Role of Toll-like receptor 8 in the development of spontaneous autoimmunity in mice

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## A

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>AIM2</td>
<td>absent in melanoma 2</td>
</tr>
<tr>
<td>ANA</td>
<td>antinuclear antibody</td>
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<tr>
<td>AP1</td>
<td>activator protein 1</td>
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## B

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BAFF</td>
<td>B cell-activating factor</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BIR</td>
<td>baculoviral inhibitor-of-apoptosis-protein repeat</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow-derived dendritic cell</td>
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## C

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CD</td>
<td>cluster differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional DC</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate recognition domain</td>
</tr>
<tr>
<td>CRP</td>
<td>C reactive protein</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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## D

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>DAI</td>
<td>DNA-dependent activator of IRFs</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Bar virus</td>
</tr>
<tr>
<td>EMCV</td>
<td>encephalo-myocarditis virus</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FeRγ</td>
<td>Fc-gamma-receptor</td>
</tr>
<tr>
<td>CpG</td>
<td>cytidine-phosphate-guanosine</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HMGB</td>
<td>high mobility group box</td>
</tr>
<tr>
<td>HIV</td>
<td>immunodeficiency virus</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IkB</td>
<td>inhibitor of nuclear factor-κB</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>nitric-oxide synthase</td>
</tr>
<tr>
<td>IPS-1</td>
<td>IFNβ promoter stimulator-1</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
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# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>JNK</td>
<td>JUN N-terminal kinase</td>
</tr>
<tr>
<td>LPDC</td>
<td>DC of the lamina propria</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeat</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88 adaptor like protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated kinases</td>
</tr>
<tr>
<td>MAVS</td>
<td>mitochondria antiviral signaling</td>
</tr>
<tr>
<td>MBL</td>
<td>mannan-binding lectin</td>
</tr>
<tr>
<td>MCMV</td>
<td>mouse cytomegalovirus</td>
</tr>
<tr>
<td>MDA5</td>
<td>melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MD2</td>
<td>myeloid differentiation factor 2</td>
</tr>
<tr>
<td>MDP</td>
<td>muramyl dipeptide</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary-response gene 88</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>NAIP</td>
<td>neuronal apoptosis inhibitory protein</td>
</tr>
<tr>
<td>NALP</td>
<td>NACHT, LRR and Pyd containing protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide oligomerization domain</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Abbreviation</td>
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<tr>
<td>O</td>
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</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>OmpA</td>
<td>outer membrane protein A</td>
</tr>
<tr>
<td>P</td>
<td></td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid DC</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PGN</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>Pol III</td>
<td>polymerase III</td>
</tr>
<tr>
<td>PRAT4A</td>
<td>protein associated with TLR4 A</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PTX3</td>
<td>pentraxin 3</td>
</tr>
<tr>
<td>PYD</td>
<td>pyrin domain</td>
</tr>
<tr>
<td>R</td>
<td></td>
</tr>
<tr>
<td>RAG</td>
<td>recombination-activating gene</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end-products</td>
</tr>
<tr>
<td>REL</td>
<td>reticuloendotheliosis viral oncogene homolog</td>
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<tr>
<td>RIG-I</td>
<td>retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RIP1</td>
<td>receptor interacting protein-1</td>
</tr>
<tr>
<td>RIP2</td>
<td>receptor interacting protein-2</td>
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<tr>
<td>RLR</td>
<td>(RIG-I)-like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
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<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
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<td>S</td>
<td></td>
</tr>
<tr>
<td>SAP</td>
<td>serum amyloid P</td>
</tr>
<tr>
<td>siRNA</td>
<td>silencing RNA</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>Sm</td>
<td>Smith antigen</td>
</tr>
<tr>
<td>SnRNP</td>
<td>small nuclear nucleoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single-stranded RNA</td>
</tr>
<tr>
<td>TAK1</td>
<td>transforming-growth-factor-β-activated kinase</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK binding kinase-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>tGPI</td>
<td>Glycosylphosphatidylinositol-anchored mucin-like glycoproteins</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR associated protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRADD</td>
<td>tumor necrosis factor-receptor 1-associated death domain protein</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain containing</td>
</tr>
<tr>
<td>UNC93B1</td>
<td>unc-93 homolog B1</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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</table>
Les récepteurs de la famille Toll-like (notés TLR pour Toll-like receptors) constituent une famille de protéines transmembranaires permettant aux mammifères de détecter des structures conservées exprimées par différents classes de microorganismes. Ils jouent ainsi un rôle majeur dans le contrôle et l’élimination des infections. Les TLRs exprimés à la membrane plasmique (TLR1, 2, 4, 5, 6, 10) reconnaissent principalement des composés d’origine bactérienne, fongique ou parasitaire, tandis que les TLRs exprimés dans les compartiments intracellulaires (TLR3, 7, 8, 9) détectent des acides nucléiques et sont ainsi spécialisés dans la reconnaissance des virus. L’activation des TLRs engage deux voies de signalisation distinctes utilisant des molécules adaptatrices. La voie dépendante de MyD88 (*Myeloid differentiation primary-response gene 88*) est utilisée par tous les TLRs sauf TLR3 (et TLR4 en partie) qui emprunte la voie dite indépendante de MyD88 qui signale via la molécule adaptatrice TRIF (*TIR-domain-containing adapter-inducing interferon-β*). *In fine* les signalisations TLRs conduisent à l’activation du facteur de transcription NF-κB, de la voie des MAP-Kinases ainsi que de la famille des facteurs régulateur d’interféron. Ces cascades de signaux induisent l’expression d’un éventail de gènes qui constitue la réponse immunitaire innée et commande également la mise en place d’une réponse adaptative.

Les TLRs intracellulaires sont spécialisés dans la détection d’acides nucléiques. Le TLR3 reconnait des ARNs « double brin » viraux et synthétiques comme le polyI:C. Le TLR7 et le TLR8 humain reconnaissent des ARNs « simple brin » viraux ainsi que des composés synthétiques d’imidazoquinoline. Le TLR9 détecte des motifs CpG non méthylés fréquemment retrouvés dans les ADNs bactériens et viraux. Les TLRs intracellulaires, dans
certaines conditions, peuvent aussi être responsable de la reconnaissance d’acides nucléiques endogènes pouvant contribuer au développement d’autoimmunité. Chez la souris, TLR7 et TLR9 peuvent être activés par des complexes immuns formés d’auto-anticorps et de ribonucléoprotéines ou d’ADN, et ainsi influencer le développement d’un lupus systémique érythématexus (SLE, pour *systemic lupus erythematosus*). Dans des modèles murins d’étude de lupus, la délétion du TLR7 joue un rôle protecteur contre le développement de la maladie, alors que paradoxalement l’absence de TLR9 présente l’effet inverse.

Bien que TLR7 et TLR8 soient phyllogénétiquement très proches l’un de l’autre, leur ligand naturel « l’ARN simple brin » stimule les TLR7 et TLR8 humains, le TLR7 murin mais pas le TLR8 murin suggérant que chez la souris le TLR8 est non fonctionnel. Une étude récente a montré que TLR8 est dynamiquement exprimé durant le développement du cerveau chez la souris et qu’il intervient à la fois dans la croissance des neurites et dans l’apoptose des neurones. D’autre part, la stimulation avec une combinaison d’imidazoquinolines et d’un oligonucléotide « polydT » de cellules dans lesquelles le TLR8 murin est exprimé de façon ectopique conduit à l’activation de la voie NF-κB, suggérant que chez la souris TLR8 est fonctionnel.

L’objectif de mon travail de thèse a été d’évaluer le rôle biologique du TLR8 dans des processus immunologiques et son implication dans le développement d’autoimmunité. Pour cela nous avons utilisé une approche basée sur l’analyse de souris défectives pour le TLR8 (TLR8−/−). Nous avons montré qu’en absence de TLR8, les cellules dendritiques (DCs) surexpriment le TLR7 et présentent une réponse accrue à une stimulation par divers ligands de TLR7 ainsi qu’une augmentation de l’activation de la voie NF-κB. *In vivo*, les souris défectives pour le TLR8 présentent une splénomégalie avec une diminution du nombre de lymphocytes B de la zone marginale (MZ B). Parallèlement à la diminution du nombre de cellules MZ B dans la rate, nous avons aussi observé une diminution de la proportion des
lymphocytes B1 (B1 B) présents dans la cavité péritonéale. L’analyse de chimères de moelle osseuse a révélé que les défauts de cellules MZ B et de B1 B étaient associés à l’absence de TLR8 dans les cellules hématopoïétiques radiosensibles, et non dans les cellules stromales radiorésistantes. La déficience en TLR8 entraîne également une augmentation des taux d’anticorps circulant (IgM, IgG, IgG2a), ainsi qu’une augmentation des autoanticorps anti ribonucléoprotéines et anti ADN double brin. Enfin, les souris TLR8−/− présentent des signes de glomérulonéphrites avec des dépôts de complexes immuns de type IgG, IgM ou C3 plus abondants au niveau des glomérules du rein. A l’inverse des souris TLR8−/−, les souris double déficientes TLR7/8−/− présentent une augmentation de la population de cellules MZ B et une diminution des symptômes lupiques avec une baisse des autoanticorps et des dépôts de complexes immuns au niveau des reins.

Nos résultats indiquent donc que chez la souris le TLR8 joue un rôle primordial dans la modulation de l’expression de TLR7, et cette régulation est cruciale dans le contrôle de la production d’autoanticorps et le développement spontané d’autoimmunité.
INTRODUCTION

The immune system detects and eliminates invading microorganisms by discriminating between self and non-self. A fundamental concept in immunology is the classification of immunity into innate and adaptive. Adaptive immunity detects non-self through recognition of peptide antigens using highly specific and randomly generated antigen receptors expressed on the surface of B and T cells. The innate immune system uses germ-line encoded receptors that recognize unique structure of molecules that are characteristic of specific classes of microorganisms and called Pattern Recognition Receptors (PRRs). The innate immune system is phyllogenetically conserved and is present in almost all multicellular organisms. The combination of these two strategies makes the mammalian immune system highly efficient on defense against microorganisms.

