2011). MAST CELL PROGENITOR TRAFFICKING AND MATURATION. *Adv Exp Med Biol* 716, 14–28. doi:10.1007/978-1-4419-9533-9_2.

Hamerman, J. A., Ogasawara, K., and Lanier, L. L. (2005). NK cells in innate immunity. *Curr. Opin. Immunol.* 17, 29–35. doi:10.1016/j.coi.2004.11.001.

Hoeksema, M. A., and de Winther, M. P. J. (2016). Epigenetic Regulation of Monocyte and Macrophage Function. *Antioxid Redox Signal* 25, 758–774. doi:10.1089/ars.2016.6695.

Hotamisligil, G. S. (2017). Foundations of Immunometabolism and Implications for Metabolic Health and Disease. *Immunity* 47, 406–420. doi:10.1016/j.immuni.2017.08.009.

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* 33 Suppl, 245–254. doi:10.1038/ng1089.

Janeway, C. A., and Medzhitov, R. (2002). Innate immune recognition. *Annu. Rev. Immunol.* 20, 197–216. doi:10.1146/annurev.immunol.20.083001.084359.

Jordana, M., Särnstrand, B., Sime, P. J., and Ramis, I. (1994). Immune-inflammatory functions of fibroblasts. *Eur. Respir. J.* 7, 2212–2222.

Kaufman, J., Graf, B. A., Leung, E. C., Pollock, S. J., Koumas, L., Reddy, S. Y., et al. (2001). Fibroblasts as Sentinel Cells: Role of the CD40-CD40 Ligand System in Fibroblast Activation and Lung Inflammation and Fibrosis. *CHEST* 120, S53–S55. doi:10.1378/chest.120.1_suppl.S53.

Kaufmann, E., Sanz, J., Dunn, J. L., Khan, N., Mendonça, L. E., Pacis, A., et al. (2018). BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. *Cell* 172, 176-190.e19. doi:10.1016/j.cell.2017.12.031.

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A. B., Ifrim, D. C., Saeed, S., et al. (2012). Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc. Natl. Acad. Sci. U.S.A.* 109, 17537–17542. doi:10.1073/pnas.1202870109.

Krasnodembskaya, A., Song, Y., Fang, X., Gupta, N., Serikov, V., Lee, J.-W., et al. (2010). Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 28, 2229–2238. doi:10.1002/stem.544.

Krystel-Whittemore, M., Dileepan, K. N., and Wood, J. G. (2016). Mast Cell: A Multi-Functional Master Cell. *Front Immunol* 6. doi:10.3389/fimmu.2015.00620.

Lelios, I., and Greter, M. (2018). Trained Microglia Trigger Memory Loss. *Immunity* 48, 849–851. doi:10.1016/j.immuni.2018.04.033.

Leoni, C., Montagner, S., Rinaldi, A., Bertoni, F., Polletti, S., Balestrieri, C., et al. (2017). Dnmt3a restrains mast cell inflammatory responses. *PNAS* 114, E1490–E1499. doi:10.1073/pnas.1616420114.

Liotta, F., Angeli, R., Cosmi, L., Filì, L., Manuelli, C., Frosali, F., et al. (2008). Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells* 26, 279–289. doi:10.1634/stemcells.2007-0454.

Liu, G.-Y., Liu, Y., Lu, Y., Qin, Y.-R., Di, G.-H., Lei, Y.-H., et al. (2016). Short-term memory of danger signals or environmental stimuli in mesenchymal stem cells: implications for therapeutic potential. *Cell Mol Immunol* 13, 369–378. doi:10.1038/cmi.2015.11.

Liu, K., and Nussenzweig, M. C. (2010). Origin and development of dendritic cells. *Immunol. Rev.* 234, 45–54. doi:10.1111/j.0105-2896.2009.00879.x.

Machado, C. de V., Telles, P. D. da S., and Nascimento, I. L. O. (2013). Immunological characteristics of mesenchymal stem cells. *Rev Bras Hematol Hemoter* 35, 62–67. doi:10.5581/1516-8484.20130017.

Marcus, A., and Raulet, D. H. (2013). Evidence for Natural Killer Cell Memory. *Curr Biol* 23. doi:10.1016/j.cub.2013.07.015.

Mariani, M. M., and Kielian, T. (2009). Microglia in Infectious Diseases of the Central Nervous System. *J Neuroimmune Pharmacol* 4, 448–461. doi:10.1007/s11481-009-9170-6.

Mehta, S., and Jeffrey, K. L. (2015). Beyond receptors and signaling: epigenetic factors in the regulation of innate immunity. *Immunol Cell Biol* 93, 233–244. doi:10.1038/icb.2014.101.

Mildner, A., Schönheit, J., Giladi, A., David, E., Lara-Astiaso, D., Lorenzo-Vivas, E., et al. (2017). Genomic Characterization of Murine Monocytes Reveals C/EBPβ Transcription Factor Dependence of Ly6C-Cells. *Immunity* 46, 849-862.e7. doi:10.1016/j.immuni.2017.04.018.

Mitroulis, I., Ruppova, K., Wang, B., Chen, L.-S., Grzybek, M., Grinenko, T., et al. (2018). Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. *Cell* 172, 147-161.e12. doi:10.1016/j.cell.2017.11.034.

Montagner, S., Leoni, C., Emming, S., Chiara, G. D., Balestrieri, C., Barozzi, I., et al. (2016). TET2 Regulates Mast Cell Differentiation and Proliferation through Catalytic and Non-catalytic Activities. *Cell Reports* 15, 1566–1579. doi:10.1016/j.celrep.2016.04.044.

Monticelli, S., and Leoni, C. (2017). Epigenetic and transcriptional control of mast cell responses. *F1000Res* 6. doi:10.12688/f1000research.12384.1.

Muralidharan, S., and Mandrekar, P. (2013). Cellular stress response and innate immune signaling: integrating pathways in host defense and inflammation. *J Leukoc Biol* 94, 1167–1184. doi:10.1189/jlb.0313153.

Naik, S. H. (2008). Demystifying the development of dendritic cell subtypes, a little. *Immunology and Cell Biology* 86, 439–452. doi:10.1038/icb.2008.28.

Naik, S., Larsen, S. B., Gomez, N. C., Alaverdyan, K., Sendoel, A., Yuan, S., et al. (2017). Inflammatory Memory Sensitizes Skin Epithelial Stem Cells to Tissue Damage. *Nature* 550, 475–480. doi:10.1038/nature24271.

Nayar, S., Dasgupta, P., and Galustian, C. (2015). Extending the lifespan and efficacies of immune cells used in adoptive transfer for cancer immunotherapies–A review. *Oncoimmunology* 4. doi:10.1080/2162402X.2014.1002720.

Naylor, A. J., Filer, A., and Buckley, C. D. (2013). The role of stromal cells in the persistence of chronic inflammation. *Clinical & Experimental Immunology* 171, 30–35. doi:10.1111/j.1365-2249.2012.04634.x.

Netea, M. G., Joosten, L. A. B., Latz, E., Mills, K. H. G., Natoli, G., Stunnenberg, H. G., et al. (2016). Trained immunity: a program of innate immune memory in health and disease. *Science* 352, aaf1098. doi:10.1126/science.aaf1098.

Netea, M. G., Latz, E., Mills, K. H. G., and O'Neill, L. A. J. (2015). Innate immune memory: a paradigm shift in understanding host defense. *Nature Immunology*. doi:10.1038/ni.3178.

Newton, K., and Dixit, V. M. (2012). Signaling in Innate Immunity and Inflammation. *Cold Spring Harb Perspect Biol* 4. doi:10.1101/cshperspect.a006049.

Nguipdop-Djomo, P., Heldal, E., Rodrigues, L. C., Abubakar, I., and Mangtani, P. (2016). Duration of BCG protection against tuberculosis and change in effectiveness with time since vaccination in Norway: a retrospective population-based cohort study. *Lancet Infect Dis* 16, 219–226. doi:10.1016/S1473-3099(15)00400-4.

Novakovic, B., and Stunnenberg, H. G. (2017). I Remember You: Epigenetic Priming in Epithelial Stem Cells. *Immunity* 47, 1019–1021. doi:10.1016/j.immuni.2017.12.005.

Ochiel, D. O., Fahey, J. V., Ghosh, M., Haddad, S. N., and Wira, C. R. (2008). Innate Immunity in the Female Reproductive Tract: Role of Sex Hormones in Regulating Uterine Epithelial Cell Protection Against Pathogens. *Curr Womens Health Rev* 4, 102–117. doi:10.2174/157340408784246395.

O'Leary, J. G., Goodarzi, M., Drayton, D. L., and von Andrian, U. H. (2006). T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat. Immunol.* 7, 507–516. doi:10.1038/ni1332.

Ospelt, C., Reedquist, K. A., Gay, S., and Tak, P. P. (2011). Inflammatory memories: is epigenetics the missing link to persistent stromal cell activation in rheumatoid arthritis? *Autoimmun Rev* 10, 519–524. doi:10.1016/j.autrev.2011.04.001.

Ostuni, R., Piccolo, V., Barozzi, I., Polletti, S., Termanini, A., Bonifacio, S., et al. (2013). Latent enhancers activated by stimulation in differentiated cells. *Cell* 152, 157–171. doi:10.1016/j.cell.2012.12.018.

Owens, B. M. J. (2015). Inflammation, Innate Immunity, and the Intestinal Stromal Cell Niche: Opportunities and Challenges. *Front Immunol* 6. doi:10.3389/fimmu.2015.00319.

Owens, B. M. J., and Simmons, A. (2013). Intestinal stromal cells in mucosal immunity and homeostasis. *Mucosal Immunol* 6, 224–234. doi:10.1038/mi.2012.125.

Owens, B. M. J., Steevels, T. A. M., Dudek, M., Walcott, D., Sun, M.-Y., Mayer, A., et al. (2013). CD90+ Stromal Cells are Non-Professional Innate Immune Effectors of the Human Colonic Mucosa. *Front. Immunol.* 4. doi:10.3389/fimmu.2013.00307.

Patel, A. A., Zhang, Y., Fullerton, J. N., Boelen, L., Rongvaux, A., Maini, A. A., et al. (2017). The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *Journal of Experimental Medicine*, jem.20170355. doi:10.1084/jem.20170355.

Paust, S., Gill, H. S., Wang, B.-Z., Flynn, M. P., Moseman, E. A., Senman, B., et al. (2010). Critical role for CXCR6 in NK cell-mediated antigen-specific memory to haptens and viruses. *Nat Immunol* 11, 1127–1135. doi:10.1038/ni.1953.

Paust, S., Senman, B., and von Andrian, U. H. (2010). Adaptive Immune Responses Mediated by Natural Killer Cells. *Immunol Rev* 235, 286–296. doi:10.1111/j.0105-2896.2010.00906.x.

Pennock, N. D., White, J. T., Cross, E. W., Cheney, E. E., Tamburini, B. A., and Kedl, R. M. (2013). T cell responses: naïve to memory and everything in between. *Adv Physiol Educ* 37, 273–283. doi:10.1152/advan.00066.2013.

Perry, V. H., and Holmes, C. (2014). Microglial priming in neurodegenerative disease. *Nat Rev Neurol* 10, 217–224. doi:10.1038/nrneurol.2014.38.

Pevsner-Fischer, M., Morad, V., Cohen-Sfady, M., Rousso-Noori, L., Zanin-Zhorov, A., Cohen, S., et al. (2007). Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood* 109, 1422–1432. doi:10.1182/blood-2006-06-028704.

Price, J. D., and Tarbell, K. V. (2015). The Role of Dendritic Cell Subsets and Innate Immunity in the Pathogenesis of Type 1 Diabetes and Other Autoimmune Diseases. *Front. Immunol.* 6. doi:10.3389/fimmu.2015.00288.

Püntener, U., Booth, S. G., Perry, V. H., and Teeling, J. L. (2012). Long-term impact of systemic bacterial infection on the cerebral vasculature and microglia. *J Neuroinflammation* 9, 146. doi:10.1186/1742-2094-9-146.

Quintin, J., Cheng, S.-C., van der Meer, J. W., and Netea, M. G. (2014). Innate immune memory: towards a better understanding of host defense mechanisms. *Current Opinion in Immunology* 29, 1–7. doi:10.1016/j.coi.2014.02.006.

Quintin, J., Saeed, S., Martens, J. H. A., Giamarellos-Bourboulis, E. J., Ifrim, D. C., Logie, C., et al. (2012). Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* 12, 223–232. doi:10.1016/j.chom.2012.06.006.

Sacco, O., Silvestri, M., Sabatini, F., Sale, R., Defilippi, A.-C., and Rossi, G. A. (2004). Epithelial cells and fibroblasts: structural repair and remodelling in the airways. *Paediatr Respir Rev* 5 Suppl A, S35-40.

Saeed, S., Quintin, J., Kerstens, H. H. D., Rao, N. A., Aghajanirefah, A., Matarese, F., et al. (2014). Epigenetic programming during monocyte to macrophage differentiation and trained innate immunity. *Science* 345, 1251086. doi:10.1126/science.1251086.

Salcedo, T. W., Azzoni, L., Wolf, S. F., and Perussia, B. (1993). Modulation of perforin and granzyme messenger RNA expression in human natural killer cells. *The Journal of Immunology* 151, 2511–2520.

Savina, A., and Amigorena, S. (2007). Phagocytosis and antigen presentation in dendritic cells. *Immunol. Rev.* 219, 143–156. doi:10.1111/j.1600-065X.2007.00552.x.

Schaefer, T. M., Desouza, K., Fahey, J. V., Beagley, K. W., and Wira, C. R. (2004). Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells. *Immunology* 112, 428–436. doi:10.1111/j.1365-2567.2004.01898.x.

Schlums, H., Cichocki, F., Tesi, B., Theorell, J., Beziat, V., Holmes, T. D., et al. (2015). Cytomegalovirus Infection Drives Adaptive Epigenetic Diversification of NK Cells with Altered Signaling and Effector Function. *Immunity* 42, 443–456. doi:10.1016/j.immuni.2015.02.008.

Shibuya, A. (2003). Development and functions of natural killer cells. *Int. J. Hematol.* 78, 1–6.

Sieburg, H. B., Cattarossi, G., and Muller-Sieburg, C. E. (2013). Lifespan Differences in Hematopoietic Stem Cells are Due to Imperfect Repair and Unstable Mean-Reversion. *PLoS Comput Biol* 9. doi:10.1371/journal.pcbi.1003006.