Twenty years ago, Charles Janeway set up a conceptual framework for the current understanding of the interactions between innate and adaptive immunity. According to his hypothesis, the adaptive immune system, since it uses randomly generated receptors for antigen recognition, cannot reliably distinguish self from non-self. Therefore, adaptive immune cells must be instructed for the origin of the antigen by a system that can discriminate with high fidelity, whether an antigen is derived from self, infectious non-self or innocuous non-self. Janeway suggested that the evolutionary ancient innate immune system might be able to provide such instruction through the activation of PRRs. We now know that the innate immune system provide all the information needed regarding the origin of pathogens, time frame and scale of the infection to tune the adaptive immune system. However, Janeway’s theory does not address certain situations, such as autoimmunity, in which the immune system is activated by self antigens.
Recently, another theory came out with Poly Matzinger (1) and the concept of “danger model”. Based on this model, danger signals released by injured or dying cells, such as those exposed to pathogen infection, toxins or mechanical damage activates immune sentinels. This concept implies that healthy cells or cells undergoing physiological death do not send out danger or alarm signals in order to avoid auto activation of the immune system. However, the danger model cannot explain why in certain circumstances, PRRs confer resistance against pathogens. From intensive studies in the last few years it is now clear that independently of the model, PRRs are crucial for instructing not only the innate but also the adaptive immune responses.

1 – PRRs and innate immunity

Innate immunity is considered to act as a sentinel for the immune system and is promptly activated upon recognition of diverse microbial pathogens such as bacteria, viruses or fungi. Innate immune cells express various PRRs, which detect evolutionary conserved molecules of microbial origin that are essential for the survival of the microorganism. These microbial derived molecules are known as Pathogen-Associated Molecular Patterns (PAMPs) (2, 3). PRRs can be broadly categorized into three classes: secreted, cytosolic and transmembrane (Figure 1).
Fluid-phase PRMs (Patter recognition molecules) belong to different molecular families, including collectins (MBL, SP-A, SP-D), ficolins, C1q, and pentraxins (CRP, SAP, PTX3). Cell-associated PRMs are strategically located in different cellular compartments (plasma membrane, endosomes, cytoplasm) and belong to different molecular classes (e.g., TLRs, scavenger receptors, lectin receptors), some of which are shown here. TLRs, NOD- and RIG-like receptors are signaling receptors that lead to activation of transcription factors, including NF-κB, IRFs, and MAPKs. C-type lectins and FcγR are capturing receptors. TLRs recognize microbial moieties. RIG-like receptors recognize viral double-strand RNA and AIM2 recognizes DNA and induce production of type I interferon. Peptidoglycan-derived units (e.g., MDP) are recognized by NODs and NALP3, a component of the inflammasome. NALPs are also activated by different danger signals, including lysosomal damage. MBL, mannan-binding lectin, MDP, muramyl dipeptide, OmpA, Outer membrane protein A, SP-A/-D, surfactant protein A/D. (4)
1-1 Secreted PRRs

Secreted PRRs (such as pentraxins, ficolins, and collectins) bind to microbial cell surfaces, activate classical and lectin pathways of the complement system, and opsonize pathogens for phagocytosis by macrophages and neutrophils (4). Pentraxins constitute a superfamily of evolutionary conserved proteins, characterized by a cyclic multimeric structure and the presence in C terminus of a 200 amino acid pentraxin domain, with an 8 amino acid-long conserved pentraxin signature (HxCxS/TWxS) (5) (6). Pentraxins bind various pathogens including fungi, yeasts, bacteria and parasites through diverse structures such as polysaccharides (7). C reactive protein (CRP) and serum amyloid P component (SAP) constitute the short pentraxin superfamily that presents divergence between human and mice in term of sequence and regulation. Pentraxin 3 (PTX3) belongs to the long pentraxin family and is highly conserved between human and mouse. Ficollins and collectins consist in a family of multimeric and multifunctional pattern recognition molecules that can recognize carbohydrate based motifs. Ficollins and collectins differ on their carbohydrate recognition domain (CRD) with C-type lectin domains for collectins and fibrinogen-like domains for ficollins (8). Upon recognition of the infectious agent, ficollins act through two distinct routes. Either they initiate the lectin pathway of complement activation cascade or they initiate a primitive opsonophagocytosis thus limiting the infection.

1-2 Cytosolic PRRs

The cytosolic PRRs include the RNA-sensing: retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs), DNA sensing: DNA-dependent activator of IRFs (DAI) and absent in
melanoma 2 (AIM2), and the nucleotide-binding domain and leucine rich repeat–containing receptors (NLRs).

Most cell types that are specialized on the detection of viral pathogens express RLRs (9). RLR members, RIG-I and melanoma differentiation factor 5 (Mda5) recognize viral RNA through their RNA-binding helicase domains and signal through their caspase activation and recruitment domains (CARD) (10) (11). RLRs interact with a common adaptor molecule called mitochondria antiviral signaling protein (MAVS) or also known as IFNβ promoter stimulator-1 (IPS1) (12). Engagement of MAVS by RLRs leads to the activation of nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3). RIG-I recognizes 5′ triphosphate–ssRNA with a dsRNA component that can be found in many ssRNA viruses like paramyxoviruses, influenza virus, vesicular stomatitis virus (VSV), or Japanese encephalitis virus (13) (Figure 2). RIG-I can also sense RNA generated by RNA polymerase III (Pol III) in the cytosol upon transcription of poly dA:dT–rich dsDNA viruses including adenovirus, herpes simplex virus-1 (HSV-1), Epstein-Bar virus (EBV) (14) (15) (13). Thus, RIG-I is a sensor for both ssRNA viruses and some dsDNA viruses. Mda5 preferentially recognizes long dsRNA structures in the cytosol, a PAMP associated with positive ssRNA viral infections like encephalo-myocarditis virus (EMCV) that belongs to the picornavirus family (12) (13) (Figure 2).
Introduction

Figure 2. RIG-I and Mda5 are cellular helicases that, upon activation stimulate IFNα/β gene expression. Upon activation, RIG-I and Mda5 are believed to undergo conformational changes that allow their N-terminal CARDs to interact with downstream signaling molecules like MAVS and lead to type I IFN production (16).

DAI functions as a cytoplasmic DNA receptor. In vitro experiments showed that DAI is an IFN-inducible protein and an inducer of proinflammatory cytokines and type I IFNs in responses to either DNA from various sources or HSV-1 infection (17). However cells derived from DAI-deficient mice responded normally to poly(dA:dT) and plasmid DNA and they also had unimpaired immune function after infection with a DNA virus (18). These results indicate that DAI has only a redundant role in DNA sensing.

AIM2 is a double-stranded DNA sensor that induces a caspase-1 dependent IL-1β maturation and subsequent directs proinflammatory cytokine maturation and secretion. Ligand
requirement for AIM2 are quite permissive, like cytosolic DNA from virus, bacteria or self (19).

NLRs represent a large family of intracellular sensors that can detect pathogens and stress signals (20). NLRs are multidomain proteins that contain a C-terminal leucine-rich repeat domain, a central nucleotide-binding oligomerization domain (NOD), and an N-terminal effector domain (21). They can be divided into three subfamilies depending on their N-terminal domains: a CARD domain present in proteins of the NOD subfamily, a pyrin domain in the NALP (NACHT, LRR and PYD containing proteins) subfamily and a BIR domain (baculoviral inhibitor-of-apoptosis-protein repeat containing domain) in the NAIP subfamily (Figure 3).

Figure 3. General domain structure of NLRs. NOD and LRRs domains are common to all NLRs. NLRs are classified into subfamilies by protein interaction domain such as CARD, pyrin or BIR domains. Adapted from (22).

NLR family members detect, in most cases indirectly, degradation products of peptidoglycans, various forms of stress, microbial products and noninfectious crystal particles (20). Within the NOD subfamily, NOD1 and NOD2 detect bacterial peptidoglycan, and
through a CARD-dependent recruitment of RIP2 drive the activation of mitogen activated kinases (MAPKs) and NF-κB (23) (24). The NALP and NAIP proteins have a role in the activation of proinflammatory caspases through formation of complexes called “inflammasomes” that lead to processing and activation of the proinflammatory cytokines IL-1β and IL-18 (25) (26).

1-3 Transmembrane PRRs

The transmembrane PRRs include the C-type lectins and the Toll-like receptor (TLR) family. C-type lectin superfamily is a large group of proteins characterized by the presence of one or more C-type lectin domains. The superfamily is divided into 17 groups based on their phylogeny and domain organization (27). Despite the presence of highly conserved domain, C-type lectin receptors (CLRs) are functionally diverse and have been implicated in various processes including cell adhesion, complement activation, endocytosis and phagocytosis. Some of them are involved in antifungal immunity and can directly induce production of cytokines, thus modulating innate and adaptive immune responses (28). They can also function as binding and uptake receptors. Dectin-1 and -2 are transmembrane receptors of the C-type lectin family that detect β-glucans and mannan, respectively, on fungal cell walls (29) (30). Dectin-1 signaling activates directly NF-κB-mediated cytokine production (31), while Dectin-2 has to associate with the ITAM-bearing signaling adaptor molecule FcRγ to induce NF-κB (32).

Mammalian TLRs are located either on the plasma membrane or in endosomal/lysosomal organelles. Cell-surface TLRs recognize conserved microbial molecules that are accessible on the cell surface of microorganisms. For example, lipopolysaccharide (LPS) of Gram-negative
bacteria is sensed by TLR4, lipoteichoic acids of Gram-positive bacteria and bacterial lipoproteins is detected by TLR1/TLR2 or TLR2/TLR6 heterodimers and bacterial flagellin is recognized by TLR5. Endosomal TLRs predominantly detect microbial nucleic acids, such as double-stranded RNA (dsRNA) by TLR3, single-stranded RNA (ssRNA) by TLR7 and TLR8, and dsDNA by TLR9.