Smith, R. J., Rao-Bhatia, A., and Kim, T.-H. (2017). Signaling and epigenetic mechanisms of intestinal stem cells and progenitors: insight into crypt homeostasis, plasticity, and niches. *Wiley Interdiscip Rev Dev Biol* 6. doi:10.1002/wdev.281.

Spicer, S. S., Simson, J. A., and Farrington, J. E. (1975). Mast cell phagocytosis of red blood cells. *Am J Pathol* 80, 481–497.

Sun, J. C., Beilke, J. N., Bezman, N. A., and Lanier, L. L. (2011a). Homeostatic proliferation generates long-lived natural killer cells that respond against viral infection. *J Exp Med* 208, 357–368. doi:10.1084/jem.20100479.

Sun, J. C., Lopez-Verges, S., Kim, C. C., DeRisi, J. L., and Lanier, L. L. (2011b). NK cells and immune "memory." *J. Immunol.* 186, 1891–1897. doi:10.4049/jimmunol.1003035.

Sun, J. C., Madera, S., Bezman, N. A., Beilke, J. N., Kaplan, M. H., and Lanier, L. L. (2012). Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J Exp Med* 209, 947–954. doi:10.1084/jem.20111760.

Torre, C., Abnave, P., Tsoumtsa, L. L., Mottola, G., Lepolard, C., Trouplin, V., et al. (2017). Staphylococcus aureus Promotes Smed-PGRP-2/Smed-setd8-1 Methyltransferase Signalling in Planarian Neoblasts to Sensitize Anti-bacterial Gene Responses During Re-infection. *EBioMedicine* 20, 150–160. doi:10.1016/j.ebiom.2017.04.031.

Tribouley, J., Tribouley-Duret, J., and Appriou, M. (1978). [Effect of Bacillus Callmette Guerin (BCG) on the receptivity of nude mice to Schistosoma mansoni]. *C. R. Seances Soc. Biol. Fil.* 172, 902–904.

Tunn, S., Nass, R., Ekkernkamp, A., Schulze, H., and Krieg, M. (1989). Evaluation of average life span of epithelial and stromal cells of human prostate by superoxide dismutase activity. *Prostate* 15, 263–271.

Turvey, S. E., and Broide, D. H. (2010). Chapter 2: Innate Immunity. *J Allergy Clin Immunol* 125, S24–S32. doi:10.1016/j.jaci.2009.07.016.

Urb, M., and Sheppard, D. C. (2012). The Role of Mast Cells in the Defence against Pathogens. *PLoS Pathogens* 8, e1002619. doi:10.1371/journal.ppat.1002619.

van der Heijden, C. D. C. C., Noz, M. P., Joosten, L. A. B., Netea, M. G., Riksen, N. P., and Keating, S. T. (2017). Epigenetics and Trained Immunity. *Antioxid. Redox Signal.* doi:10.1089/ars.2017.7310.

Van Linthout, S., Miteva, K., and Tschöpe, C. (2014). Crosstalk between fibroblasts and inflammatory cells. *Cardiovasc Res* 102, 258–269. doi:10.1093/cvr/cvu062.

Van Zant, G., and Liang, Y. (2012). Concise Review: Hematopoietic Stem Cell Aging, Life Span, and Transplantation. *Stem Cells Transl Med* 1, 651–657. doi:10.5966/sctm.2012-0033.

Vecchiarelli, A., Mazzolla, R., Farinelli, S., Cassone, A., and Bistoni, F. (1988). Immunomodulation by Candida albicans: crucial role of organ colonization and chronic infection with an attenuated agerminative strain of C. albicans for establishment of anti-infectious protection. *J. Gen. Microbiol.* 134, 2583–2592. doi:10.1099/00221287-134-9-2583.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. (2008). Functions of natural killer cells. *Nat. Immunol.* 9, 503–510. doi:10.1038/ni1582.

Wake, H., and Fields, R. D. (2011). Physiological Function of Microglia. *Neuron glia biology* 7, 1. doi:10.1017/S1740925X12000166.

Weissman-Shomer, P., and Fry, M. (1975). Chick embryo fibroblasts senescence in vitro: Pattern of cell division and life span as a function of cell density. *Mechanisms of Ageing and Development* 4, 159–166. doi:10.1016/0047-6374(75)90017-2.

Wickramasinghe, S., Porwit, A., and Erber, W. (2011). "CHAPTER 2 - Normal bone marrow cells: Development and cytology," in *Blood and Bone Marrow Pathology (Second Edition)*, eds. A. Porwit, J. McCullough, and W. N. Erber (Edinburgh: Churchill Livingstone), 19–44. doi:10.1016/B978-0-7020-3147-2.00002-X.

Wiencke, J. K., Butler, R., Hsuang, G., Eliot, M., Kim, S., Sepulveda, M. A., et al. (2016). The DNA methylation profile of activated human natural killer cells. *Epigenetics* 11, 363–380. doi:10.1080/15592294.2016.1163454.

Wong, T., McGrath, J. A., and Navsaria, H. (2007). The role of fibroblasts in tissue engineering and regeneration. *Br. J. Dermatol.* 156, 1149–1155. doi:10.1111/j.1365-2133.2007.07914.x.

Yokoyama, W. M. (2003). "The Role of Natural Killer Cells in Innate Immunity to Infection," in *Innate Immunity* Infectious Disease. (Humana Press, Totowa, NJ), 321–339. doi:10.1007/978-1-59259-320-0_18.

Zak, D. E., and Aderem, A. (2009). Systems biology of innate immunity. *Immunol Rev* 227, 264–282. doi:10.1111/j.1600-065X.2008.00721.x.

Zawislak, C. L., Beaulieu, A. M., Loeb, G. B., Karo, J., Canner, D., Bezman, N. A., et al. (2013). Stage-specific regulation of natural killer cell homeostasis and response against viral infection by microRNA-155. *Proc Natl Acad Sci U S A* 110, 6967–6972. doi:10.1073/pnas.1304410110.

1

2 **Table 1: Characteristics of the trained immunity compared to cell involved**

	Types of cells	Lifespan	Priming agents	Mechanism underlying	Consequence	References
Innate immune cells	Monocytes/ Macrophages	7 days	-BCG -β-glucan -LPS -*Candida albicans*	-Epigenetics modifications (H3K4me3 persistancy) -Immunometabolic induction (Pathways promotor dectin-1-Akt-mTOR-HIF-pathways activated	-Reprograming cells -Production of pro-inflammatory cytokine (IL-1, TNF-a, IFN-g, GM-CSF) -Recrutement of effector cells	(Vecchiarelli et al., 1988) , (Patel et al., 2017) , (Bekkering et al., 2018), (Bistoni et al., 1988)
	NK cells	6 Months	-Hapten-induced contact -MCMV	-Trained immunity carried by NK-cells (Ly49C-I+) -miRNA (miR155, DNAM, CXCR6 play an important role in	-Reprograming NK-cell with pro-inflammatory cytokines -Signals operating through IL-12 and STAT4 -Secretion of perforin and	(Sun et al., 2011a), (O'Leary et al., 2006a), (Zawislak et al., 2013), (Gabrielli

			-Vaccinia virus	the function of NK cell memory)	granzymes to lyse virus-infected cells	et al., 2016), (Paust et al., 2010a), (Netea et al., 2016)
Stem cells	Hematopietic cells	Indefinite lifespan	-BCG -β-glucan	-Epigenetic modifications (H3K4me3 persistancy) -Immunometabolic (glycolysis, Cholesterol metabolism)	-Myelopoiesis expansion -Inflammatory signaling (IL-1b, GM-CSF) -Enhanced response to chemotherapy -Benefice response to systemic inflammation	(Sieburg et al., 2013), (Van Zant and Liang, 2012), (Mitroulis et al., 2018), (Kaufmann et al., 2018)
	Mesenchymal	Indefinite	-LPS	-Epigenetic modifications, miRNAs (miR146a, miR150 and	-Increased expression of pro-inflammatory cytokine (IL-6, IL-8	(Liu et al., 2016)

	stem cells	lifespan	-TNF-α	miR155, along with the modicifation of DNA by 6hydroxymethylcytosine (5hmC)	and MCP-1)	
	Epithelial stem cells	Indefinite lifespan	-imiquimod (IMQ)-induced model of skin inflammation	-Epigenetic modifications: Induced epithelial stem cells maintains chromosomal accessibility of both epidermal and inflammation genes after the first stimulus. In the second stimulus genes were transcribed rapidly.	-Inflammatory memory in a non-immune in epithelial stem cells (EpSCs) of the skin - Accelerating wound repair in induced mice 2.5 times faster than naive	(Naik et al., 2017)

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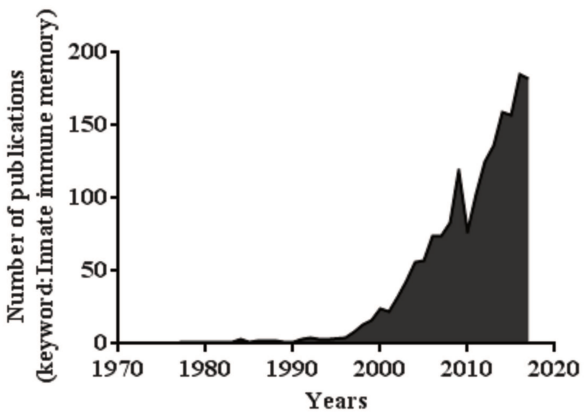
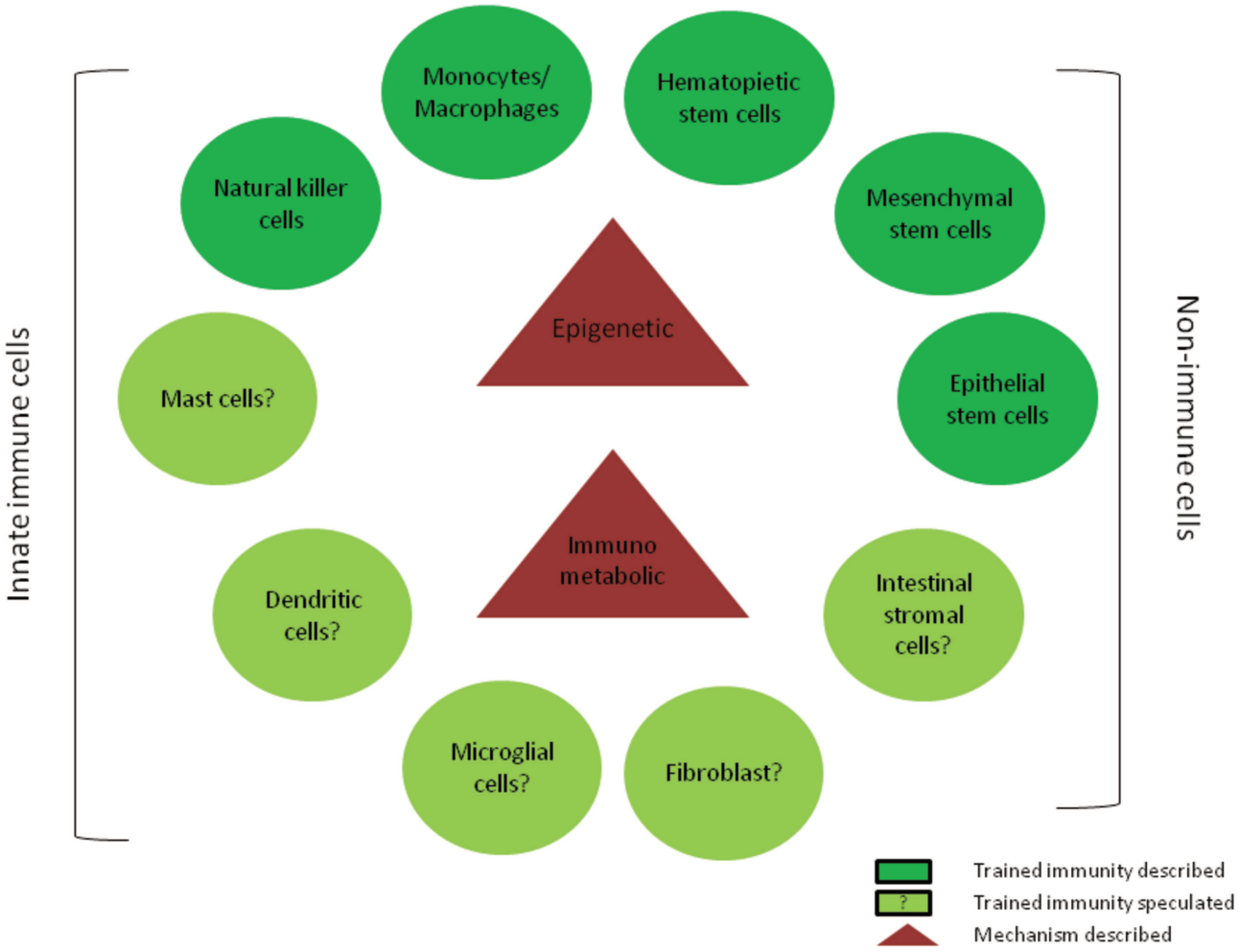

Number of publications
(keyword:Innate immune memory)
200
150
100
50
0
1970
1980
1990
2000
2010
2020
Years


Monocytes/ Macrophages
Hematopietic stem cells
Natural killer cells
Mesenchymal stem cells
Epigenetic
Mast cells?
Epithelial stem cells
Immuno metabolic
Dendritic cells?
Intestinal stromal cells?
Microglial cells?
Fibroblast?
Innate immune cells
Non-immune cells
Trained immunity described
?
Trained immunity speculated
Mechanism described

Article de recherche:

Human Dental pulp Stem Cells (hDPSC) adopt an immunological memory phenotype after exposure to bacterial stimuli

Attoumani Hamada, Michel Drancourt, Eric Ghigo

(Unpublished data : subjecting to PLoS ONE)

Préambule de l'article

Outre leurs capacités régénératives, les données de la littérature indiquent que les hDPSCs possèdent certaines caractéristiques des cellules immunitaires, notamment la capacité de percevoir un signal de danger grâce à l'expression de protéines réceptrices de type Toll, TLR 1-10 sur leurs surface cellulaire mais également d'émettre des signaux de danger en présence d'une infection (Fawzy El-Sayed et al., 2016b). Ainsi une fois activés, les DPSC peuvent migrer jusqu'au site d'infection où ils peuvent se différencier en odontoblastes afin de reconstruire les tissus lésés (Gunzer, 2007; Li et al., 2014) .Il est démontré aussi que les DPSC sont également capables de produire des médiateurs inflammatoires (un phénotype pro-inflammatoire, un phénotype immunosuppresseur) pour interagir avec les cellules immunitaires afin de les attirer sur les site infectieux lors de la phase précoce d'infection ou de résoudre l'inflammation afin de rétablir les conditions physiologiques de base lors de la phase tardive d'une infection (Bindal et al., 2018; Chang et al., 2005; He et al., 2013). Les résultats exposés au chapitre 4 de notre Thèse, ajoutent une autre caractéristique immunitaire qui est d'internaliser un agent infectieux puis d'initier une réponse immunitaire.