2-Toll-like receptors

The involvement of the TLRs in innate immunity was first described in Drosophila. Stimulation of adult Drosophila Toll by the secreted Spätzle factor, a Toll ligand, activates the production of the antimicrobial peptide drosomycin (33). A year after this discovery, a homologue of the Drosophila Toll was identified in human which in our days is known to be TLR4 (34). The murine homologue of TLR4 was shown to be able to recognize lipopolysaccharide, a constituent of the external membrane of the Gram negative bacteria (35) (36). Subsequently, a family of proteins structurally related to Drosophila Toll was identified, collectively referred as Toll-like receptors. The TLR family is now one of the best-characterized PRR families and by now, ten members have been identified in humans and twelve in mice.

TLRs are type I integral membrane glycoproteins composed of extracellular, transmembrane and intracellular signaling domains (37). The extracellular N-terminal domain is specialized in the ligand recognition and consists of 16–28 leucine-rich repeats (LRRs), and each LRR consists of 20–30 amino acids with the conserved motif “LxxLxLxxN” (38) (39). The intracellular C-terminal domain is known as the Toll/IL-1 receptor (TIR) domain and shows homology with that of the IL-1 receptor (Figure 4). This domain is required for the interaction and recruitment of various adaptor molecules that lead to the activation of downstream signaling pathways.
TLRs recognize specific structures derived from viruses, bacteria, fungi or parasitic protozoa and induce the production of inflammatory cytokines and type I interferon. Depending on the pathogen encountered, certain TLRs are activated, induce specific signaling pathways and lead to adequate immune responses. TLRs also induce antimicrobial peptides or proteins such as nitric-oxide synthase (iNOS) (40). TLRs also indirectly elicit an antimicrobial response. They induce the production of tumor necrosis factor (TNF) and IL-1β that in turn activate the local endothelium to induce vasodilatation and increase the permeability of the blood vessel, allowing serum proteins and leukocyte infiltration to be recruited at the inflammation site. Moreover IL-1β in combination with IL-6 activate hepatocytes to produce acute phase proteins such as collectins and pentraxins that activate complement and opsonize pathogens for phagocytosis by macrophages and neutrophils.

TLRs are expressed in distinct cellular compartments. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 (only expressed in mice) are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular vesicles such as the endosomes, lysosomes, endolysosomes and endoplasmic reticulum.
2-1 Plasma membrane TLRs

TLR1, TLR2 and TLR6 are closely related. TLR2 senses various components from bacteria, mycoplasma, fungi, and viruses. These components include the lipoproteins of bacteria and mycoplasma and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, tGPI-mucin from Trypanosoma cruzi and the hemagglutinin from measles virus. TLR2 functions as heterodimer with either TLR1 or TLR6. The resulting TLR1/TLR2 and TLR6/TLR2 complexes recognize triacyl and diacyl lipoproteins, respectively (41) (42). The crystal structures of the extracellular domains of TLR2, TLR1, and TLR6 revealed that they form “m”-shaped structures and that their cognate ligands interact with internal pockets formed by the TLR1/TLR2 or TLR6/TLR2 heterodimers (43) (Figure 5).

![Structure of the TLR1-TLR2-ligand complex](image)

Figure 5. Structure of the TLR1-TLR2-ligand complex. TLR1-TLR2 dimers result from binding of an agonist. The extracellular domain of TLR1 and TLR2 form a ”m”-shaped heterodimer with the two N-termini extending outwards in opposite direction and the LRR C-termini modules converging at the center (44).

Moreover TLR2 has the ability to act together with other coreceptors on the cell surface to detect microbial components. CD36 acts together with the complex TLR2/TLR6 to sense some, but not all, TLR2 agonists (41). Stimulation with TLR2 ligands induces the production
of various proinflammatory cytokines in macrophages and DCs. In addition, TLR2 is expressed in inflammatory monocytes and induces type I IFNs in response to viral infection, suggesting that the cellular responses to TLR2 ligands differ depending on the cell type that is involved (45).

TLR4 recognizes lipopolysaccharide (LPS) together with myeloid differentiation factor 2 (MD2) on the cell surface. LPS is a component derived from the outer membrane of Gram negative bacteria and is known to be a cause of septic shock (3). The crystal structure of a complex comprising TLR4, MD2, and LPS revealed that two complexes of TLR4-MD2-LPS interact symmetrically to form a homodimer (46) (Figure 6).

**Figure 6. Structure of the TLR4-MD2-LPS complex.** LPS induces dimerization of the TLR4-MD2 complex. The structure formed resemble the “m”-shaped dimers of TLR1-TLR2 (44).

Additional proteins such LPS binding protein (LBP) and CD14 are also required for LPS binding and delivering to the TLR4 MD2 complex (47). TLR4 stimulation induces the production of various chemokines and cytokines (36, 48) and can also induces production of type I IFNs, albeit usually at low levels (49). LPS is the most documented ligand for TLR4, but it is not the only one. TLR4 is also involved in the recognition of viruses by binding to viral envelope proteins such as respiratory syncytial virus (RSV) (50). In addition, TLR4 modulates the pathogenesis of H5N1 avian influenza virus infection by recognizing a danger
associated molecular pattern (DAMP) rather than the virus itself. Acute lung injury caused by avian influenza virus infection produces endogenous oxidized phospholipids, which stimulate TLR4 and mice lacking TLR4 are resistant to avian flu induced lethality (51).

TLR5 recognizes a conserved domain of the flagellin monomer and triggers proinflammatory and adaptive immune responses (52) (53). TLR5 is highly expressed by DCs of the lamina propria (LPDCs) in the small intestine, where it recognizes flagellin from flagellated bacteria. In response to flagellin, LPDCs induce B cells to differentiate into IgA-producing plasma cells and trigger the differentiation of naive T cells into antigen-specific Th17 and Th1 cells (54). TLR5 also cooperates with TLR4 for the recognition and immune response against in vivo infection with *Pseudomonas aeruginosa* and *Salmonella typhimurium* (52).

TLR11 is present in mice but not in humans and shows close homology to TLR5. TLR11 recognizes uropathogenic bacteria and a profilin-like molecule derived from the intracellular protozoan *Toxoplasma gondii* (55). Mice lacking TLR11 are highly susceptible to kidney infection by uropathogenic bacteria, indicating a potentially important role for TLR11 in preventing infection of internal organs of the urogenital track (56).

TLR10 is related to TLR1 and TLR6 based on sequence similarity. TLR10 seems to be functional in humans, although mouse TLR10 is disrupted by insertion of an endogenous retrovirus. The ligands for human TLR10 as well as for murine TLR12 and TLR13 have not been identified yet.
2-2 Intracellular TLRs

In contrast to TLR2 or TLR4, the spectrum of recognition of endosomal TLRs is far more restricted to the sensing of nucleic acids. Endosomal TLRs are specialized in nucleic acid, one signature of invading viruses or certain bacteria.

TLR3 recognizes dsRNA and triggers an antiviral immune responses through the production of type I interferon and proinflammatory cytokines. Its role was demonstrated in a study of TLR3-deficient mice, which show reduced production of type I IFN and inflammatory cytokines in response to genomic dsRNA from reovirus and polyI:C, a synthetic analog of dsRNA (57). The recognition mechanism was elicited by structural analysis of TLR3 ectodomain bound to dsRNA (58) and implies the formation of an TLR3–TLR3 homodimer that forms a horseshoe-like structure (‘‘m” shape) (59) (Figure 7).

![Figure 7](image)

**Figure 7. Structure of the TLR3-dsRNA complex.** TLR3 dimerization is induced by the ligand and the complex adopt a “m”-shaped structure in which the two C-termini of the extracellular domains are in close approximation (44).

TLR3 is also involved in recognizing dsRNA produced during the course of replication of ssRNA viruses, such as the respiratory syncytial virus (RSV) (60), EMCV (61) and West Nile
virus (WNV) (62). Therefore, a protective role of TLR3 against viral infection has been suggested in mice and humans. Deficiency of the TLR3 protein in humans was associated with susceptibility to HSV-1 infection (63). Moreover, TLR3 is implicated in the recognition of small interfering (si) RNA. A recent report has shown that siRNA can activate TLR3 in a sequence-independent manner and induce the production of IL-12 and IFNγ (64). TLR3 is expressed by conventional DCs (cDCs) and macrophages as well as non immune cells, including fibroblasts and epithelial cells. TLR3 is localized on intracellular vesicles in cDCs, while in fibroblasts and epithelial cells it can be found on the cell surface or in intracellular vesicles (65). The intracellular vesicles wherein TLR3 is localized are suggested to be the early endosomes (66). Strong expression of TLR3 mRNA is found in CD8α+CD4− DCs, a DC subpopulation with high phagocytic activity. CD8α+ DCs can take up apoptotic bodies of virally-infected or dsRNA-loaded cells, allowing dsRNA to reach intracellular compartments where TLR3 is located. Phagocytosis associated with TLR3 activation promotes cross-presentation, which is important for the induction of CTL response against viruses that do not infect DCs (67).

TLR7 was initially identified to recognize imidazoquinoline derivatives such as imiquimod, resiquimod (R-848), and guanine analogues like loxoribine (which have anti tumor properties) (68). TLR7 recognizes guanosine- and uridine-rich ssRNA derived from RNA viruses, such as VSV, influenza A virus and human immunodeficiency virus (HIV) (69) (70) (71). TLR7 is also involved in the recognition of Borrelia burgdorferi (72) and RNA released into endolysosomes by phagacytosed bacteria (73) (74). In addition, TLR7 senses synthetic polyU RNA and certain small interfering siRNAs (69) (75). TLR7 is highly expressed in plasmacytoid dendritic cells (pDCs) that are able to produce large amount of type I IFNs after viral infection. Cytokine induction by pDCs in response to ssRNA is totally TLR7-dependent.
and TLR7-mediated recognition of RNA viruses by pDCs can occur in a replication-independent manner. Viruses are internalized and recruited to the endolysosomes, where they trigger TLR7 anti viral response. TLR7 can also sense replicating VSV that enter the cytoplasm via autophagy (76), a process for lysosomal degradation of cellular organelles involving the formation of double membrane vesicles called autophagosomes. Such mechanism is important for the delivery of cytosolic replicating RNA viruses to the lysosomes where TLR7 is present.

TLR9 was originally identified to recognize unmethylated 2-deoxyribo (cytidine-phosphate-guanosine) (CpG) DNA motifs that are frequently present in bacteria, but are rare in vertebrates (77). TLR9 is found on DCs, macrophages and B cells, but like TLR7, it is highly expressed in pDCs and its stimulation induces the secretion of large amounts of type I interferons. By now, we know that TLR9 detects dsDNA viruses such as mouse cytomegalovirus (MCMV) (78), HSV-1 (79) and HSV-2 (80) as well as nucleic acids from protozoa including *Trypanosoma cruzi* (81). Over the last few years, synthetic CpG oligodeoxynucleotides (ODNs) that activate TLR9 can be classified in three categories A, B and C, based on their biological properties. A-type ODNs are strong inducers of type I IFNs, but have low ability to induce B cell activation and IL-12 production. By contrast, B-type ODNs simulate B cell activation and IL-12 production, but poorly induce type I IFNs (82). C-type ODNs have features of both classes A and B, and induce both type I IFN and proinflammatory cytokine secretion. TLR9 exists in two forms: either full length or cleaved. Full length TLR9 is mainly found in the endoplasmic reticulum and upon CpG activation, it is translocated to endosomes where it is processed to give a cleaved and biological active form (83) (84). Both full length and cleaved TLR9 can bind CpG but only the truncated form can
interact with MyD88 to induce signaling. The cleaving of TLR9 is done by peptidases like cathepsins (85), (86) and asparagine endopeptidase (87).

Both TLR8 and TLR7 are located on the X chromosome, 70kb apart, and TLR8 has been derived from duplication of the TLR7 gene. Human TLR8 preferentially recognizes imidazoquinoline compounds, including R848 and ssRNA derived from HIV (70). TLR8 is one of the less studied TLRs because it functions in a species-specific manner and initial studies suggested that murine TLR8 was non-functional. Indeed, transfection of HEK293 cells with human TLR7, human TLR8, murine TLR7, but not murine TLR8, induce NF-κB activation upon R848 stimulation (88). Moreover, murine TLR8-deficient macrophages show normal response upon stimulation with human TLR8 agonists including R848 and ssRNA (70). Unresponsiveness of rodent TLR8 to human TLR8 ligands has been attributed to the lack of five amino acids in the ectodomain of the receptor suggesting that its unresponsiveness is related to a sensing defect rather than to a signaling defect (89). Indeed, we and others showed the TIR domain of murine TLR8 is functional (Figure 18 A) (90). TLR8 expression has been reported in a variety of murine cell types including embryonic fibroblasts (91), DCs, B cells, mesenchymal stem cells, Glia, neural progenitor cells, cortical neurons, granule neurons and axons. Nevertheless, recent studies suggest that mTLR8 is functional. Actually, Vartanian’s group described a role for mTLR8 as a suppressor of neurite outgrowth as well as an inducer of neuronal apoptosis in an NF-κB-independent mechanism (92). Cotransfection experiments of murine TLR7 with murine TLR8 revealed that TLR8 inhibits TLR7 functions (93). In HEK293 cells transfected with mouse TLR8 and stimulated, a combination of imidazoquinoline and polyT oligodeoxynucleotides is able to activate murine TLR8 (94). In addition TLR8 is required for sensing poly(A)/T-rich DNA in pDCs.
Upon Vaccina Virus infection IFN-α production and virus clearance in the spleen are impaired in mice reconstituted with TLR8-deficient pDCs (95).

**Figure 8. PAMP recognition by TLRs.** TLR4 in a complex with MD-2 recognizes LPS. During the early-activation phase the complex TLR4-MD2 signals from the plasma membrane. It is then internalized and retained in endosomes, where it triggers signaling through TRIF during the late activation response. TLR2/1 and TLR2/6 heterodimers recognize triacylated and triacylated lipopeptides, respectively. TLR5 binds flagellin. In endosomes, TLR3 recognizes dsRNA derived from virus or infected cells. TLR7 binds ssRNA and TLR9 recognizes DNA derived from both viruses and bacteria. Human TLR8 binds ssRNA while the ligand for murine TLR8 is still unknown.

### 2-3 Intracellular localization and trafficking of nucleic acid sensing TLRs

Physical separation of host nucleic acids from foreign nucleic acids and the intracellular receptors that detect them is a key strategy to distinguish self from non-self. Proper trafficking of nucleic acids sensing TLRs is essential to prevent receptor activation by self components. Nucleic acid-sensing TLRs localize to various intracellular compartments within endoplasmic reticulum (ER), endosomes, multivesicular bodies and lysosomes. However, TLR signaling
occurs only within acidified endolysosomal compartments. Blockade of endolysosome acidification with different chemicals prevents TLR7- and TLR9-induced responses suggesting that the delivery of internalized nucleic acids to endolysosomes is pivotal for interaction with these TLRs (96). TLR7 and TLR9 are sequestered in the endoplasmic reticulum and upon activation are rapidly translocate to endosomes (97) (98). This translocation is regulated by the ER-localizing protein UNC93B1, a 12 membrane spanning molecule (99). In mice, a single non sense mutation called “3d” in the gene coding for the UNC93B1 protein disrupts TLR3, TLR7, TLR9 signaling and exogenous antigen presentation rendering ”3d” mice highly susceptible to viral and bacterial infection (100). Interestingly, UNC93B1 deficiency in humans is also correlated with HSV encephalitis (101). A physical association of intracellular TLRs and UNC93B1 is mandatory for their export from the ER (102). The N-terminal cytoplasmic portion of UNC93B1 has been shown to interact with TLR9 and TLR7 and to affect negatively TLR7 responsiveness in dendritic cells (103). Trafficking of TLRs is also controlled by two other molecules: PRAT4A and gp96. PRAT4A interacts with TLR9 and is essential to its location in endosomes (104) while gp96 functions as a general chaperone for most of the TLRs (105) (Figure 9).
**Figure 9. Intracellular TLRs localization and trafficking.** TLR3, TLR7 and TLR9 localize mainly to the ER in the steady state and traffic to endolysosomes when they are engaged by their ligands. UNC93B1 interacts with these TLRs in the ER and is essential for this trafficking. Proteolytic cleavage of TLR9 by cellular proteases is required for downstream signal transduction. Adapted from (106).

### 2-4 Distinct signaling pathways

Ligand binding to the leucine rich repeats (LRRs) in the ectodomain of TLRs results in allosteric changes in the cytoplasmic signaling domain. These conformational changes induce an apposition of the cytoplasmic domains that are probably required for association between TIR domains of the TLRs and TIR domains of adaptor molecules and signaling (107). Various adaptor molecules have been described downstream of TLRs and their interaction leads to proinflammatory cytokines, as well as type I IFN production. TLR adaptor molecules are MyD88 (Myeloid differentiation primary-response gene 88), TRIF (TIR domain-
containing adaptor inducing IFN-β) also known as TICAM-1 (TIR domain-containing molecule-1), MAL (MyD88 adaptor like protein) also known as TIRAP (TIR associated protein), TRAM (TRIF-related adaptor molecule). In general, TLR signaling pathways are classified as either MyD88-dependent pathways, which drives mainly the production of inflammatory cytokines, or MyD88-independent pathways, which are responsible for the induction of type I IFN as well as inflammatory cytokines. MAL and TRAM act as sorters for the recruitment of MyD88 or TRIF by TLRs. Hence, MAL links MyD88 with TLR4 and TLR2, while TRAM bridges TRIF to TLR4.

2-4-1 MyD88

All TLRs, with the exception of TLR3, depend at least in part on the MyD88 adaptor molecule for signaling activity. MyD88 signaling activates the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce the production of inflammatory cytokines such as IL-6 and TNF (108). Upon TLR stimulation, MyD88 interacts with IL-1R associated kinase 4 (IRAK4). IRAK4 phosphorylates IRAK1 and IRAK2 that in turn activate TNF receptor-associated factor 6 (TRAF6). TRAF6, a RING-domain ubiquitin ligase activates transforming-growth-factor-β-activated kinase (TAK1) through polyubiquitination. TAK1 signaling goes through two different pathways. On one hand, it phosphorylates Iκκ complex (including NEMO and Iκκβ) that in turn phosphorylates IκB leading to its polyubiquitination, subsequent degradation and resultant nuclear translocation of NFκB. On the other hand, TAK1 activates also a cascade of mitogen associated kinases (MAPKs), starting with MAPKK3 and MAPKK6 leading to phosphorylation of Jun kinases (JNKs), p38, extracellular regulated kinases (ERK1/2) and leading to the activation of the transcription factor AP1 (3) (109) (Figure 10).
MyD88-dependent signaling is also involved in type I IFN production by pDCs in the case of viral recognition by TLR7 or TLR9. In this context, a multiprotein complex including MyD88, TRAF6, TRAF3, IRAK1, Iκκα and IRF7 is formed (110). IRF7 is phosphorylated by IRAK1 and Iκκα, dissociates from the complex and translocates into the nucleus to drive the expression of type I IFNs (111) (Figure 10).