Cependant, beaucoup de choses restent à découvrir. Une analogie peut être faite dans la découverte des propriétés immunitaires des macrophages et celles des cellules souches. En 1883, Elie Metchnikoff décrit les macrophages, cellules immunitaires capables d'internaliser des bactéries et les tuer par phagocytose (Metchnikoff, 1883). En 2012, Quintin *et al.* découvrent la propriété de mémoire immunologique des macrophages (Quintin et al., 2012). En parallèle, nous avons montré que les hDPSCs avaient la capacité d'internaliser une bactérie et d'initier une réponse protectrice (Hamada A. *et al*., soumis pour publication) et

nous suggérons que les hDPSCs possèdent une mémoire immunologique. Dans cette étude, nous nous sommes basés sur les propriétés inflammatoires des hDPSCs en réponse à des ligands bactériens LPS et PGN. Il a été préalablement montré que les hDPSCs sont un modèle d'inflammation: lorsque ces cellules sont stimulées par du LPS, elles développent une reaction pro-inflammatoire avec production de cytokines IL-6, IL-8, MCP-1 (Bindal *et al.*, 2018). Notre intention était de savoir si les hDPSCs stimulées par du LPS ou par du PGN avaient la capacité de répondre 30 jours après un premier contact, de façon significativement augmentée lors d'un second stimulus. Nous avons montré dans cette étude que, comme les cellules immunitaires, les hDPSCs ont la capacité de mémoriser des signaux de ligands bactériens pendant 30 jours pour indure une réaction immunitaire rapide et exacerbée lors d'un second contact. Cette mémoire se caractérise par une augmentation de l'expression et production de cytokines pro-inflammatoires IL-6 et MCP-1 lors du second stimulus par LPS ou par PGN. Ce résultat vient conforter une étude montrant que des MSCs ont la capacité de mémoriser des signaux de danger durant une semaine et de développer une réaction exacerbée lors du second contact **Figure 8** (Liu et al., 2016).

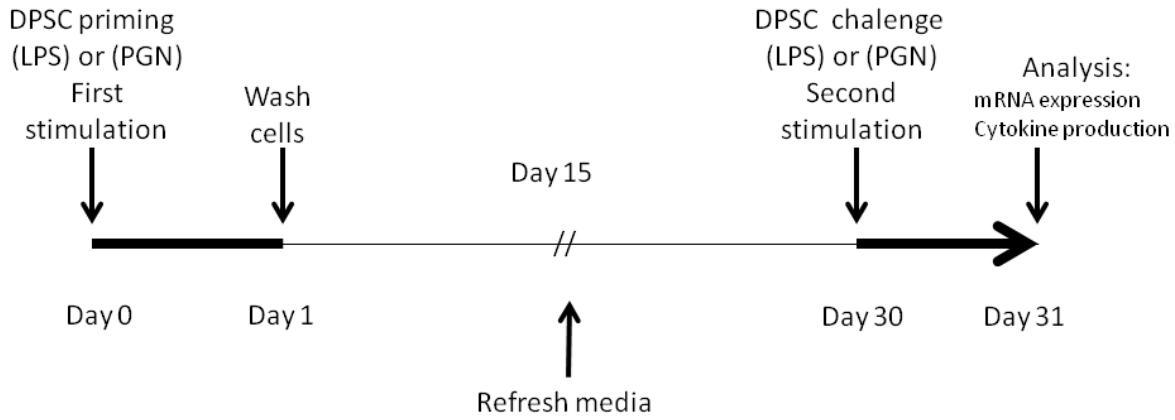


Figure 8 : Schéma expérimental pour l'exploration de l'immunité entrainée chez les cellules hDPSCs.

Human Dental pulp Stem Cells adopt an immunological memory phenotype after exposure to bacterial ligand stimuli

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Abstract

Dental pulp stem cells (DPSCs) are mesenchymal stem cells derived from the dental pulp tissue. Cumulative data indicate that DPSCs possess characteristics of immune cells including the expression of Toll-like receptors, the production of antimicrobial peptide along with phagocytic properties. Previous reports have shown that DPSCs induce an inflammatory phenotype by releasing pro-inflammatory cytokines IL-6, IL-8, TNF-alpha and MCP-1 in response to signal of danger such as bacterial ligands. In this study, we investigated whether the DPSCs can keep in memory this inflammatory phenotype and act faster upon a secondary challenge. Our results show that DPSCs primed with bacterial lipopolysaccharide (LPS) or peptidoglycan (PGN) develop an inflammatory memory by transcribing faster IL-6 and MCP-1 genes and producing a high level of IL-6 and MCP-1 protein. We found also that histone methyl transferase is involved in the establishment of the inflammatory memory induced by PGN. Taken together, these data show that DPSCs possess trained immunity and suggest that a LPS or PGN pre-conditioned DPSCs could have a better therapeutic efficacy in wound healing and pulp regeneration than non-primed cells.

Keywords: DPSC, inflammatory, memory, cytokine, epigenetic

Introduction

Dental pulp stem cell (DPSCs) are mesenchymal stem cells initially isolated from the dental pulp tissue eighteen years ago (Gronthos et al., 2000). Under physiological conditions, the principal function of DPSCs is to produce odontoblasts to maintain the homeostasis of dentin, therefore protecting the dental pulp (Tatullo et al., 2015). However, the dental pulp may be injured by mechanical, chemical and microbial factors (Alkharobi et al., 2017; El-Gendy et al., 2015; Gronthos et al., 2002). Such injuries eventually activate the DPSCs which may then express Toll-like receptors (TLRs), produce antimicrobial peptides and exhibit a protective inflammatory response (Attoumani et al., 2018; Fawzy El-Sayed et al., 2016). In addition, it has been reported that DPSCs stimulated with the Gram-negative bacteria ligand lipopolysaccharide (LPS) induce an inflammatory response associated with the migration of DPSCs towards the dentin surface and the differentiation into odontoblasts to fix the dentin(Li et al., 2014; Widbiller et al., 2018). Also, LPS-activated DPSCs release pro-inflammatory cytokines including IL-6, MCP-1, IL-1b, IL-8 and TNF to recruit immune cells at the site of infection (Bindal et al., 2018; He et al., 2013, 2017; Tomic et al., 2011). The way DPSCs support infection and respond to a second infection remain unknown.

Also, pre-conditioning strategies have been proposed to improve DPSCs function and therapeutic potential in regenerative medicine (Ahmed et al., 2016; Gnanasegaran et al., 2016). For example, transplantation of hypoxic pre-conditioned (HP) DPSCs have the capacity to memorise HP-promoted recruitment and osteogenesis ability to boost their therapeutic potential (Ahmed et al., 2016).

However, there is no study relating the immune response of bacteria-pre-conditioned DPSCs. Therefore, we investigated whether dental pulp stem cells exhibit an immunological memory.

Materials and methods

DPSCs culture.

DPSCs obtained from cell lines service (CLS, Eppelheim, Germany) were cultured in Dulbecco's Modified Eagle Medium F-12 (DMEM/F12, Invitrogen, Villebon-sur-Yvette, France) supplemented with 5% heat-inactivated fetal calf serum (FBS, qualified, EU-approved, South America origin, Gibco, Paisley, UK) at 37°C under a 5% CO_2 atmosphere. DPSCs viability was determined by using the Trypan blue exclusion assay. This assay is distinctively differentiates non-viable from viable cells based on the analysis of the integrity of the cell membrane(Samyuktha et al., 2014). Briefly, trypsinized DPSCs were suspended into 20 µL of basic medium and mixed with 20 µL of a 0.4% solution of Trypan blue dye (Eurobio, Les Ulis, France) for 1 min at room temperature. Cells were immediately counted using a Neubauer microchamber (Brand GmbH, Wertheim, Germany) with a light microscope using a 100 X magnification. All experiments were done in triplicate to assure reproducibility.

DPSCs Morphology.

DPSCs were seeded onto glass coverslips at a density of $5x10^4$ cells/mL into 24-well plates (Greiner Bio-One, United Kingdom) until they achieve a 80% confluence. Cells were then stimulated with *Escherichia coli* LPS (100 ng/µL) or with *Staphylococcus aureus* PGN (100 ng/µL) (Sigma, St Louis, MO, USA) for 24 hours at 37°C. Then, DPSCs were washed with phosphate buffered saline (PBS) (Life Technologies Ldt., Paisley, UK) to remove the bacterial ligand (LPS or PGN) and fixed with 4% paraformaldehyde for 15 minutes.
Actin filaments were red-stained with phalloidin-fluor 555 (Invitrogen, Eugene, Oregon, USA) and the nucleus was stained in blue using 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen). Finally, glass coverslips were mounted on object slide with Mowiol (Calbiochem, San Diego, USA). Imaging and evaluation of the number of internalized bacteria per cell was

performed on a 63X oil objective using a Zeiss LSM 800 confocal inverted microscopy system.

Induction of trained immunity

DPSCs ($5*10^4$ cells/mL) were cultured in a volume of 500 µL/well in 12-well plates and divided into two groups (LPS or PGN-primed DPSCs and non-primed DPSCs). For the primed group DPSCs, DPSCs were stimulated with the bacterial ligand for 24 hours (h) and then placed with fresh complete culture medium for 30 days before the second challenge. After the second challenge, DPSCs were collected for 30min, 1 h, 2 h, 4 h, 6 h, 8 h and 24 hours to quantify gene expression. In order to measure the production of the cytokines, the supernatants were collected 24 hours after the second challenge. To induce the inhibition of methylation, DPSCs were incubated for 1 hour with the methyltransferase inhibitor 5′-deoxy-5′-(methylthio) adenosine (MTA, 1mM). DPSCs were pre-treated in parallel with the same volume of DMSO were used as negative controls.

Gene expression analysis

Total RNA was extracted from Primed or No-Primed DPSC and DPSC non-exposed to bacterial ligand (as negative control cells) using Trizol reagent according to the instructions of the manufacturer (Invitrogen) and quantified using a spectrophotometer at 260 nm (Nanodrop 1000 spectrophotometer, Thermo Scientific, Cambridge Scientific, Watertown, MA). All the samples were then normalized at 100 ng RNA before reverse-transcription to the first strand of cDNA by using oligo (dT) and Moloney murine leukemia virus–reverse transcriptase M-MLV Reverse Transcriptase, according to the manufacturer's instructions (Invitrogen). Gene expressions were evaluating using an ABI7900 Fast Real-Time PCR System, the SYBR Green Fast Master Mix (Roche Applied Science, Meylan, France) and primers listed in **Supplementary Table 1**. The data were obtained by calculating the fold change = $2^{-\Delta\Delta Ct}$,

corresponding to $\Delta\Delta Ct = (Ct_{Target} - Ct_{AGAPDH})_{assay} - (Ct_{Target} - Ct_{GAPDH})_{control.}$ Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to normalize the relative expression of each gene under investigation.

Flow cytometry analysis

DPSCs were investigated using flow cytometry. Briefly, DPSCs were cultured to 80% confluence and primed with LPS (100 ng/µL) or PGN (100 ng/µL). Thereafter, DPSCs were washed two times in ice-cold PBS supplemented with 2% FBS and 2 mM EDTA. A total of 10^6 DPSCs were incubated with antibodies against positive and negative mesenchymal stem cell markers listed in **Supplementary Table 2** in accordance with the manufacturer's protocol.

Cytokine quantification

Un-primed and primed DPSCs were treated with LPS or PGN for 24 hours. Then, supernatants were collected to evaluate the concentration of MCP-1 and IL-6 by using ELISA kits according to the manufacturer's protocols (R&D Systems, Lille, France). Concentrations were calculated using standard curves.

Statistical analysis

Data were analyzed using GraphPad Prism 5.0 c software using the unpaired *t*-test. $P < 0.05$ was considered as statistically significant.

Results

Viability of DPSCs primed with LPS or PGN.

First, we determined if the cells viability is affected by incubation with LPS or PGN. The percentage of viable DPSCs primed with LPS and PGN compare to the control re reported in **Figure 1A**. LPS (100 ng/µL) or PGN (100 ng/µL) did not induce a significant change in the viability compared to control DPSCs: the percentage of viability varied from 100% at T0 to 96% at T120 ($P < 0.05$). In addition, confocal microscopy observation of DPSCs at 80% confluence primed with LPS (100 ng/ µL) or PGN (100 ng/ µL) for 3 days, and then stained for actin and nucleus, do not revealed disorder in the distribution of actin; and as well modification of nuclei (**Figure 1B)** compare to the control.

Immunophenotype of DPSC primed with LPS or PGN.

Mesenchymal immunophenotype analysis of DPSCs revealed that priming with LPS or PGN did not affect the MSC markers. Indeed, un-primed mock DPSCs, LPS- or PGN-primed DPSCs were positive for the mesenchymal stem cells markers CD29, CD44, CD 90 and CD 105 and negative for CD117 and hematopoietic stem cell markers CD14, CD34 and CD 45 (**Figure 1C).** These results suggest that priming does not induced differentiation of DPSCs.

LPS and PGN induce inflammatory memory in DPSCs.

LPS-stimulated DPSCs and PGN-stimulated DPSCs exhibit a significant increase expression of IL-6 and MCP-1 mRNA compared to negative control cells. Then, to determine whether DPSC can keep in memory this priming and respond faster in a second challenge, we performed an assessment by following the next procedure **(Figure 2A).** Interestingly, priming influences the cell response to a secondary stimulation. Indeed, IL-6 and MCP-1 mRNA expression were increased significantly in both LPS and as well PGN-primed DPSC **(Figure**

2B). Next, by ELISA we have analysed the protein level of MCP-1 and IL-6 in DPSC supernatant collected 24 hours after the second stimuli LPS or PGN. Similarly to the mRNA expression, the level of MCP-1 and IL-6 production were significantly increased (**Figure 2C).**

Involvement of histone methylation for PGN training in DPSCs.