2-4-2 TRIF

TRIF is exclusively used by TLR3 and TLR4. TLR3 signals only through TRIF while TLR4 shares a part of its signaling with MyD88. TRIF leads to the production of inflammatory cytokines and type I IFNs in particular IFNβ. TRIF forms a multi protein complex along with TRAF6, TRADD, Pellino-1 and RIP1 for the activation of TAK1, which in turn activates the NF-κB and MAPK pathways for inflammatory cytokine production. It also associates with a complex involving TANK binding kinase-1 (TBK1) to induce the phosphorylation and translocation of IRF3, leading to IFN-β expression (Figure 10).
Figure 10. Simplified scheme of TLR signaling in cDC or macrophage (left panel) and pDC (right panel). MyD88, myeloid differentiation primary-response gene 88; IRAK, interleukin-1 receptor associated kinase; TRAF6, tumor necrosis factor-receptor-associated factor 6; TAK-1, transforming-growth-factor-β activated kinase 1; MAPK, mitogen activated protein kinase; IκK, inhibitor of NF-κB kinase; TRIF, Toll/Il-1 receptor-domain containing adaptor protein inducing IFNβ; TBK1, TRAF-family-member-associated NF-κB activator-binding kinase; IRF, IFN-regulatory factor (112).

2-4-3 NF-κB signaling

The nuclear factor-κB (NF-κB) pathway has many components that positively or negatively regulate its activity. In mammals, the NF-κB family of transcription factors consists of five members: RELA (or p65), REL (c-Rel), RELB, p50 and p52 which can form homodimers or heterodimers. In resting cells, NF-κB dimers are normally kept in an inactive state by
association with proteins of the IκB family. Activation of NF-κB is controlled by the IκB (Ikk) complex. Following cell stimulation, Ikk complex phosphorylates IκB proteins which induce their subsequent polyubiquitination and proteasomal degradation, allowing NF-κB dimers to translocate in the nucleus and activate gene transcription.

Two distinct NF-κB activation pathways have been described. The canonical (classical) NF-κB pathway is activated by a large number of stimuli, including proinflammatory cytokines as well as bacterial or viral products. These stimuli induce the degradation of IκBα and the nuclear accumulation of mainly p50-RELA dimers, which regulate the expression of immunoregulatory and anti-apoptotic genes (Figure 11). The non-canonical (alternative) NF-κB pathway is activated by receptors that are involved in lymphoid tissue organogenesis and lymphocyte development, such as the lymphotoxin-β receptor and the receptor for B cell-activating factor (BAFF), which induce the processing of p100 to p52 and the nuclear translocation of p52-RELB dimers (Figure 11).

Figure 11. Classical and alternative pathway of NF-κB activation. Canonical pathway (left panel): NF-κB dimers are normally sequestered in the cytoplasm of resting cells. Pro-inflammatory
signals stimulate receptors such as TNFR or TLR which activate the IKK complex (NEMO-Iκκα-Iκκβ). The Iκκ complex phosphorylates IκB on specific serine residues, thereby triggering their polyubiquitination and proteasome-dependent degradation. Removal of IκB allows NF-κB dimers to accumulate in the nucleus and bind to the promoter of targeted genes. **Alternative pathway** (right panel): In unstimulated cells, NIK is destabilized by bound TRAF3. Activation through a subset of receptors such as BAFFR, CD40, RANK or Lymphotoxin-βR leads to the recruitment of TRAF3. TRAF3 is inactivated and active NIK is thus released. NIK then phosphorylates and activates Iκκ; it also recruits p100 bound to RELB, which is phosphorylated by Iκκα. This triggers polyubiquitination and subsequent processing of p100 to form the mature subunit p52. Predominantly RELB/p52 heterodimers are generated, which migrate to the nucleus.

### 2-5 TLR cooperation

Certain TLRs have a synergistic effect in vitro and this is particularly marked by the production of IL-12p70 which is the bioactive form of IL-12 composed of IL-12p35 and IL-12p40 (113). Single TLR stimulation in DCs induces in general an important production of IL-12p40, but little, if any, of IL-12p70. On the contrary, stimulation of human or mouse DCs with TLR7/8 ligand (R848) plus either TLR3 ligand (polyI:C) or TLR4 ligand (LPS) drives 10 to 100 fold higher IL-12p70 quantities than a single stimulation (114) (115). These results have been extended with MyD88−/− and TRIF−/− mice and concluded that MyD88-associated TLRs synergize with TRIF-associated TLRs for IL-12p70 production.

The in vivo cooperation between TLR5 and TLR4 has also been evidenced in our laboratory in the antibacterial immune response against *Pseudomonas aeruginosa*. Indeed, upon a high dose of *Pseudomonas aeruginosa* infection, TLR4−/− and TLR5−/− showed similar rate of survival than WT while TLR4/5 double-deficient mice showed increased susceptibility (52). In addition, TLR7-TLR9 cooperation in host defense against MCMV infection has also been enlightened. TLR7 and TLR9 were shown to be each able to confer a significant protection against MCMV infection and double-deficiency in TLR7 and TLR9 dramatically increased mice susceptibility compare to TLR7−/− or TLR9−/− mice (116). Recently, genetic data
demonstrated a negative cooperation of TLR9 on TLR7 with implication in autoimmunity development. Indeed, TLR9 regulates TLR7 and MyD88-dependent autoantibodies production by suppressing RNA-associated autoantibodies generation and by controlling TLR7 expression (117) (118).

3- Adaptive immune system

Adaptive immune responses are mediated by two types of antigen receptors: T- and B-cell receptors. The genes encoding antigen receptors are assembled from variable and constant fragments through recombination-activating gene (RAG)-protein-mediated somatic recombination called VDJ leading to the expression of a diverse repertoire of antigen receptors (119). Antigen receptor activation triggers clonal selection and expansion of rare T and B cells that is the hallmark of adaptive immunity. There are two types of lymphocytes that express antigen receptor: conventional lymphocytes and innate-like lymphocytes. In the case of conventional lymphocytes, αβ T cells and B2 cells, antigen receptors are essentially randomly assembled. In contrast, innate-like lymphocytes like B1 B cells, marginal zone B cells, natural killer T cells and γδ T cells possess a recurrent expression of families of germ-line-encoded BCRs and TCRs with limited diversity, suggesting that they recognize conserved structures. The effector functions of these lymphocytes and the site where they reside are often predetermined and the effector responses of innate-like lymphocytes therefore do not generally require the same type of instruction that are provided by the innate immune system to conventional lymphocytes.

3-1 DCs orchestration of conventional lymphocytes

Dendritic cells are described as professional antigen presenting cells. They are like sentinels, residing within the tissue, at an immature state (unable to activate T cell) expressing low
amounts of MHC and costimulatory molecules but with a high capacity of endocytosis. On one hand, it is clear that resident DCs in the secondary lymphoid tissues are able to induce T cell tolerance when antigen is provided in the absence of microbial stimuli (120). On another hand, DC maturation leads to several changes mandatory for efficient T cell priming. Once they encounter a pathogen, they egress to the secondary lymphoid organs (spleen and lymph nodes) where they complete their maturation by expressing at their surface high amounts of MHC-peptide complexes (121). Recognition of the MHC-peptide complexes on DCs by Ag-specific TCR constitutes the first signal of DC-T cell interaction. Mature DCs express also co-stimulatory molecules such as CD80, CD86 or CD40 that interact with their ligands expressed on T cells and provide a second signal for an amplified and sustained T cell response. A third signal is the cytokine cocktail produced by mature DCs that educate and differentiate T cells into effector cells such as CTLs, Th1, Th2, Th17 or regulatory in order to trigger an adapted and efficient response (Figure 12). A given DC needs stimulation by both TLR ligands and proinflammatory cytokines to be fully matured (122). Indeed, cytokine-activated DCs, although upregulating CD86, CD40 and chemokine receptor CCR7, are only poorly immunogenic because they fail to produce IL-12. This phenomenon, termed “licensing” of DCs, is necessary for the induction of T-cell responses (123). Th1 cells promote cell-mediated immunity, while Th2 cells promote humoral immunity. The function of Th1 and Th2 cells lies in their ability to produce reciprocal sets of cytokines. For example, IL-12 induces the generation of Th1 cells by activating STAT4, which result in the sequential induction of the master regulator of Th1 lineage differentiation T-bet and subsequently IFN-γ expression (124) (125). Induction of Th2 cells is initiated by IL-4-mediated activation of STAT6, which induces expression of the transcription factor GATA3 leading to the production of IL-4, IL-5 and IL-13 (126). Th17 cells are induced by TGF-β plus IL-6 or IL-21 and produce IL-17 and IL-21 cytokines. RORγt is considered to be the transcription factor responsible for guiding the
development of Th17 cells (126). These cells have been recognized to be key mediators of inflammation and tissue damage in several animal models. However, TGF-β is also important for the generation of the inhibitory Treg cells.

**Figure 12. T cell lineage differentiation in mice.** When stimulated by APCs and cognate peptide, undifferentiated mouse T helper cells develop into lineages defined by the cytokines they secrete.

T cell lineage differentiations are regulated through the recognition of pathogens via the PRRs expressed by DCs (127). Indeed, mice temporarily deprived of CD11c+ DCs cannot mount efficient and specific CD8+ T cell-responses to infections with *Listeria monocytogenes*, *Plasmodium yoelii*, LCMV or antigen immunization (128) (129). DC-primed Helper T cells (CD4+) produce cytokines (Table 1) that allow activation of cytotoxic T lymphocytes (CD8+) (CTLs), NK cells or macrophages. In the case of B lymphocytes, DC-activated CD4+ T cells provide co-stimulation through cytokine cocktail that in addition to the BCR and antigen
association lead to the maturation in plasma cells secreting antibodies (130). However, a T-independent activation could occur when B lymphocytes encounter an antigen that combines a ligand that activates BCR and PRRs at the same time.

<table>
<thead>
<tr>
<th>CD4 T-cell effector subset</th>
<th>Polarizing factors</th>
<th>Transcription factors</th>
<th>Signature cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IL-12, IFNγ</td>
<td>T-bet</td>
<td>IFNγ, TNF, IL-2</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4</td>
<td>GATA-3</td>
<td>IL-4, IL-5, IL-13</td>
</tr>
<tr>
<td>Th17</td>
<td>TGF-β, IL-6</td>
<td>ROR-γt</td>
<td>IL-17, IL-21, IL-22</td>
</tr>
<tr>
<td>Treg</td>
<td>TGF-β, RA</td>
<td>Foxp3</td>
<td>IL-10</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of CD4 T cell subsets. Adapted from (131).

3-2 Innate B lymphocytes: MZ and B1 B cells

Characteristically, MZ and B1 B lymphocytes have a natural activated or effector phenotype. In mice they express high level of the IgM isotype and low levels of IgD isotype on their surface, a pattern of activated B cells. B1 B cells are predominantly distributed in the peritoneal and pleural cavities while MZ B cells are located in the marginal zone of the spleen. Through their intimate contact with recirculating blood and in conjunction with specialized macrophages and DCs subsets, MZ B cells screen blood-borne antigens and particularly those bound to complement as they highly express CD21 (complement receptor). Once they encounter antigens, MZ B cells rapidly egress to the T-B junction of the spleen, where they proliferate and generate large clones of IgM-secreting plasma cells. The B1 B cells that reside in the peritoneal cavity give rise to plasma secreting IgA in the gut and the mesenteric lymph nodes (132). Both MZ and B1 B cells exhibit a high antigen-presenting capacity (133, 134).
3-3 Specialization of DC subsets

Human and mouse DC subsets differ phenotypically by several markers but in accordance with the objectives of our study, only mouse DC subsets will be mentioned. There are multiple subsets of DCs that differ by their location and TLR expression pattern, suggesting that specialization of DC populations may be related to certain type of pathogen detection and involved in distinct effector cell type induction. In mouse secondary lymphoid organs, there are two main subtypes of DCs, conventional and plasmacytoid DCs. Conventional DCs express high level of CD11c and can be divided in resident and migratory DC. The blood-derived lymphoid tissue DCs comprise different subsets such as CD8α⁺ DCs, CD8α⁻ DCs or pDCs.

CD8α⁺ DCs reside in the deep T cell areas of secondary lymphoid tissue. CD8α⁺ DCs efficiently cross-present exogenous cell bound and soluble antigens on major histocompatibility complex class I. Upon activation, CD8α⁺ DCs are major producers of interleukin-12 and stimulate inflammatory responses. In steady state, they have immune regulatory properties and help in maintaining tolerance toward self tissues. During infection with intracellular pathogens, they become major presenters of pathogen antigens, promoting CD8⁺ T cell responses against invading microbes (135) (136). The murine CD8α⁺ DCs express almost all TLRs except TLR7 (Table 2) (137).

The other main population of blood-derived DCs resident in lymphoid tissue is constituted of CD8α⁻CD11b⁺ DCs that are localized in the marginal zone of the spleen and in the outer edges of the paracortex in the lymph nodes. These cells express all the TLRs, however TLR3 expression is very low (Table 2) (137). Their role is not really clear, but CD8α⁻ DCs generally do not cross-present antigen but they present antigen to CD4 T cells. Freshly isolated CD11b⁺
DCs from secondary lymphoid tissues induce Th-2 differentiation when transferred to a naïve host (138) (139).

Plasmacytoid DCs (pDCs) constitute another significant population of DCs in the spleen. They are called “plasmacytoid” because of their “plasma cell-like” morphological appearance. pDCs are present in lymphoid and non lymphoid organs and are characterized by being CD11c\textsuperscript{int} B220\textsuperscript{+} PDCA-1\textsuperscript{+} MHCII\textsuperscript{int}. They express several TLRs, mostly TLR7 and TLR9 (Table 2) and upon viral infection they are the main producers of IFN-α. In the steady state, pDCs present poorly antigen on MHC complexes, however, following activation with microbial stimuli, they are converted in potent APCs (140).

![Table 2. Expression of TLRs among primary human and mouse DC subsets. Adapted from (141).](image)

In the skin, two main subsets of DCs exists: Langerhans cells and Dermal DCs that reside in peripheral tissue and migrate constitutively into lymph nodes, even in the absence of inflammatory stimuli thereby participating to peripheral tolerance (142).
4-Autoimmunity and nucleic acid sensing--TLRs

4-1 Autoimmunity and tolerance

Briefly, autoimmunity occurs when immune system reacts against self molecules and leads to tissue damage. Immune system has the difficult task to maintain organism integrity and to balance between fighting invaders and limiting inflammation to prevent tissue damage and development of autoimmune disease. This fine control exists whereby deviations can result in one side to immune deficiency or on the other to autoimmunity. Tolerance represents an immunological state in which potentially autoaggressive cells are prevented from creating damage to the host and B and T lymphocytes are selected on the basis to be non autoreactive (143). Several mechanisms have been postulated to explain the tolerant state. They can be broadly classified into two groups central tolerance and peripheral tolerance. Central tolerance refers to clonal deletion of self-reactive B and T lymphocytes during their maturation in the central lymphoid organs (thymus for T lymphocytes and bone marrow for B lymphocytes) (144). Therefore, developing lymphocytes that express high affinity receptor for self antigen are negatively selected or deleted. Another tolerogenic process occurring during the maturation of B cells in the bone marrow is called “receptor editing” and is a modification in the light chain of the antigen receptor expressed by the autoreactive B cells in order to change its specificity (145). However, clonal deletion of self reactive lymphocytes is far from perfect and few autoreactive cells can access the periphery. Clonal deletion by activation-induced cell death is a mechanism happening in the periphery to prevent uncontrolled T-cell activation during a normal immune response and involves apoptotic cell death by the Fas-Fas ligand system (146). Clonal anergy is another mechanism and refers to prolonged or irreversible functional inactivation of lymphocyte. Clonal anergy involves unactivated DCs that do not or
weakly express costimulatory molecules. Therefore, resting DCs present antigens without costimulation and lead T-lymphocytes to unresponsiveness or anergy (147). B cells are also affected by clonal anergy in the tissue. If B cells encounter antigen in the absence of specific helper T cells, the antigen-receptor complex is down-regulated, and such cells never re-express their immunoglobulin receptors (Figure 13). Lastly, the normal immune system generates CD25⁺CD4⁺ Tregs that are engaged in suppressing immune responses toward self, quasi-self (such as autologous tumor cells), and non-self (such as microbes and allografts). Mature Tregs persist in the periphery and exert dominant control over the self-reactive T cells (148) (Figure 13).

Figure 13. Mechanism of regulation between tolerance and autoimmunity.
4-2 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus is a chronic autoimmune disorder, classically depicted as a systemic autoimmune disease caused by the production of pathogenic autoantibodies against to a spectrum of nuclear antigens. Clinical manifestations of human SLE are various and may involve several organs. A typical patient with SLE is a young woman (since 90% of lupus patient are female) in her child-bearing age who presents intermittent fatigue, joint pain and swelling, skin rashes, low white blood cell count, and chest pain due to pleuritis. Approximately, fifty percents of lupus patients will manifest the more severe complications of the disease, which can include nephritis, central nervous system vasculitis, pulmonary hypertension, interstitial lung disease, and stroke. The diagnosis of lupus is complicated by this extensive variation of symptoms. Current diagnosis guideline requires that patient fulfill any four of eleven criteria to be diagnosed with systemic form of SLE. The criteria are listed in Table 3.

<table>
<thead>
<tr>
<th>Clinical criteria</th>
<th>Laboratories criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin:</td>
<td></td>
</tr>
<tr>
<td>• Butterfly-shaped rash over the cheeks</td>
<td>Antibodies:</td>
</tr>
<tr>
<td>• Raised red rashes that scar</td>
<td>ANA</td>
</tr>
<tr>
<td>• Rashes appearing in areas exposed to the sun</td>
<td>Else (anti-dsDNA, anti-Sm)</td>
</tr>
<tr>
<td>• Mouth or nose ulcers</td>
<td></td>
</tr>
<tr>
<td>Else:</td>
<td></td>
</tr>
<tr>
<td>• Fever, fatigue and weight loss</td>
<td></td>
</tr>
<tr>
<td>• Arthritis involving multiple joints for several weeks</td>
<td></td>
</tr>
<tr>
<td>• Joint pain with swelling and tenderness</td>
<td></td>
</tr>
<tr>
<td>• Heart and lung lining inflammation (pericarditis and/or pleuritis)</td>
<td></td>
</tr>
<tr>
<td>• Seizures or other neurologic disorder</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Generalities:</th>
<th>Antibodies:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine: blood or protein detected (kidney problem)</td>
<td>ANA</td>
</tr>
<tr>
<td>Blood: low blood counts (anemia, low white blood or low platelets)</td>
<td>Else (anti-dsDNA, anti-Sm)</td>
</tr>
</tbody>
</table>

Table 3. Criteria for SLE diagnostic.
However, the gold standard for diagnosis of lupus remains the blood test which allows the measurement of antinuclear autoantibodies (ANA). The prevalence of lupus ranges from approximately 40 cases per 100,000 persons among Northern Europeans to more than 200 per 100,000 persons among African Americans. Genetic factors are for sure implicated in lupus pathology but environmental factors have also a key role. Indeed the concordance rate for lupus is 25% among homozygote twins and approximately 2% among dizygotic twins (149). Moreover, a temporal association with EBV infection and lupus has been reported. Anti-EBV antibodies were present in 99% of a cohort of young patients as compare with only 70% in healthy controls (150).