Epigenetic modification of DNA-associated histone proteins known to be required for the establishment of trained immunity and thus to improve the response to a second stimuli (Netea et al., 2016). Indeed it has been shown that activation of histone methyltransferase promote the expression of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and Il-6 (Garcia-Valtanen et al., 2017). We questioned the involvement of the methyltransferases in our model. To this purpose, we have treated DPSCs with the methyltransferase inhibitor 5′-deoxy-5′-(methylthio) adenosine MTA (Quintin et al. 2012) for 1 hour, wash-out. Then cells were primed with LPS or PGN during 24 hours. Supernatant were collected 24 hours after re-stimulation with three same bacterial ligands and ELISA assays were performed for MCP-1 and IL-6. In the case of the PGN stimulus, we observed that MTA treatment significantly reduced the production of IL-6 which decreased by 33.8%; significantly reduced the production of MCP-1 by 68.2% compared to negative control cells. These results suggested a role of histone methyl transferase in establishment of the inflammatory memory.

Discussion

Recently, Liu showed that DPSCs exposed by danger signal such as LPS, responded by increasing the expression of pro-inflammatory genes and producing cytokines IL-6, IL-8, MCP-1 and TNF-alpha, in a way similar to that described for the immune cells monocytes/macrophages and T cells (Liu et al., 2016). Here, we show that this inflammatory

response can kept in memory by DPSCs and respond intensely to the same stimuli upon a second stimulation.

Firstly, we unravelled that priming did not affect the viability, morphology and mesenchymal phenotype of DPSCs. Second, we demonstrated that DPSCs primed with LPS or PGN induced a trained immunity as measured by the expression of genes coding for inflammatory factors and cytokine production of IL-6 and MCP-1 30 days after the first stimulation. We have interpreted this phenotype as characteristic of inflammatory memory. The increase of the inflammatory cytokines MCP-1 and IL-6 has been previously observed in LPS-primed adipose tissue-derived mesenchymal stem cells. However in later model, the memory effect lasted for seven days only (Liu et al., 2016) whereas in our study, the memory effect lasted for 30 days. Our study adds new piece of information following John Pereira' suggestion to study trained immuniy in stem cell cells (Pereira et al., 2016).

Histone methyltransferases are involved in the establishment of trained immunity (Rizzetto et al., 2016). Interestingly, it was reported that dental pulp stem cell possesses different types of methyltransferases which are involved in the control of cell phenotype and regulate the renewal and pluripotency of stem cell population (Duncan et al., 2016; Rodas-Junco et al., 2017). Here, we observed that DPSCs previously treated with the histone methyltransferase inhibitor MTA, secreted significantly less cytokines IL-6 and MCP-1 after the second stimulation compared to non-treated, negative control DPSCs. However the pre-treatment of MTA do not inducing a change in the release of IL-6 and MCP-1 for inflammatory memory inducing by LPS.

Il-6 and MCP-1 are two mediators of inflammation synthesized in response to stimuli such as trauma of the dental pulp or tooth decay (Alkharobi et al., 2017). Both cytokines are well known to play an important role in initiating and maintaining the inflammatory responses by stimulating several processes including B-cell differentiation, T-cell activation,

haematopoiesis and increasing vascular permeability (Sabir and Sumidarti, 2017). MCP-1 contributes to the inflammatory response by attracting monocytes and T-cells at the site of infection. It promotes the recruitment of circulating immunocompetent blood cells and their migration through the endothelial barrier to gain access to damaged sites to eliminate pathogens (Martínez-Sarrà et al., 2017). Accordingly, inflammation is regarded as a prerequisite for pulp healing and regeneration (Goldberg et al., 2008).

In response to stimuli, DPSCs are activated and differentiate into odontoblasts to fix dentin. These findings suggest that this inflammatory memory could permit an increasing of wound healing in dental pulp tissue (Martínez-Sarrà et al., 2017). Transplanted DPSCs were shown to induce a complete regeneration in a root canal therapy after pulpectomy in dogs (Iohara et al., 2011). Recently, Misako Nakashima reported the first clinical trial showing that DPSCs were safe and effective in complete pulp regeneration in five patients diagnosed with with irreversible pulpitis (Nakashima et al., 2017). Therefore, pre-conditioning of DPSCs may improve the biological response by enhancing faster production of cytokines and the differentiation of DPSCs facilitating inflammatory response and dentine regeneration.

Conclusion

In this study, we have observed that DPSCs are susceptible to stimulation with LPS and PGN and respond faster after a second infection by producing inflammatory cytokines IL-6 and MCP-1. It is termed memory inflammatory. Our data bring a new immune characteristic of DPSCs. As DPSCs are now used for the complete regeneration of dental pulp after pulpectomy (Nakashima et al., 2017), pre-conditioned DPSCs with bacterial ligands could improved this therapeutic process.

Author contributions

A.H and E.G conceived and designed the experiments. A.H. performed experiments and analyzed the data. A.H, E.G and M.D wrote the paper.

Declaration of interests

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.

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References:

Ahmed, N. E.-M. B., Murakami, M., Kaneko, S., and Nakashima, M. (2016). The effects of hypoxia on the stemness properties of human dental pulp stem cells (DPSCs). *Sci Rep* 6. doi:10.1038/srep35476.

al, T. O., et Activation of human dental pulp progenitor/stem cells in response to odontoblast injury. - PubMed - NCBI. Available at: https://www.ncbi.nlm.nih.gov/pubmed/15721135 [Accessed April 30, 2018].

Alkharobi, H., Beattie, J., Meade, J., Devine, D., and El-Gendy, R. (2017). Dental Pulp Cells Isolated from Teeth with Superficial Caries Retain an Inflammatory Phenotype and Display an Enhanced Matrix Mineralization Potential. *Front Physiol* 8. doi:10.3389/fphys.2017.00244.

Attoumani, H., Drancourt, M., and Ghigo, E. (2018). Immune Properties of Human Dental Pulp Stem Cells and Interactions with the Immune System. 2, 5.

Bindal, P., Ramasamy, T. S., Kasim, N. H. A., Gnanasegaran, N., and Lin, C. W. (2018). Immune responses of human dental pulp stem cells in lipopolysaccharide induced microenvironment. *Cell Biol*. *Int*. doi:10.1002/cbin.10938.

Bronckaers, A., Hilkens, P., Fanton, Y., Struys, T., Gervois, P., Politis, C., et al. (2013). Angiogenic Properties of Human Dental Pulp Stem Cells. *PLoS One* 8. doi:10.1371/journal.pone.0071104.

Duncan, H. F., Smith, A. J., Fleming, G. J. P., and Cooper, P. R. (2016). Epigenetic modulation of dental pulp stem cells: implications for regenerative endodontics. *Int Endod J* 49, 431–446. doi:10.1111/iej.12475.

El-Gendy, R., Kirkham, J., Newby, P. J., Mohanram, Y., Boccaccini, A. R., and Yang, X. B. (2015). Investigating the Vascularization of Tissue-Engineered Bone Constructs Using Dental Pulp Cells and 45S5 Bioglass® Scaffolds. *Tissue Eng Part A* 21, 2034–2043. doi:10.1089/ten.tea.2014.0485.

Fawzy El-Sayed, K. M., Klingebiel, P., and Dörfer, C. E. (2016). Toll-like Receptor Expression Profile of Human Dental Pulp Stem/Progenitor Cells. *J Endod* 42, 413–417. doi:10.1016/j.joen.2015.11.014.

Garcia-Valtanen, P., Guzman-Genuino, R. M., Williams, D. L., Hayball, J. D., and Diener, K. R. (2017). Evaluation of trained immunity by β-1, 3 (d)-glucan on murine monocytes in vitro and duration of response in vivo. *Immunol Cell Biol* 95, 601–610. doi:10.1038/icb.2017.13.

Gnanasegaran, N., Govindasamy, V., Musa, S., and Abu Kasim, N. H. (2016). ReNCell VM conditioned medium enhances the induction of dental pulp stem cells into dopaminergic like cells. *Cytotechnology* 68, 343–353. doi:10.1007/s10616-014-9787-z.

Goldberg, M., Farges, J.-C., Lacerda-Pinheiro, S., Six, N., Jegat, N., Decup, F., et al. (2008). Inflammatory and immunological aspects of dental pulp repair. *Pharmacol Res* 58, 137–147. doi:10.1016/j.phrs.2008.05.013.

Gronthos, S., Brahim, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., et al. (2002). Stem cell properties of human dental pulp stem cells. *J. Dent. Res.* 81, 531–535. doi:10.1177/154405910208100806.

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 97, 13625–13630.

He, W., Qu, T., Yu, Q., Wang, Z., Lv, H., Zhang, J., et al. (2013). LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells. *Int Endod J* 46, 128–136. doi:10.1111/j.1365-2591.2012.02096.x.

He, X., Jiang, W., Luo, Z., Qu, T., Wang, Z., Liu, N., et al. (2017). IFN-γ regulates human dental pulp stem cells behavior via NF-κB and MAPK signaling. *Sci Rep* 7. doi:10.1038/srep40681.

Iohara, K., Imabayashi, K., Ishizaka, R., Watanabe, A., Nabekura, J., Ito, M., et al. (2011). Complete Pulp Regeneration After Pulpectomy by Transplantation of CD105 Stem Cells with Stromal Cell-Derived Factor-1. *Tissue Engineering Part A* 17, 1911–1920. doi:10.1089/ten.tea.2010.0615.

Li, D., Fu, L., Zhang, Y., Yu, Q., Ma, F., Wang, Z., et al. (2014). The effects of LPS on adhesion and migration of human dental pulp stem cells in vitro. *J Dent* 42, 1327–1334. doi:10.1016/j.jdent.2014.07.007.

Liu, G.-Y., Liu, Y., Lu, Y., Qin, Y.-R., Di, G.-H., Lei, Y.-H., et al. (2016). Short-term memory of danger signals or environmental stimuli in mesenchymal stem cells: implications for therapeutic potential. *Cell Mol Immunol* 13, 369–378. doi:10.1038/cmi.2015.11.

Martínez-Sarrà, E., Montori, S., Gil-Recio, C., Núñez-Toldrà, R., Costamagna, D., Rotini, A., et al. (2017). Human dental pulp pluripotent-like stem cells promote wound healing and muscle regeneration. *Stem Cell Res Ther* 8. doi:10.1186/s13287-017-0621-3.

Nakashima, M., Iohara, K., Murakami, M., Nakamura, H., Sato, Y., Ariji, Y., et al. (2017). Pulp regeneration by transplantation of dental pulp stem cells in pulpitis: a pilot clinical study. *Stem Cell Research & Therapy* 8, 61. doi:10.1186/s13287-017-0506-5.

Netea, M. G., Joosten, L. A. B., Latz, E., Mills, K. H. G., Natoli, G., Stunnenberg, H. G., et al. (2016). Trained immunity: a program of innate immune memory in health and disease. *Science* 352, aaf1098. doi:10.1126/science.aaf1098.

Pereira, J. M., Hamon, M. A., and Cossart, P. (2016). A Lasting Impression: Epigenetic Memory of Bacterial Infections? *Cell Host Microbe* 19, 579–582. doi:10.1016/j.chom.2016.04.012.

Rizzetto, L., Ifrim, D. C., Moretti, S., Tocci, N., Cheng, S.-C., Quintin, J., et al. (2016). Fungal Chitin Induces Trained Immunity in Human Monocytes during Cross-talk of the Host with Saccharomyces cerevisiae. *J. Biol. Chem.* 291, 7961–7972. doi:10.1074/jbc.M115.699645.

Rodas-Junco, B. A., Canul-Chan, M., Rojas-Herrera, R. A., De-la-Peña, C., and Nic-Can, G. I. (2017). Stem Cells from Dental Pulp: What Epigenetics Can Do with Your Tooth. *Front Physiol* 8. doi:10.3389/fphys.2017.00999.

Sabir, A., and Sumidarti, A. (2017). Interleukin-6 expression on inflamed rat dental pulp tissue after capped with Trigona sp. propolis from south Sulawesi, Indonesia. *Saudi J Biol Sci* 24, 1034–1037. doi:10.1016/j.sjbs.2016.12.019.

Samyuktha, V., Ravikumar, P., Nagesh, B., Ranganathan, K., Jayaprakash, T., and Sayesh, V. (2014). Cytotoxicity evaluation of root repair materials in human-cultured periodontal ligament fibroblasts. *J Conserv Dent* 17, 467–470. doi:10.4103/0972-0707.139844.

Tatullo, M., Marrelli, M., Shakesheff, K. M., and White, L. J. (2015). Dental pulp stem cells: function, isolation and applications in regenerative medicine. *J Tissue Eng Regen Med* 9, 1205–1216. doi:10.1002/term.1899.

Tomic, S., Djokic, J., Vasilijic, S., Vucevic, D., Todorovic, V., Supic, G., et al. (2011). Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. *Stem Cells Dev*. 20, 695–708. doi:10.1089/scd.2010.0145.

Widbiller, M., Eidt, A., Wölflick, M., Lindner, S. R., Schweikl, H., Hiller, K.-A., et al. (2018). Interactive effects of LPS and dentine matrix proteins on human dental pulp stem cells. *Int Endod J* 51, 877–888. doi:10.1111/iej.12897.

Figure 1. Functional characteristic of DPSC at different stimulation.

Stimulation of DPSCs to bacterial ligand LPS and PGN comparing to the unstimulated cells (as control). **(A) Morphology** : Cells had spindle-shaped morphology (fibroblast like) at all conditions (LPS, PGN and control) **(B) Viabilty** : Upon exposure to LPS or PGN the viability of DPSC were similar for the three conditions **(C) Mesenchymal stem cell immunophenotype of DPSC**. Expression of negative and positif MSC markers of DPSC stimulated with LPS or PGN during 24 hours. Data were obtained from three independently performed experiments with mean ± SEM.

Figure 2. Gene expression using PCR and cytokine production using ELISA analysis for IL-6 and MCP-1. (A). Experimental procedure for the exploration of trained immunity in hDPSC **(B).** The level of mRNA expression in relative fold change was evaluated for IL-6 and MCP-1 in DPSC primed and no-primed for different time 30 days after the first stimulation. These genes were normalized by using the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **(C)** Quantification of cytokine production by using ELISA. Data were obtained from three independently performed experiments with mean ± SEM.