4-3 Autoantibodies

SLE is categorized in the class of systemic disease and is characterized by the production of a big range of autoantibodies. Almost all the patients present elevated titers of nuclear autoantigens and most of them are directed against DNA or RNA-associated structure such as histones or small nuclear ribonucleoproteins (snRNPs). Usually the presence of such antibodies in sera of patients is determined by ANA staining on the human epithelial cell line Hep-2, that is useful to detect globally antinuclear autoantibodies. In addition to the ANA test, practitioners prescribe tests to determine the presence of specific antibodies: anti-dsDNA, anti-Sm (Smith antigen) and anti-ribonucleoproteins. Interestingly, specificity of autoantibodies plays a crucial role in clinical manifestations and they are listed in Table 4.
Most studies of autoantibody-mediated tissue damage in patients with lupus have focused on the role of anti-dsDNA in patients with lupus nephritis. Two theories are emerging on the pathogenic role of these antibodies. The first propose that anti-dsDNA autoantibodies bind to nucleosomes present in the bloodstream and then these complexes aggregate in the renal glomerular basement membrane where they activate complement and initiate glomerulonephritis (152). The second hypothesis is based on molecular mimicry or antibody polyreactivity. Anti-dsDNA or nucleosomes could react with proteins in the kidney. They thus may exert their pathogenic effect on renal cells. Indeed, studies have shown that anti-dsDNA could interact with α-actinin, a major protein involved in the function of renal podocytes and therefore in the glomerular filtration barrier (153).

Table 4. Pathogenic autoantibodies in Systemic Lupus Erythematosus. Adapted from (151).
4-4 Cell activation by endogenous ligands through endosomal TLRs

4-4-1 TLR7 and TLR9 implication in SLE

Implication of TLRs in autoinflammatory diseases has emerged since autoimmune-prone mice lacking the TLR adaptor molecule MyD88 have markedly reduced anti-chromatin, Sm and rheumatoid factor autoantibodies titers (154) (Table 5). Intracellular TLRs and especially TLR7 and TLR9 have been shown to be activated by self ligands and implicated in the production of autoantibodies against RNA and DNA. Indeed, Leadbetter et al and Viglianti et al demonstrated that autoreactive B cells could be activated via a sequential engagement of the BCR by DNA–associated antigens (anti-chromatin immune complexes) and TLR9 by DNA (155) (156). The role of TLR7 in autoreactive B cells with co-activation of BCR/TLR7 by RNA and RNA-associated antigens has also been highlighted (154). However, there is a discrepancy between TLR7 and TLR9 in their role in promoting autoinflammatory disease. TLR9-deficient mice backcrossed on an autoimmune-prone background present an impaired generation of DNA autoantibodies but more severe disease symptoms including skin disease, increased immune activation, and accelerated lupus nephritis leading to mortality (157) (Table 5). In contrast, TLR7-deficient mice showed impaired generation of RNA-antibodies and less severe disease symptoms (157). Evidences on the fact that the presence of DNA/RNA-containing immune-complexes are important for the establishment of autoimmune responses are coming from the fact that humans and mice lacking functional nucleases, which digest excess of RNA and DNA molecules, or even with a defect in clearance of apoptotic materials develop inflammatory disorders (158, 159). Hence, clearance of potential self ligands circulating in the body is a crucial step in order to avoid disease occurrence (Figure 14).
However, the threshold of detection of these self ligands by immune cells has also to be tightly regulated (Figure 14).

![Figure 14. Balance between self-ligand and receptors.](image)

Indeed, in several studies, TLR7 expression has been implicated to be a pivotal element in accelerating autoimmune phenotype. In mice, a genomic translocation of the Y chromosome into the X of at least 17 genes including TLR7, known as Yaa (Y linked accelerator locus) is associated with a hyperactive phenotype in B cells and DCs (160) (161). This translocation induces a striking acceleration of autoimmunity when the mice are bred on an autoimmune-prone background (162) (Table 5). The fact that TLR7-deficient male bearing the Yaa translocation develop less severe disease emphasizes the potential of TLR7 in triggering autoimmunity (163, 164). Another confirmation of the role of TLR7 as an inducer of autoimmune phenotype came with the study of TLR7 transgenic mice on the non autoimmune background C57/Bl6. These mice display a high inflammatory environment, SLE-like
symptoms and the severity depends on the number of TLR7 genes integrated in their genome (163). However, independently of the expression level, the threshold of detection of the receptor plays also an important role. Thus, repeated TLR7 agonist stimulation leading to desensitization attenuates TLR7-induced inflammatory responses. This TLR7 tolerance is involved in prevention of autoimmune disease such as experimental autoimmune encephalomyelitis (EAE) or arthritis (165).

To date, TLR9 over-expression has never been associated with induction of autoinflammatory damages. However, recent publications report an unexpected role of TLR9 in autoimmunity by delineating why TLR9-deficient mice do develop more disease than WT animals. It seems that TLR9 regulates TLR7-dependent autoantibody production leading to a higher production of RNA-associated autoantibodies (117) through the regulation of TLR7 expression by TLR9 in B cells (118). Therefore, in TLR9\(^{-/-}\) B cells, TLR7 is overexpressed and leads to a stronger autoantibody production.
### Table 5. Evidence for the role of TLRs in mouse models of lupus. Adapted from (112).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Model</th>
<th>Autoantibodies</th>
<th>Glomerulo nephritis</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR9 (^{-/-})</td>
<td>Mixed MRL-Fas (^{br})</td>
<td>↓ dsDNA-specific, ↑ Sm-specific</td>
<td>←</td>
<td>ND</td>
</tr>
<tr>
<td>C57/Bl6 (Fcgr2b (^{-/-}), 56R transgene encoding polyspecific antibody MRL-Fas (^{br})</td>
<td>↓ Switch in polyspecific IgG2a and IgG2b</td>
<td>↑</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>MRL-Fas (^{br})</td>
<td>↓ dsDNA-specific, ↑ Sm-specific</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>C57/Bl6-Fas (^{br})</td>
<td>↑ dsDNA-specific, ↑ Nucleolus-specific, Nucleosome specific</td>
<td>↑</td>
<td>←</td>
<td></td>
</tr>
</tbody>
</table>

| TLR3 \(^{-/-}\) | Mixed MRL-Fas \(^{br}\) | ← | ← | ← |
| MyD88 \(^{-/-}\) | Mixed MRL-Fas \(^{br}\) | ↓ dsDNA-specific, ↓ Sm-specific | ND | ND |
| C57/Bl6 (Fcgr2b \(^{-/-}\)) | ↓ Switch in polyspecific IgG2a and IgG2b | ND | ND |
| C57/Bl6 (Fcgr2b \(^{-/-}\), 56R transgene encoding polyspecific antibody | ↓ Switch in polyspecific IgG2a and IgG2b | ND | ND |

| TLR7 \(^{-/-}\) | MRL-Fas \(^{br}\) | ↓ Sm-specific | ↓ | ND |
| C57/Bl6 (564 transgene encoding nucleic acid-specific antibody) | ↓ 564 nucleic acids specific | ND | ND |

| Yaa TLR7 duplication | BXSB | ↑ diverse specificities | ↑ | ↑ |
| C57/Bl6 (Fcgr2b \(^{-/-}\)) | ↑ Nucleolus-specific | ↑ | ↑ |
| C57/Bl6 (Sle1) | ↑ ds-DNA-specific, ↑ snRNP-specific, ↑ Glomerulus-specific | ↑ | ↑ |

| TLR7 transgenic | C57/Bl6 | ↑ diverse specificities | ↑ | ↑ |
4-4-2 How self-ligand can reach endosomal TLRs

Although systemic autoimmunity is characterized by multiple syndromes with extremely variable clinical manifestations and different genetic dispositions, the loss of self tolerance at the level of B cells and autoantibodies is remarkably focused on a small group of autoantigens. The commonly targeted autoantigens can be divided into three general categories: DNA-related, RNA-related and immune complexes. Self-nucleic acids released by apoptotic or damaged cells are rapidly degraded by serum nucleases. However, dominant autoantigens are released from apoptotic cells and found in apoptotic blebs. These self-nucleic acids could interact with cellular proteins such as High Mobility Group Box proteins (HMGBs), ribonucleoproteins, antimicrobial peptides or autoantibodies to form complexes thus increasing their protection against nucleases and their endocytosis to induce TLR7 or TLR9 activation.

HMGBs proteins are highly expressed in the nucleus, where they regulate chromatin structure and transcription, but they are also present in the cytosol and in extracellular fluids. HMGB1 has been shown to participate in the activation of several immune receptors including TLRs (167). HMGB1-self nucleic complexes could interact with the receptor for advanced glycation end-products (RAGE) on the cell surface and lead to the recruitment and activation of the TLR9-MyD88 pathway (168). Because the lack of HMGBs impairs severely the activation of TLR3, TLR7 and TLR9 by their cognate ligands, it has been suggested that HMGBs molecules act as universal sentinels for nucleic-acids (169) (Figure 15).

Antimicrobial peptides such as LL37 belong to one of the first evolved chemical defense mechanism of eukaryotes against bacteria, protozoa, fungi and viruses (170). Antimicrobial peptides are produced at damaged epithelial cell surfaces, where they prevent microbial
invasion by direct killing pathogens. LL37 is also able to bind self-DNA or RNA and form aggregates that are protected from nucleases. These complexes are able to enter pDCs through proteoglycan-mediated attachment of LL37 to cell membrane and lipid-raft-mediated endocytosis (171). Thus, by binding self-DNA, LL37 forms condensed structures that are delivered and retained in early endocytic compartments to trigger TLR9 and plays a role in breaking innate tolerance to self-DNA (172) (Figure 15).

Nucleic acids bound to autoantibodies are endocytosed through B cell receptors interaction in B cells (156). In DCs, FCγRIIA (CD32) delivers nucleic-acid containing immune complexes to intracellular lysosomes, where they could activate TLRs and induce a signaling cascade (173) (Figure 15).