Figure 3. Methyl transferase involve in the establishment of inflammatory memory

DPSC were treated with MTA (1 mM) or untreated (control), primed (primo-infection) with LPS or PGN and then re-stimauled with same stimulis 30 days after. Supernatant were recolted 24 hours afters the second stimulis and cytokine quantification were performed by using ELISA. Data were obtained from three independently performed experiments with mean ± SEM.

Supplementary Table 1: Sequence of primers used for q-RTPCR analyses.

The gene symbol and sequences are listed for the selected genes.

Gene symbol	**Forward**	**Reverse**	**References**
GAPDH	CATCATCCCTGCCTCTACTG	GCCTGCTTCACCACCTTC	(He et al., 2013)
IL-6	CTAGAGTACCTCCAGAACAG	TGACCAGAAGAAGGAATGC	(He et al., 2013)
MCP-1	AAGCAGAAGTGGGTTCAGGA	GCAATTTCCCCAAGTGTCTG	(Bronckaers et al., 2013)

Supplementary Table 2: Antibodies used for flow cytometry analysis.

Antibodies	**Conjugate**	**Clone**	**Manufacturer**
Positive mesenchymal stem cell markers			
CD 29	FITC	HMβ1-1	Biolegend
CD90	AF-647	5E10	Biolegend
CD105	PE	43A3	Biolegend
CD 44	FITC	J.173	Beckman Coulter
Negative mesenchymal stem cell markers			
CD 34	FITC	581	Beckman Coulter
CD 45	ECD	J33	Beckman Coulter
CD 117	PE	104D2D1	Beckman Coulter
CD 14	APC	RMO52	Beckman Coulter

Figure 1A.

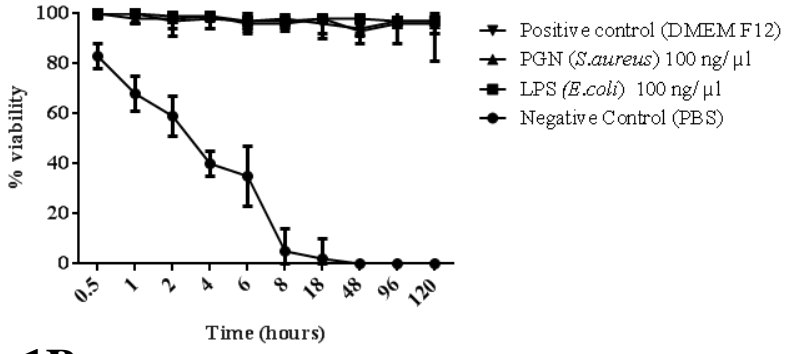


Figure 1B.

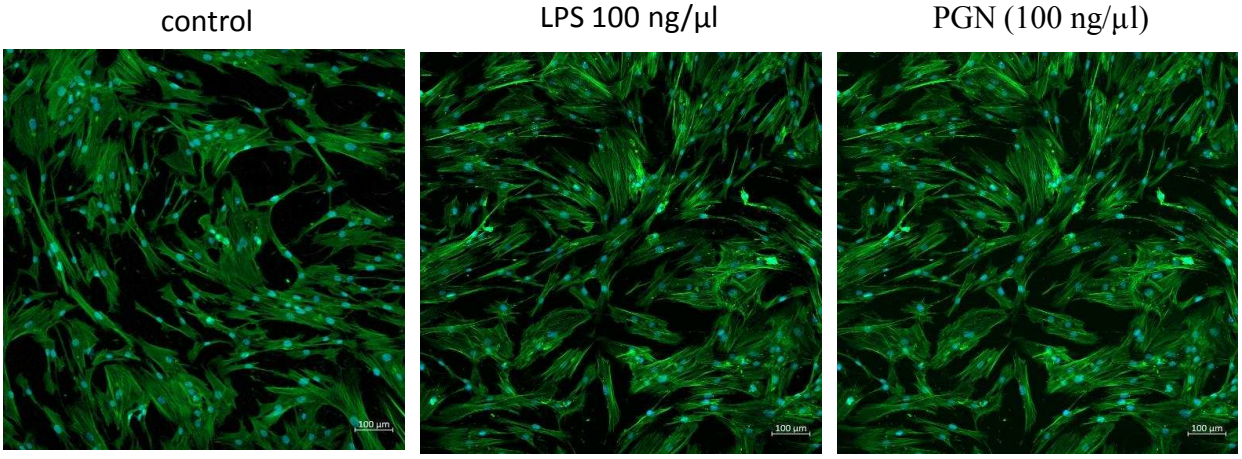


Figure 1C.

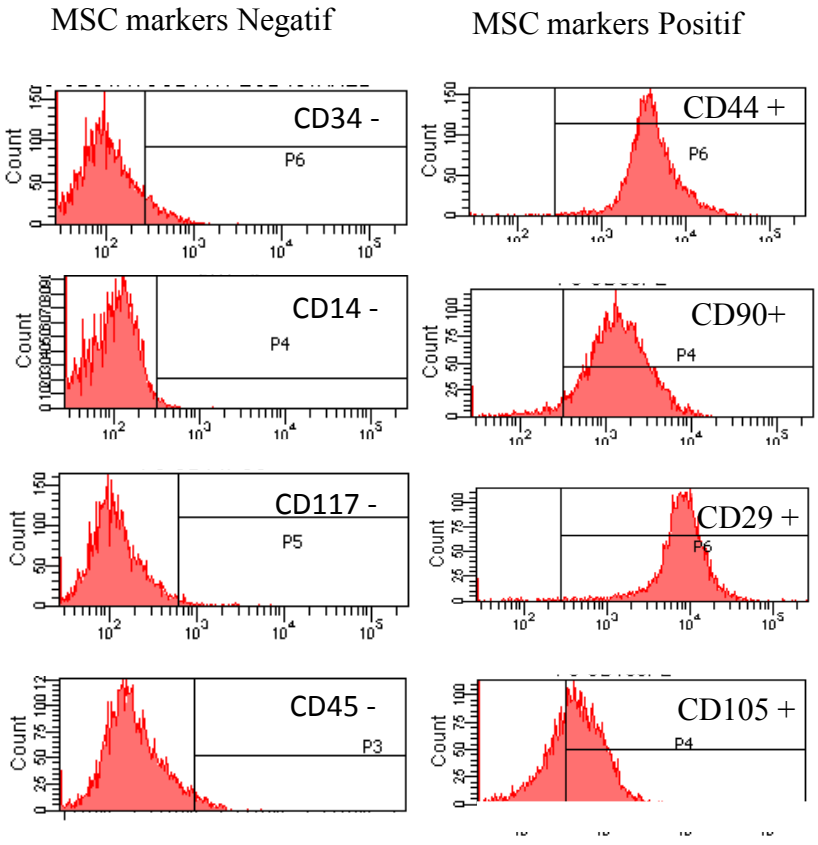


Figure 2A.

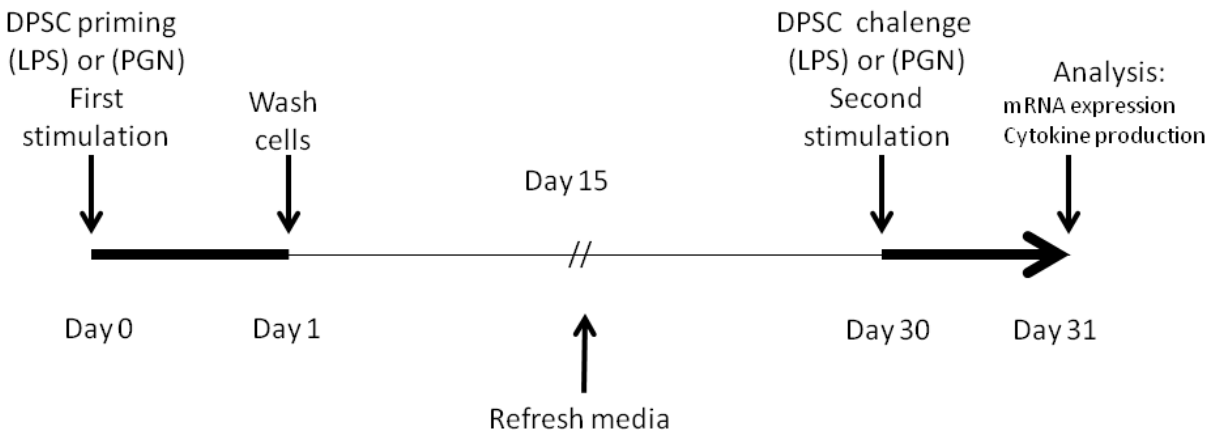


Figure 2B.

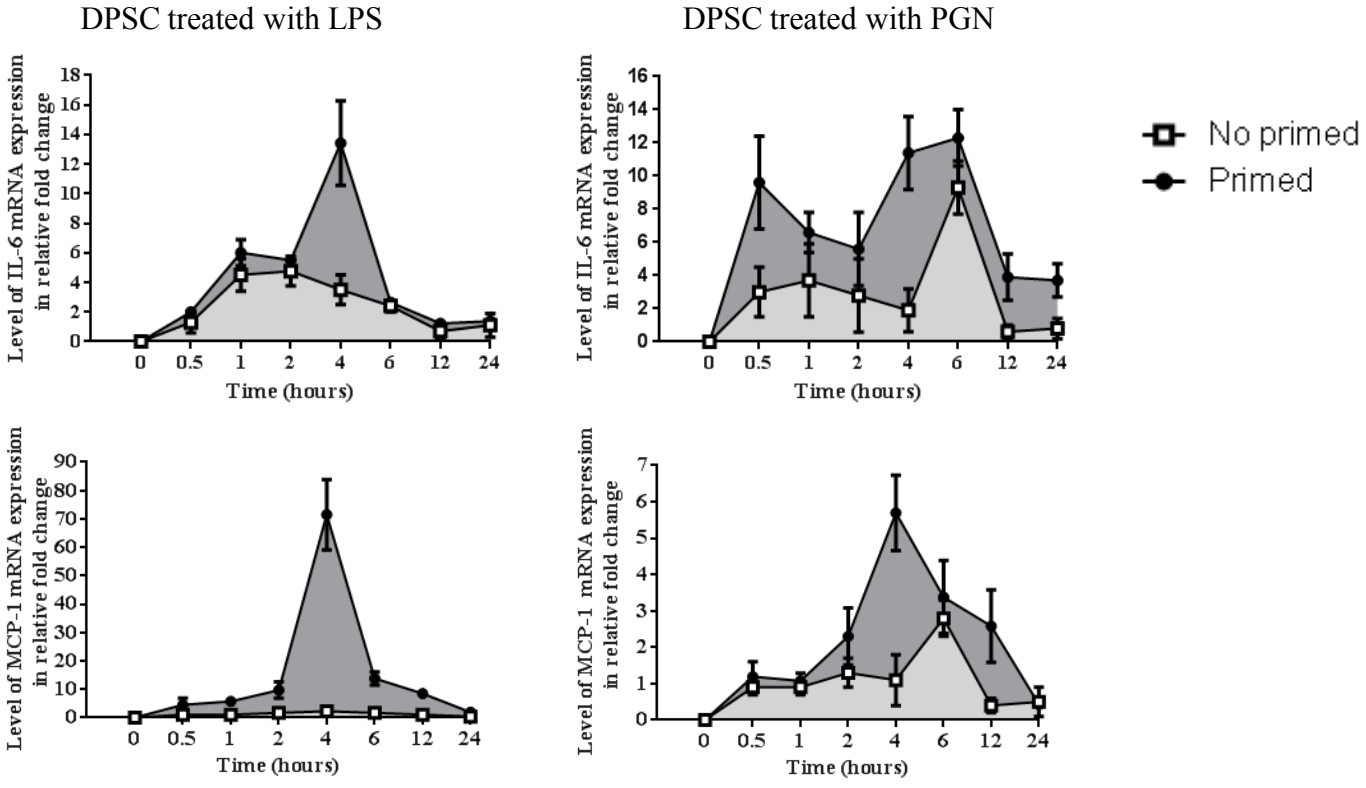
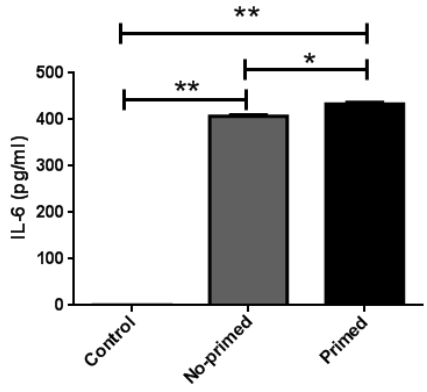
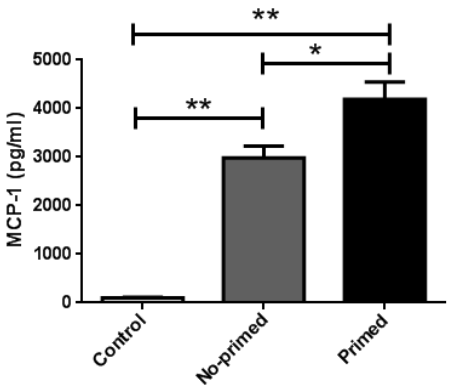


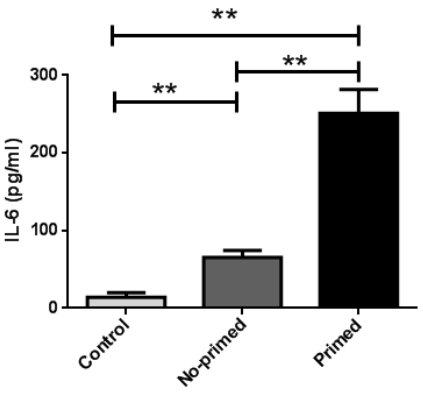
Figure 2C.

DPSC treated with LPS





DPSC treated with PGN



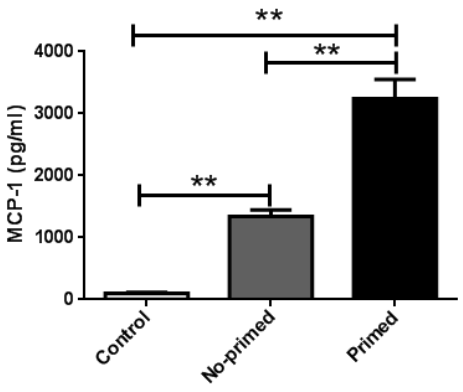
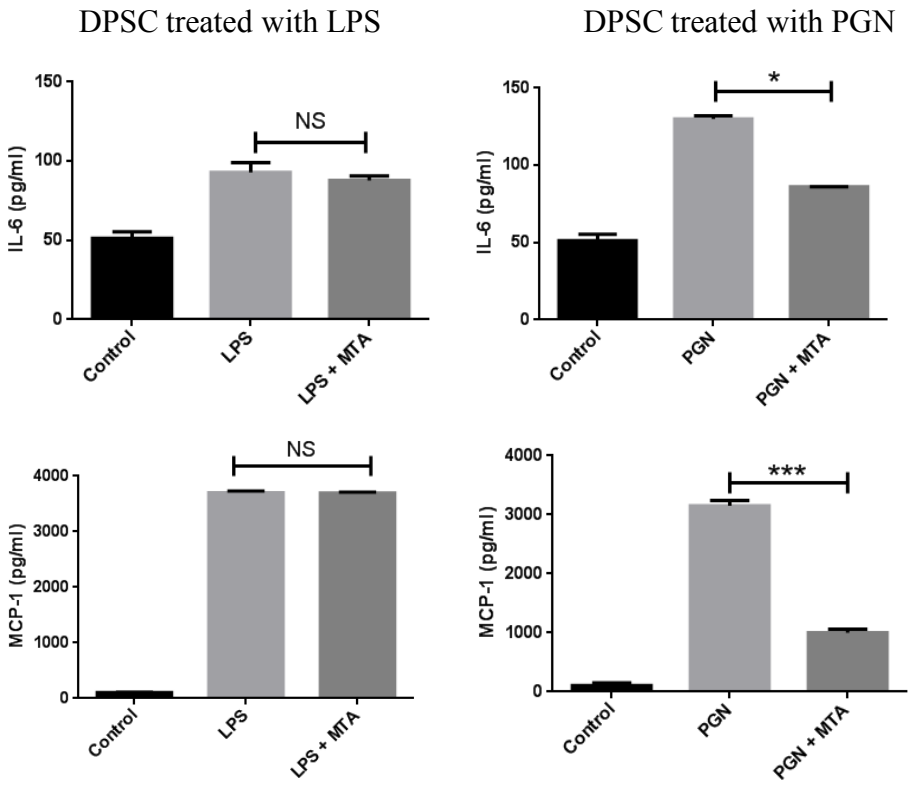


Figure 3.


DPSC treated with LPS
DPSC treated with PGN
IL-6 (pg/ml)
MCP-1 (pg/ml)
NS
*
***
Control
LPS
LPS + MTA
PGN
PGN + MTA
0
50
100
150
1000
2000
3000
4000

Discussion/ Perpspectives

Discussion :

En 2000, Gronthos et ces collaborateurs ont isolé pour la première fois les hDPSCs dans la pulpe dentaire. Depuis ce premier travail, les DPSCs ont fait l'objet d'une attention particulière dans le cadre de la médecine régénérative grâce à leur grande plasticité et leur capacité à se différencier en plusieurs types cellulaires.

En 2002, Gronthos a publié la première étude concernant l'utilisation thérapeutique des DPSCs chez des souris immunodéprimées. Cette transplantation *in vivo* a démontré la capacité des DPSCs à générer un tissu dentaire fonctionnel sous la forme de complexe dentine/pulpe (Gronthos 2002). Récemment, une autre étude vient conforter les données de Gronthos, montrant le potentiel thérapeutique des hDPSCs pour une régénération complète de la pulpe chez des patients atteints de pulpite irréversible (Nakashima et al., 2017). Par la suite d'autres études ont montré le potentiel thérapeutique de ces cellules dans d'autres tissus que les tissus dentaires ; tel que la régénération de tissu osseux (Leyendecker Junior et al., 2018), du tissu neuronal (Chalisserry et al., 2017; Luo et al., 2018) et du tissu œsophagien (Zhang et al., 2018).

Depuis une dizaine d'années, les caractéristiques immunitaires des hDPSCs sont de plus en plus décryptées. En 2002, Fawzy a mis en évidence pour la première fois, la présence de PRRs sur des hDPSC, plus particulièrement les récepteurs de type Toll. Les hDPSCs expriment TLR 1-10 (Fawzy El-Sayed et al., 2016b). La plupart de ces récepteurs répondent à des signaux de danger suggérant que la biologie des hDPSCs peut être influencée par les bactéries et ligands bactériens tels que le LPS. En effet, il a été montré que le ligand bactérien LPS a un impact sur la prolifération, la différentiation, et la migration des hDPSCs.

Le LPS est un composant majeur de la paroi cellulaire des bactéries Gram-négatif qui jouent un rôle important dans les maladies infectieuses telles que la parodontite et la pulpite (Huang, 2011; Rutherford and Gu, 2000). Le LPS peut induire l'expression de cytokines pro-inflammatoires, telles que l'interleukine-6 (IL-6) et l'IL-8 et le tumor necrosing factor (TNF-a) via l'activation de TLR-4 dans les cellules souches de la pulpe dentaire (Bindal et al., 2018; Chang et al., 2005; He et al., 2013, 2015).

Les hDPSCs peuvent également produire des facteurs immunomodulateurs et anti-inflammatoires tels que l'IL-10, hepatocyte growth factor (HGF), transforming growth factor-*β* (TGF-*β*) indoleamine 2,3-dioxygenase (IDO) et la prostaglandine E2 (Luo et al., 2018). Ainsi les hDPSCs exercent leurs effets biologiques en favorisant la production de divers médiateurs inflammatoires (pro- et anti-inflammatoires) suggérant leur utilisation en thérapie cellulaire. Cette activité immuno-modulatrice a été rapidement utilisée dans un contexte thérapeutique.

Les premières études relatant les caractéristiques immunitaires des hDPSCs reposent principalement sur leur activité immuno-modulatrice, en effet il a été montré que les hDPSCs induisaient une suppression de la prolifération de peripheral blood mononuclear cells (PBMC) et inhibaient la fonction des lymphocytes T par conséquent induisent une tolérance. Ainsi les hDPSCs peuvent agir comme des agents immunosuppresseurs en modulant la réponse immunitaire dans les maladies inflammatoires ou auto-immunes et permettraient également d'augmenter l'immunotolérance au cours de transplantations hétérologues (Haiqi et al., 2011; Hong et al., 2017, 2017; Tomic et al., 2011).

La connaissance de la technologie des hDPSCs augmente rapidement dans le domaine dentaire et autres spécialités telle que l'immunité. Cependant dans le domaine immunitaire,

la connaissance des différentes voies est au stade expérimental et nécessite une meilleure connaissance de la biologie de ces cellules face à un agent infectieux. C'est dans cette optique que nous avons décidé d'investiguer les propriétés immunitaires des cellules souches de la pulpe dentaire.

Ce travail a permis de mettre en évidence que les cellules souches de la pulpe dentaire avaient la capacité d'internaliser un agent infectieux tel que *Bartonella quintana* et d'initier une réponse immunitaire protectrice. Nous avons également montré que les hDPSCs possèdent une mémoire entrainée leur permettant d'agir de manière efficace lors d'un second contact avec l'agent infectieux.

En premier, nous avons montré que les hDPSCs avaient la capacité d'internaliser l'agent pathogène *Bartonella quintana*. Après 30 minutes de contact, nous avons retrouvé les bactéries à l'intérieur des cellules hDPSCs et de façon croissante au cours des 24 heures de nos observations. Cette étude soutient le concept émergent selon lequel les cellules souches mésenchymateuses sont des cellules non-immunitaires qui possèdent des caractéristiques de cellules immunitaires professionnels (Owens, 2015; Owens et al., 2013; Owens and Simmons, 2013).

Une étude récente a mis en évidence que les cellules souches mésenchymateuses avaient la capacité d'internaliser l'agent infectieux *Mycobacterium tuberculosis* et de contrôler sa réplication par autophagie (Khan et al., 2017). Dans notre étude, nous avons également observé que cette internalisation est associée à une activité anti-bactérienne. Une analyse d'expression génique montre que les hDPSCs infectées par *Bartonella quintana* expriment le peptide antimicrobien beta-defensine 2 BD2. BD2 est un agent microbicide à large spectre qui tue les microorganismes en formant des micropores de type canal qui perturbent

l'intégrité de la membrane et induisent une fuite du contenu cellulaire (Farges et al., 2015; Pazgier et al., 2006).

Les hDPSCs peuvent agir de manière indirecte via une activité immunomodulatrice ; nous avons noté un profil pro-inflammatoire avec une expression des gènes tel que l'IL-6, IL-8, MCP-1 et TNF-alpha connus pour activer et attirer des cellules immunitaires dans le site infectieux afin de contrôler l'infection. Une observation semblable a était faite dans des cellules endothéliales infectées par une autre espèce appartenant au genre *Bartonella*, *Bartonella henselae*. En effet ces dernières, une fois infectés induisent expriment le gène MCP-1 permettant d'attirer les monocytes/macrophages afin de contrôler l'infection (McCord *et al.*, 2005).

Cela suggère que l'effet combiné de ces deux mécanismes (internalisation direct et expression de médiateurs inflammatoires) contribue dans une moindre mesure au contrôle précoce de l'infection à *Bartonella quintana*. Des études récentes montrent que si les cellules non-immunitaires n'agissent pas directement contre l'agent pathogène, elles peuvent utiliser d'autres mécanismes permettant d'initier un processus d'élimination via un signal « eat-me ». Le signal « eat -me » est un processus purement biologique permettant aux phagocytes (macrophages) d'internaliser et d'éliminer des cellules apoptotiques afin d'assurer l'homéostasie mais également pour éliminer des cellules infectées (Wei li 2012).

En perspective, il serait intéressant d'étudier si les hDPSCs produisent des signaux « eat-me » afin d'initier leur phagocytose par des macrophages et autres phagocytes professionnels. Egalement, il serait intéressant d'étudier d'autres agents pathogènes et d'investiguer les éventuels mécanismes de défenses déployés par les hDPSCs pour le contrôle de l'infection bactérienne, tel que l'autophagie.

Nous avons observé que l'agent pathogéne *Bartonella quintana* induit des changements structurels à l'intérieur des cellules souches de la pulpe dentaire, une production d'IL-10 et une réplication au sein de la cellule suggérant que hDPSCs pourraient être des sanctuaires pour les infections chroniques de *Bartonella quintana*.

Ces travaux nous ont permis également de mettre en évidence une mémoire immunologique des cellules souches de la pulpe dentaire en réponse aux ligands bactériens LPS et PGN. Il s'agit d'une mémoire basée sur les caractéristiques de médiateurs inflammatoires des DPSC concernant deux cytokines IL-6 et MCP-1 connu pour jouer un rôle très important dans la lutte contre les pathogènes (Barkhordar et al., 1999; Turner et al., 2014). Cela vient conforter la suggestion récente de Pereira et collaborateurs. Cette suggestion était issue du constat selon lequel, la mémoire immunologique a été mise en évidence pour des cellules de l'immunité innée macrophages et naturel killer, de durée de vie très courte par rapport au concept de « trained immunity ». De ce fait il a émis l'hypothèse que la mémoire entrainée est portée par des cellules ayant une durée de vie longue comme les cellules souches (Pereira et al., 2016).

Bien que n'étant pas classées comme des cellules immunitaires, de nombreux travaux décrits précédemment démontrent les capacités immunitaires des cellules souches de la pulpe dentaire. Sur la base de ses propriétés immunomodulatrices, telle que la production de molécules pro-inflammatoires, nous avons montré que les cellules souches de la pulpe dentaire exposées aux ligands bactériens LPS et PGN induisaient une réponse améliorée lors d'un second contact via une production exacerbée de molécules pro-inflammatoires, ici IL-6 et MCP-1. Cette mémoire immunologique a été mise en évidence pour une durée de 30 jours.

Par ailleurs, un mécanisme épigénétique a été mis en évidence pour expliquer cette mémoire immunologique. Lorsqu'on pré-traite les cellules souches avec une histone methyltransferase inhibitor (5'-deoxy-5'-methylthioadenosine (MTA) avant le priming, on observe une perte de la mémoire immunologique pour les cellules stimulées par du PGN et non pour celles stimulées par du LPS, suggérant que la mémoire inflammatoire est régie par un mécanisme épigénétique plus précisément une activité histone méthyl transferesae sur un gène spécifique. De manière intéressante, il a été déjà montré dans notre équipe, que les cellules souches de la planaire, équivalent des cellules mésenchymateuse des vertébrés étaient dotées d'une mémoire immunologique qui était spécifique à *Staphylococcus aureus*. Le gene peptidoglycan recognition receptor 2 (PGRP 2) était impliqué dans cette mémoire. Cette mémoire inflammatoire des cellules souches de la pulpe dentaire aux ligands bactériens LPS et PGN renforce le rôle et l'importance dans la réponse immunitaire des hDPSC dans un contexte infectieux.

Ce type de mémoire vient d'être mis en évidence dans un autre type de cellules non-immunitaires qui possèdent des caractéristiques de cellules immunitaires, notamment les cellules souches épithéliales. Les auteurs ont montré que les cellules souches épithéliales : epithelial stem cells (EpSCs) possédaient une mémoire inflammatoire. Des EpSCs pré-conditionnées avec l'imiquimod (IMQ) (un inducteur de l'inflammation sur la peau) ont la capacité de mémoriser l'inflammation et d'accélérer le processus de guérison lorsqu'on les transplante sur des souris naïves (Naik et al., 2017). Cette observation donne des idées intéressantes quant à l'utilisation des cellules souches pré-conditionnées pour l'amélioration de thérapie cellulaire de la même façon. En effet, il a été montré que le pré-conditionnement hypoxique des cellules souches de la pulpe dentaire peut améliorer la réparation biologique dans les défauts osseux d'origine infectieuse (Ahmed et al., 2016). De

ce fait le pré-conditionnement des cellules souches de la pulpe dentaire par du LPS ou PGN pourrait améliorer la puissance thérapeutique des cellules souches greffées dans le domaine dentaire mais également d'autres domaines médicaux concernant l'ostéogénèse, la chondrogenèse et le renouvellement neuronal.

Perspectives :

Notre travail de thèse a apporté des observations intéressantes, contribuant à de nouvelles connaissances concernant la biologie et les propriétés immunitaires des cellules souches de la pulpe dentaire en interaction avec un pathogène.

L'étude portant sur l'interaction entre les cellules souches de la pulpe dentaire et l'agent *Bartonella quintana*, nous a permis de montrer pour la première fois une capacité phagocytaire des DPSCs *in vitro*. Suite à cela nous avons voulu investiguer si les cellules souches de la pulpe dentaire étaient également capables d'internaliser cette bactérie *ex vivo*. Pour cela nous avons amélioré un modèle de culture de dent entière conçu par Odile TECLES en rajoutant une circulation du milieu de culture afin de se rapprocher au maximum de la réalité clinique. Nous avons injecté 10^6 bactéries *Bartonella quintana* par mL dans le milieu culture DMEMF12 complémenté avec 5% de sérum de veau fœtal, le tout placé dans un incubateur à 37°c sous 5% de CO_2. Les dents sont ensuite prélevées à différents temps pour réaliser l'isolement de la pulpe dentaire en vue d'une PCR spécifique à *Bartonella quintana*, coupe histologique pour investiguer la co-localisation des DPSCs avec *Bartonella quintana* par immuno-histochimie / immuno-fluorescence et l'isolement des cellules souches de la pulpe dentaire pour observer la présence des bactéries après culture *in vitro*.

Nos résultats préliminaires montrent une détection de l'ADN spécifique de *Bartonella quintana* dans la pulpe dentaire après 9h d'infection.

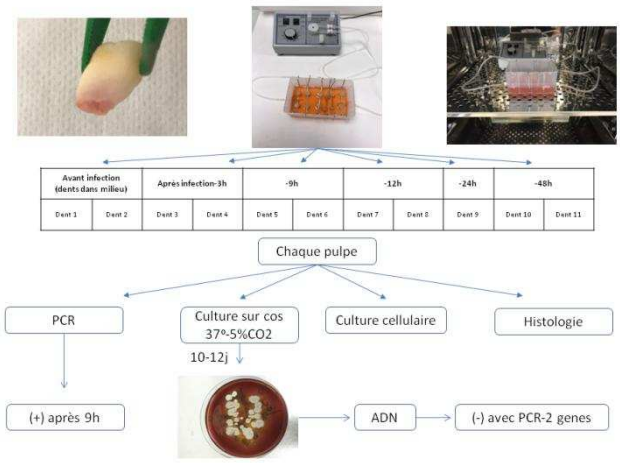


Figure 9 : Modèle de culture de dent entière dans un contexte infectieux *ex vivo*.

La seconde partie de ce travail consistera à enlever le milieu de culture contenant les bactéries et à remettre en culture les dents. Les mêmes expérimentations présentées préalablement seront effectuées. Cela nous permettra de confirmer que les cellules souches internalisent *Bartonella quintana* dans un modèle *in vivo*, de mesurer le temps de persistance de *Bartonella quintana* dans les cellules et de confirmer l'hypothèse que les DPSCs sont un sanctuaire pour les infections à *Bartonella quintana.*

Dans ce même travail on peut étudier l'interaction avec d'autres bactéries provenant de carries dentaires mais également des bactéries impliquées dans les infections chroniques telles que les bactéries *Coxiella burnetii* ou *Mycobacterium tuberculosis ;* le parasite *Plasmodium falciparum* ; et des virus tels que les virus du genre Herpes.

D'un point de vue mécanique, un mécanisme anti-apoptotique peut être étudié comme c'est le cas dans d'autres types de cellules telles que les cellules endothéliales pour *Bartonella*

quintana (Liberto et al., 2004) et monocytes pour l'infection a *Bartonella henselae* (Kempf et al., 2005).

Les cellules souches de la pulpe dentaire se présentent de plus en plus comme les « gold standard » pour les applications thérapeutiques chez les patients. Récemment, une équipe japonaise a montré une première étude portant sur l'essai clinique de transplantation de cellules souches de la pulpe dentaire sur 5 patients atteints de pulpite irréversible. Le résultat est un succès car les cellules ont permis une régénération complète de la pulpe chez l'homme. Suite à la suggestion précédente que les cellules souches de la pulpe dentaire sont des sanctuaires pour certaines bactéries, il serait judicieux de contrôler si les cellules ne comportent pas de microorganismes dans une perspective de sécurité sanitaire pour les receveurs.

Le deuxième volet concerne la mémoire inflammatoire des cellules souches de la pulpe dentaire, cette mémoire inflammatoire se matérialise pour une production accrue des cytokines IL-6 et MCP-1. A court terme il serait intéressant de mesurer la durée de cette mémoire immunologique et d'étudier l'implication de ce phénotype inflammatoire en interaction avec des cellules immunitaires telles que les monocytes. Ce dernier permettra peut être d'améliorer les propriétés fonctionnelles des cellules immunitaires telles que la différentiation des monocytes, l'augmentation de l'activité phagocytaire et l'activation du profil inflammatoire ou anti-inflammatoire des macrophages.

Enfin sur le long terme, on pourrait également étudier l'impact de cette mémoire inflammatoire dans un contexte thérapeutique. Notre hypothèse est que ce pré-conditionnement améliore l'activité thérapeutique des cellules souches de la pulpe dentaire.

En conclusion générale, l'ensemble de notre thèse a montré deux nouvelles propriétés immunitaires des cellules souches de la pulpe dentaire, phagocytose et immunité entrainée. Ceci est entièrement nouveau et ouvre des perspectives de recherches fondamentales et de recherches appliquées dans le domaine de la médecine régénérative.

REFERENCES

Références :

Abnave, P., Mottola, G., Gimenez, G., Boucherit, N., Trouplin, V., Torre, C., et al. (2014). Screening in Planarians Identifies MORN2 as a Key Component in LC3-Associated Phagocytosis and Resistance to Bacterial Infection. *Cell Host & Microbe* 16, 338–350. doi:10.1016/j.chom.2014.08.002.

Aboudharam, G., Fournier, P.-E., Drancourt, M., Raoult, D., Foucault, C., and Brouqui, P. (2004). Molecular detection of Bartonella quintana DNA in the dental pulp of a homeless patient. *Eur. J. Clin. Microbiol. Infect. Dis.* 23, 920–922. doi:10.1007/s10096-004-1244-z.

Ahmed, N. E.-M. B., Murakami, M., Kaneko, S., and Nakashima, M. (2016). The effects of hypoxia on the stemness properties of human dental pulp stem cells (DPSCs). *Sci Rep* 6. doi:10.1038/srep35476.

Arango Duque, G., and Descoteaux, A. (2014). Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. *Front Immunol* 5. doi:10.3389/fimmu.2014.00491.

Arthur, A., Rychkov, G., Shi, S., Koblar, S. A., and Gronthos, S. (2008). Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. *Stem Cells* 26, 1787–1795. doi:10.1634/stemcells.2007-0979.

Barkhordar, R. A., Hayashi, C., and Hussain, M. Z. (1999). Detection of interleukin-6 in human dental pulp and periapical lesions. *Endod Dent Traumatol* 15, 26–27.

Bindal, P., Ramasamy, T. S., Kasim, N. H. A., Gnanasegaran, N., and Lin, C. W. (2018). Immune responses of human dental pulp stem cells in lipopolysaccharide induced microenvironment. *Cell Biol. Int.* doi:10.1002/cbin.10938.

Bistoni, F., Vecchiarelli, A., Cenci, E., Puccetti, P., Marconi, P., and Cassone, A. (1986). Evidence for macrophage-mediated protection against lethal Candida albicans infection. *Infect. Immun.* 51, 668–674.

Brouqui, P., and Raoult, D. (1996). Bartonella quintana invades and multiplies within endothelial cells in vitro and in vivo and forms intracellular blebs. *Research in Microbiology* 147, 719–731. doi:10.1016/S0923-2508(97)85119-4.

Capo, C., Amirayan-Chevillard, N., Brouqui, P., Raoult, D., and Mege, J.-L. (2003). Bartonella quintana Bacteremia and Overproduction of Interleukin-10: Model of Bacterial Persistence in Homeless People. *J Infect Dis* 187, 837–844. doi:10.1086/368384.

Chalisserry, E. P., Nam, S. Y., Park, S. H., and Anil, S. (2017). Therapeutic potential of dental stem cells. *J Tissue Eng* 8. doi:10.1177/2041731417702531.

Chang, J., Zhang, C., Tani-Ishii, N., Shi, S., and Wang, C.-Y. (2005). NF-κB Activation in Human Dental Pulp Stem Cells by TNF and LPS. *J Dent Res* 84, 994–998.

d'Aquino, R., Papaccio, G., Laino, G., and Graziano, A. (2008). Dental Pulp Stem Cells: A Promising Tool for Bone Regeneration. *Stem Cell Reviews* 4, 21–26. doi:10.1007/s12015-008-9013-5.

Drancourt, M., Tran-Hung, L., Courtin, J., Lumley, H. de, and Raoult, D. (2005). Bartonella quintana in a 4000-year-old human tooth. *J. Infect. Dis.* 191, 607–611. doi:10.1086/427041.

Farges, J.-C., Alliot-Licht, B., Renard, E., Ducret, M., Gaudin, A., Smith, A. J., et al. (2015). Dental Pulp Defence and Repair Mechanisms in Dental Caries. *Mediators of Inflammation*. doi:10.1155/2015/230251.

Fawzy El-Sayed, K. M., Klingebiel, P., and Dörfer, C. E. (2016a). Toll-like Receptor Expression Profile of Human Dental Pulp Stem/Progenitor Cells. *J Endod* 42, 413–417. doi:10.1016/j.joen.2015.11.014.

Fawzy El-Sayed, K. M., Klingebiel, P., and Dörfer, C. E. (2016b). Toll-like Receptor Expression Profile of Human Dental Pulp Stem/Progenitor Cells. *J Endod* 42, 413–417. doi:10.1016/j.joen.2015.11.014.

Foucault, C., Brouqui, P., and Raoult, D. (2006). Bartonella quintana Characteristics and Clinical Management. *Emerg Infect Dis* 12, 217–223. doi:10.3201/eid1202.050874.

Galler, K. M., Eidt, A., and Schmalz, G. (2014). Cell-free Approaches for Dental Pulp Tissue Engineering. *Journal of Endodontics* 40, S41–S45. doi:10.1016/j.joen.2014.01.014.

Goldberg, M., Farges, J.-C., Lacerda-Pinheiro, S., Six, N., Jegat, N., Decup, F., et al. (2008). Inflammatory and immunological aspects of dental pulp repair. *Pharmacol Res* 58, 137–147. doi:10.1016/j.phrs.2008.05.013.

González-Estévez, C. (2009). Autophagy meets planarians. *Autophagy* 5, 290–297.

Gordon, S. (2016). Phagocytosis: An Immunobiologic Process. *Immunity* 44, 463–475. doi:10.1016/j.immuni.2016.02.026.

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 97, 13625–13630.

Gunzer, M. (2007). Migration, cell-cell interaction and adhesion in the immune system. *Ernst Schering Found Symp Proc*, 97–137.

Haiqi, H., Yong, Z., and Yi, L. (2011). Transcriptional regulation of Foxp3 in regulatory T cells. *Immunobiology* 216, 678–685. doi:10.1016/j.imbio.2010.11.002.

Hamada, A., Torre, C., Lepolard, C., and Ghigo, E. (2016). Inhibition of LTA4H expression promotes Staphylococcus aureus elimination by planarians. *Matters* 2, e201604000011. doi:10.19185/matters.201604000011.

He, W., Qu, T., Yu, Q., Wang, Z., Lv, H., Zhang, J., et al. (2013). LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells. *Int Endod J* 46, 128–136. doi:10.1111/j.1365-2591.2012.02096.x.

He, W., Wang, Z., Luo, Z., Yu, Q., Jiang, Y., Zhang, Y., et al. (2015). LPS promote the odontoblastic differentiation of human dental pulp stem cells via MAPK signaling pathway. *J. Cell. Physiol.* 230, 554–561. doi:10.1002/jcp.24732.

He, W., Wang, Z., Zhou, Z., Zhang, Y., Zhu, Q., Wei, K., et al. (2014). Lipopolysaccharide Enhances Wnt5a Expression through Toll-like Receptor 4, Myeloid Differentiating Factor 88, Phosphatidylinositol 3-OH Kinase/AKT and Nuclear Factor Kappa B Pathways in Human Dental Pulp Stem Cells. *Journal of Endodontics* 40, 69–75. doi:10.1016/j.joen.2013.09.011.

He, X., Jiang, W., Luo, Z., Qu, T., Wang, Z., Liu, N., et al. (2017). IFN-γ regulates human dental pulp stem cells behavior via NF-κB and MAPK signaling. *Sci Rep* 7. doi:10.1038/srep40681.

Henderson, W. R. (1994). The Role of Leukotrienes in Inflammation. *Annals of Internal Medicine* 121, 684. doi:10.7326/0003-4819-121-9-199411010-00010.

Hong, J. W., Lim, J. H., Chung, C. J., Kang, T. J., Kim, T. Y., Kim, Y. S., et al. (2017). Immune Tolerance of Human Dental Pulp-Derived Mesenchymal Stem Cells Mediated by CD4+CD25+FoxP3+ Regulatory T-Cells and Induced by TGF-β1 and IL-10. *Yonsei Med J* 58, 1031–1039. doi:10.3349/ymj.2017.58.5.1031.

Huang, G. T.-J. (2011). Dental Pulp and Dentin Tissue Engineering and Regeneration – Advancement and Challenge. *Front Biosci (Elite Ed)* 3, 788–800.

Iohara, K., Zheng, L., Ito, M., Tomokiyo, A., Matsushita, K., and Nakashima, M. (2006). Side Population Cells Isolated from Porcine Dental Pulp Tissue with Self-Renewal and Multipotency for Dentinogenesis, Chondrogenesis, Adipogenesis, and Neurogenesis. *STEM CELLS* 24, 2493–2503. doi:10.1634/stemcells.2006-0161.

Keating, S. T., Riksen, N. P., and Netea, M. G. (2017). Planarians SET New Paths for Innate Immune Memory. *EBioMedicine* 20, 7–8. doi:10.1016/j.ebiom.2017.05.007.

Kempf, V. A. J., Schairer, A., Neumann, D., Grassl, G. A., Lauber, K., Lebiedziejewski, M., et al. (2005). Bartonella henselae inhibits apoptosis in Mono Mac 6 cells. *Cell. Microbiol.* 7, 91–104. doi:10.1111/j.1462-5822.2004.00440.x.

Khan, A., Mann, L., Papanna, R., Lyu, M.-A., Singh, C. R., Olson, S., et al. (2017). Mesenchymal stem cells internalize Mycobacterium tuberculosis through scavenger receptors and restrict bacterial growth through autophagy. *Scientific Reports* 7, 15010. doi:10.1038/s41598-017-15290-z.

Krasnodembskaya, A., Song, Y., Fang, X., Gupta, N., Serikov, V., Lee, J.-W., et al. (2010). Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 28, 2229–2238. doi:10.1002/stem.544.

Lacy-Hulbert, A. (2009). "Comparative Characterization of Non-professional and Professional Phagocyte Responses to Apoptotic Cells," in *Phagocytosis of Dying Cells: From Molecular Mechanisms to Human Diseases*, eds. D. V. Krysko and P. Vandenabeele (Dordrecht: Springer Netherlands), 189–215. doi:10.1007/978-1-4020-9293-0_7.

Leyendecker Junior, A., Gomes Pinheiro, C. C., Lazzaretti Fernandes, T., and Franco Bueno, D. (2018). The use of human dental pulp stem cells for in vivo bone tissue engineering: A systematic review. *J Tissue Eng* 9. doi:10.1177/2041731417752766.

Li, D., Fu, L., Zhang, Y., Yu, Q., Ma, F., Wang, Z., et al. (2014). The effects of LPS on adhesion and migration of human dental pulp stem cells in vitro. *J Dent* 42, 1327–1334. doi:10.1016/j.jdent.2014.07.007.

Liberto, M. C., Matera, G., Lamberti, A. G., Barreca, G. S., Focà, D., Quirino, A., et al. (2004). Bartonella quintana-induced apoptosis inhibition of human endothelial cells is associated with p38 and SAPK/JNK modulation and with stimulation of mitosis. *Diagn. Microbiol. Infect. Dis.* 50, 159–166. doi:10.1016/j.diagmicrobio.2004.06.016.

Liu, G.-Y., Liu, Y., Lu, Y., Qin, Y.-R., Di, G.-H., Lei, Y.-H., et al. (2016). Short-term memory of danger signals or environmental stimuli in mesenchymal stem cells: implications for therapeutic potential. *Cell Mol Immunol* 13, 369–378. doi:10.1038/cmi.2015.11.

Liu, L., Ling, J., Wei, X., Wu, L., and Xiao, Y. (2009). Stem Cell Regulatory Gene Expression in Human Adult Dental Pulp and Periodontal Ligament Cells Undergoing Odontogenic/Osteogenic Differentiation. *Journal of Endodontics* 35, 1368–1376. doi:10.1016/j.joen.2009.07.005.

Luo, L., He, Y., Wang, X., Key, B., Lee, B. H., Li, H., et al. (2018). Potential Roles of Dental Pulp Stem Cells in Neural Regeneration and Repair. *Stem Cells International*. doi:10.1155/2018/1731289.

Madhu, B. P., Singh, K. P., Saminathan, M., Singh, R., Shivasharanappa, N., Sharma, A. K., et al. (2016). Role of nitric oxide in the regulation of immune responses during rabies virus infection in mice. *Virusdisease* 27, 387–399. doi:10.1007/s13337-016-0343-7.

Martens, W., Wolfs, E., Struys, T., Politis, C., Bronckaers, A., and Lambrichts, I. (2012). Expression Pattern of Basal Markers in Human Dental Pulp Stem Cells and Tissue. *Cells Tissues Organs* 196, 490–500. doi:10.1159/000338654.

Mbongue, J. C., Nicholas, D. A., Torrez, T. W., Kim, N.-S., Firek, A. F., and Langridge, W. H. R. (2015). The Role of Indoleamine 2, 3-Dioxygenase in Immune Suppression and Autoimmunity. *Vaccines (Basel)* 3, 703–729. doi:10.3390/vaccines3030703.

Monks, J., Rosner, D., Geske, F. J., Lehman, L., Hanson, L., Neville, M. C., et al. (2005). Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell Death Differ.* 12, 107–114. doi:10.1038/sj.cdd.4401517.

Morita, M. (1991). Phagocytic response of planarian reticular cells to heat-killed bacteria. *Hydrobiologia* 227, 193–199. doi:10.1007/BF00027602.

Nagl, M., Kacani, L., Müllauer, B., Lemberger, E.-M., Stoiber, H., Sprinzl, G. M., et al. (2002). Phagocytosis and Killing of Bacteria by Professional Phagocytes and Dendritic Cells. *Clin Diagn Lab Immunol* 9, 1165–1168. doi:10.1128/CDLI.9.6.1165-1168.2002.

Naik, S., Larsen, S. B., Gomez, N. C., Alaverdyan, K., Sendoel, A., Yuan, S., et al. (2017). Inflammatory Memory Sensitizes Skin Epithelial Stem Cells to Tissue Damage. *Nature* 550, 475–480. doi:10.1038/nature24271.

Nakashima, M., Iohara, K., Murakami, M., Nakamura, H., Sato, Y., Ariji, Y., et al. (2017). Pulp regeneration by transplantation of dental pulp stem cells in pulpitis: a pilot clinical study. *Stem Cell Research & Therapy* 8, 61. doi:10.1186/s13287-017-0506-5.

Netea, M. G., Quintin, J., and van der Meer, J. W. M. (2011). Trained Immunity: A Memory for Innate Host Defense. *Cell Host & Microbe* 9, 355–361. doi:10.1016/j.chom.2011.04.006.

Nuti, N., Corallo, C., Chan, B. M. F., Ferrari, M., and Gerami-Naini, B. (2016). Multipotent Differentiation of Human Dental Pulp Stem Cells: a Literature Review. *Stem Cell Rev* 12, 511–523. doi:10.1007/s12015-016-9661-9.

Odelberg, S. J. (2004). Unraveling the Molecular Basis for Regenerative Cellular Plasticity. *PLOS Biology* 2, e232. doi:10.1371/journal.pbio.0020232.

Owens, B. M. J. (2015). Inflammation, Innate Immunity, and the Intestinal Stromal Cell Niche: Opportunities and Challenges. *Front Immunol* 6. doi:10.3389/fimmu.2015.00319.

Owens, B. M. J., and Simmons, A. (2013). Intestinal stromal cells in mucosal immunity and homeostasis. *Mucosal Immunol* 6, 224–234. doi:10.1038/mi.2012.125.

Owens, B. M. J., Steevels, T. A. M., Dudek, M., Walcott, D., Sun, M.-Y., Mayer, A., et al. (2013). CD90+ Stromal Cells are Non-Professional Innate Immune Effectors of the Human Colonic Mucosa. *Front. Immunol.* 4. doi:10.3389/fimmu.2013.00307.

Özdemir, A. T., Özgül Özdemir, R. B., Kırmaz, C., Sarıboyacı, A. E., Ünal Halbutoğlları, Z. S., Özel, C., et al. (2016). The paracrine immunomodulatory interactions between the human dental pulp derived mesenchymal stem cells and CD4 T cell subsets. *Cellular Immunology* 310, 108–115. doi:10.1016/j.cellimm.2016.08.008.

Pazgier, M., Hoover, D. M., Yang, D., Lu, W., and Lubkowski, J. (2006). Human beta-defensins. *Cell. Mol. Life Sci.* 63, 1294–1313. doi:10.1007/s00018-005-5540-2.

Pereira, J. M., Hamon, M. A., and Cossart, P. (2016). A Lasting Impression: Epigenetic Memory of Bacterial Infections? *Cell Host Microbe* 19, 579–582. doi:10.1016/j.chom.2016.04.012.

Potdar, P. D., and Jethmalani, Y. D. (2015). Human dental pulp stem cells: Applications in future regenerative medicine. *World J Stem Cells* 7, 839–851. doi:10.4252/wjsc.v7.i5.839.

Quintin, J., Saeed, S., Martens, J. H. A., Giamarellos-Bourboulis, E. J., Ifrim, D. C., Logie, C., et al. (2012). Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. *Cell Host Microbe* 12, 223–232. doi:10.1016/j.chom.2012.06.006.

Rabinovitch, M. (1995). Professional and non-professional phagocytes: an introduction. *Trends in Cell Biology* 5, 85–87. doi:10.1016/S0962-8924(00)88955-2.

Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R., and Sánchez Alvarado, A. (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev. Cell* 8, 635–649. doi:10.1016/j.devcel.2005.02.014.

Robb, S. M. C., Gotting, K., Ross, E., and Sánchez Alvarado, A. (2015). SmedGD 2.0: The Schmidtea mediterranea genome database. *Genesis* 53, 535–546. doi:10.1002/dvg.22872.

Rolain, J.-M., Foucault, C., Guieu, R., La Scola, B., Brouqui, P., and Raoult, D. (2002). Bartonella quintana in human erythrocytes. *Lancet* 360, 226–228.

Rombouts, C., Jeanneau, C., Bakopoulou, A., and About, I. (2016). Dental Pulp Stem Cell Recruitment Signals within Injured Dental Pulp Tissue. *Dent J (Basel)* 4. doi:10.3390/dj4020008.

Rutherford, R. B., and Gu, K. (2000). Treatment of inflamed ferret dental pulps with recombinant bone morphogenetic protein-7. *Eur. J. Oral Sci.* 108, 202–206.

Sánchez Alvarado, A., Newmark, P. A., Robb, S. M., and Juste, R. (2002). The Schmidtea mediterranea database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development* 129, 5659–5665.

Semple, F., and Dorin, J. R. (2012). β-Defensins: Multifunctional Modulators of Infection, Inflammation and More? *JIN* 4, 337–348. doi:10.1159/000336619.

Serhan, C. N., Dalli, J., Karamnov, S., Choi, A., Park, C.-K., Xu, Z.-Z., et al. (2012). Macrophage proresolving mediator maresin 1 stimulates tissue regeneration and controls pain. *FASEB J.* 26, 1755–1765. doi:10.1096/fj.11-201442.

Shamri, R., Xenakis, J. J., and Spencer, L. A. (2011). Eosinophils in innate immunity: an evolving story. *Cell Tissue Res* 343, 57–83. doi:10.1007/s00441-010-1049-6.

Silva, M. T., and Correia-Neves, M. (2012). Neutrophils and Macrophages: the Main Partners of Phagocyte Cell Systems. *Front Immunol* 3. doi:10.3389/fimmu.2012.00174.

Téclès, O., Laurent, P., Zygouritsas, S., Burger, A.-S., Camps, J., Dejou, J., et al. (2005). Activation of human dental pulp progenitor/stem cells in response to odontoblast injury. *Arch. Oral Biol.* 50, 103–108. doi:10.1016/j.archoralbio.2004.11.009.

Tobin, D. M., Vary, J. C., Ray, J. P., Walsh, G. S., Dunstan, S. J., Bang, N. D., et al. (2010). The lta4h Locus Modulates Susceptibility to Mycobacterial Infection in Zebrafish and Humans. *Cell* 140, 717–730. doi:10.1016/j.cell.2010.02.013.

Tomic, S., Djokic, J., Vasilijic, S., Vucevic, D., Todorovic, V., Supic, G., et al. (2011). Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists. *Stem Cells Dev.* 20, 695–708. doi:10.1089/scd.2010.0145.

Torre, C., Abnave, P., Tsoumtsa, L. L., Mottola, G., Lepolard, C., Trouplin, V., et al. (2017a). Staphylococcus aureus Promotes Smed-PGRP-2/Smed-setd8-1 Methyltransferase Signalling in Planarian Neoblasts to Sensitize Anti-bacterial Gene Responses During Re-infection. *EBioMedicine* 20, 150–160. doi:10.1016/j.ebiom.2017.04.031.

Torre, C., Abnave, P., Tsoumtsa, L. L., Mottola, G., Lepolard, C., Trouplin, V., et al. (2017b). Staphylococcus aureus Promotes Smed-PGRP-2/Smed-setd8-1 Methyltransferase Signalling in Planarian Neoblasts to Sensitize Anti-bacterial Gene Responses During Re-infection. *EBioMedicine* 20, 150–160. doi:10.1016/j.ebiom.2017.04.031.

Tso, G. H. W., Law, H. K. W., Tu, W., Chan, G. C. F., and Lau, Y. L. (2010). Phagocytosis of Apoptotic Cells Modulates Mesenchymal Stem Cells Osteogenic Differentiation to Enhance IL-17 and RANKL Expression on CD4+ T cells. *STEM CELLS*, N/A-N/A. doi:10.1002/stem.406.

Tsoumtsa, L. L., Torre, C., Trouplin, V., Coiffard, B., Gimenez, G., Mege, J.-L., et al. (2017). Antimicrobial capacity of the freshwater planarians against S. aureus is under the control of Timeless. *Virulence* 8, 1160–1169. doi:10.1080/21505594.2016.1276689.

Turner, M. D., Nedjai, B., Hurst, T., and Pennington, D. J. (2014). Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1843, 2563–2582. doi:10.1016/j.bbamcr.2014.05.014.

Varga, I., Hollý, D., Vojtaššák, J., Böhmer, D., Polák, Š., and Danišovič, Ľ. (2011). Morphological characterization of in vitro expanded human dental pulp-derived stem cells. *Biologia* 66. doi:10.2478/s11756-011-0069-3.

Visan, I. (2013). Nonprofessional phagocytes. *Nature Immunology*. doi:10.1038/ni.2531.

Wang, Y.-X., Ma, Z.-F., Huo, N., Tang, L., Han, C., Duan, Y.-Z., et al. (2011). Porcine tooth germ cell conditioned medium can induce odontogenic differentiation of human dental

pulp stem cells. *Journal of Tissue Engineering and Regenerative Medicine* 5, 354–362. doi:10.1002/term.321.

Zhang, C., Zhang, Y., Feng, Z., Zhang, F., Liu, Z., Sun, X., et al. (2018). Therapeutic effect of dental pulp stem cell transplantation on a rat model of radioactivity-induced esophageal injury. *Cell Death & Disease* 9, 738. doi:10.1038/s41419-018-0753-0.

Zhao, Y., Wang, L., Jin, Y., and Shi, S. (2012). Fas Ligand Regulates the Immunomodulatory Properties of Dental Pulp Stem Cells. *J Dent Res* 91, 948–954. doi:10.1177/0022034512458690.