**Figure 15. Self ligands can reach endolysosomes.** 1. TLR7 and TLR9 are activated upon stimulation with viral ssRNA and CpG DNA, respectively. 2. Viruses that have entered the cytoplasm are engulfed by autophagosomes and deliver viral nucleic acids to endolysosomes. 3. An HGMB1-DNA complex released from damaged cells is captured by RAGE. 4. Autoantibodies recognizing self-DNA or RNA bind to FCγRIIA. 5. LL37, an antimicrobial peptide, associates with endogenous DNA. These
proteins are responsible for the delivery of endogenous nucleic acids to endolysosomes where they are recognized by TLR7 or TLR9. Adapted from (174).

4-5 SLE: a disease of TLR-driven amplification

SLE disease establishment is dependent of the microbial environment. It has been shown that SLE symptoms in lupus-prone mice (MRL-FAS\textsuperscript{lpr}) could be decreased once the animals are fed with ultrafiltered PAMP free diet (175). However, the impact of TLRs on lupus is evocated by the fact that Myd88-deficient mice on the MRL/lpr background are protected from the disease (176).

4-5-1 TLR-driven B cell activation

Nuclear antigens activate antinuclear B cells through BCR/TLR engagement. The use of B cells bearing a transgenic BCR specific for IgG2a allotype called AM14 has been useful to investigate the role of BCR/TLR co-engagement. AM14 B cells are able to proliferate upon stimulation by immunocomplexes composed of either DNA-associated or RNA-associated proteins and IgG2a antibodies (155) (154). Nevertheless, BCR is required for the delivery of the relevant autoantigen to intracellular compartments that contain TLR7 and TLR9. Autoreactive B cells are uniquely stimulated by these nuclear antigens released from dying cells because they express receptors of both the innate (TLR7 and TLR9) and the adaptive immune system (BCR). As discussed above, co-ligation of the BCR and TLRs induces proliferation and plasmablast differentiation. This dual stimulation may be sufficient to overcome the regulatory mechanism occurring in absence of T cell help that normally prevent activation of autoreactive B cells in the periphery. Thus, the recognition of endogenous nuclear antigens by TLRs within the endosomal compartments of autoreactive B cells is
necessary for antibody production and facilitates the generation of antinuclear antibody-secreting cells. Autoantibodies produced in SLE are thought to have a direct pathogenic effect, and may promote ongoing tissue damage and cell death in target organs. This release of additional endogenous TLR ligands from necrotic or apoptotic cells could then promote further activation and expansion of autoreactive B cells, creating a cell-autonomous activation cycle.

4-5-2 TLR-driven pDCs activation

Type I IFNs have many effects on almost every cell type in the immune system. In pDCs, TLR7 or TLR9 signaling results in a massive and rapid production of type I IFNs (177). Type I IFNs have been implicated in the pathogenesis of SLE, since deficiency of type I interferon receptor (IFNRI) in lupus-prone mice reduces lupus-like disease (178, 179). In lupus patients interferon level in the sera correlates with disease activity (180). Given these associations, activation of nuclear acid-sensing TLRs in pDCs by endogenous ligands is likely to occur in the context of SLE. RNA or DNA associated immune complexes are potent stimulators of type I IFN production. Stimulation of murine pDCs with U1 small nuclear ribonucleoprotein immune complexes leads to a TLR7-dependent type I IFN production (181). In humans, the stimulation of pDCs by immune-complexes is dependent of the FcγRIIa at least (173). One important outcome of type I IFN secretion is the promotion of B cell activation. In addition, type I IFNs facilitate antibody production, particularly of the inflammatory immunoglobulin IgG2a and IgG3 (182, 183). This allows a B cell-pDC activation cycle in SLE, whereby type I IFNs and autoantibodies provide positive feedback to pDCs and autoreactive B cells (Figure 16).
Figure 16. Current model according virus-induced IFN-α initiates a self perpetuating feedback loop to drive autoantibody production. a. Viral infection induces pDCs to produce IFN-α. b. IFN-α upregulates TLR7 expression by B cells, promotes cell death and increased release of certain RNA autoantigens, prime pDCs to respond more efficiently to immune complexes. c. Autoreactive B cells that have upregulated TLR7 bind the autoantigens released from apoptotic cells. d. The BCRs of these cells deliver RNA autoantigens to TLR7, and engagement of TLR7 leads to proliferation and differentiation of these autoreactive B cells. e. These B cells produce autoantibodies, which combine with autoantigens form immunocomplexes. f. Immunocomplexes bind receptors of the Fc portion of IgG (FcγRs) at the surface of pDCs that are derived from circulating monocytes. g. The internalized autoantigen engages TLR7, and more IFN-α is then produced by pDCs (184).
**4-5-3 TLR-driven cDCs activation**

TLR signaling in conventional DCs induces their maturation in efficient APCs and leads to cytokine production. cDCs bear Fc receptor at the surface that can bind nucleic acid-containing immuno-complexes. This ligation facilitates import of the immune-complex in the endosomal compartments leading to the activation of TLRs (185). Type I IFNs are also potent activators of conventional DCs. Thus, immune complexes can directly activate TLRs in myeloid DCs, and these cells can be further stimulated by type I IFNs that are produced by pDCs. DC activation induces their maturation and allows efficient T cell priming. Polarized CD4+ T cells can then provide cognate help to autoreactive B cells completing an activation cycle between B cells, T cells and DCs.

**4-6 Treatments for SLE**

There is no permanent cure for SLE. The aim of the treatment is to decrease symptoms and protect organs by lowering inflammation and/or the level of autoimmune activity in the body. Many individuals displaying mild symptoms may need no treatment or only intermittent courses of anti-inflammatory medications. Those with a more serious disease, involving damage to internal organs may require high doses of corticosteroids in combination with other medications that suppress the immune system.

Nonsteroidal anti-inflammatory drugs, such as aspirin or ibuprofen are helpful in reducing inflammation and pain in muscles, joints and other tissues. Corticosteroids as methylprednisolone are more potent than nonsteroidal anti-inflammatory drugs in reducing and restoring function when the disease is the most active. Corticoids inhibit NF-κB activity, thus leading to an anti-inflammatory effect. However, SLE treatment requires much higher corticosteroid
dose than other autoimmune diseases. This resistance of lupus patient to corticosteroid treatment is in part explained by a recent study that showed that TLR stimulation in pDCs by self nucleic acids-containing immuno-complexes reduces therapeutic activity of glucocorticoids by protecting pDCs from apoptosis (186) (187). Antimalarial medications are also found to be particularly effective for SLE patients with fatigue, skin involvement and joint disease (188). Usually, hydroxychloroquine alone or in combination with chloroquine or quinacrine is recommended in cases of resistant skin disease. For more severe forms of lupus, immunosuppressive or cytotoxic treatment could be considered. Most recent research indicates benefits of Rituximab (189) or Abetimus (189). Rituximab is an antibody against CD20, which is found at the surface of all mature B cells and is used to decrease circulating B cells number. Abetimus sodium has been designed to deplete specifically B lymphocytes that produce double-stranded DNA autoantibodies.

A new way to treat patients with strong brain or kidneys manifestations is plasmapheresis, a process of removing and treating the blood to get rid of antibodies and immunocomplexes before it is returned into the body (190, 191).
RESULTS

TLR8 deficiency leads to autoimmunity in mice
TLR8 deficiency leads to autoimmunity in mice

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Introduction

TLRs, mammalian homologs of the Drosophila receptor Toll, detect evolutionarily conserved structures expressed by different groups of microbes and play a major role in the elimination of infections by coactivation of the immune system (1). TLRs are composed of an ectodomain of leucine-rich repeats, which are involved in ligand binding, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that interacts with TIR domain–containing adaptor molecules. The signaling pathways activated by different TLRs involve a family of 5 adaptor proteins, which couple to downstream protein kinases and ultimately lead to the activation of transcription factors such as NF-κB, MAPKs, and members of the IFN-regulatory factor family (2).

The family of TLRs consists of 10 members in humans (TLR1–TLR10) and 12 members in mice (TLR1–TLR9 and TLR11–TLR13). The mammalian TLRs that are located in the plasma membrane recognize bacterial membrane components, whereas the TLRs that detect nucleic acid–based ligands are predominately located within endosomal compartments (3). The nucleic acid–sensing TLRs include TLR3, TLR7, TLR8, and TLR9. TLR3 senses viral and synthetic double-stranded RNA, TLR7 recognizes viral single-stranded RNA (ssRNA) and synthetic imidazoquinoline immune response modifiers and polyT oligonucleotides, and TLR9 detects unmethylated CpG containing DNA motifs, found in bacterial and viral DNA (3). Although TLR7 and TLR8 are phylogenetically very close, their natural ligand viral ssRNA stimulates human TLR7 and TLR8 and mouse TLR7, but not mouse TLR8, leading to the belief that TLR8 is biologically inactive in mice (4). However, recent studies have shown that TLR8 is dynamically expressed during mouse brain development and functions as a negative regulator of neurite outgrowth and an inducer of neuronal apoptosis (5). Moreover, stimulation of murine TLR8–transfected HEK293 cells with a combination of imidazoquinoline immune response modifiers and polyT oligonucleotides leads to NF-κB activation, which suggests that mouse TLR8 is functional (6). Upon ligand binding, most TLRs form homodimers; however, some ligands require TLR cooperation of 2 different TLRs in activating downstream signaling cascades. For example, TLR1/2 and TLR2/6 heterodimers are required for the detection of bacterial cell wall components (7–9).

TLRs are expressed on many cell types, including human and mouse DCs and B cells (1, 10, 11). The recognition of invading microbes by TLRs on DCs induces the activation and maturation of DCs, which instruct and support T cell activation and lead to cell-mediated adaptive immune response (12). Interaction between activated antigen-specific T cells and naive B cells promotes B cell expansion and differentiation, leading to a humoral immune response. Recent studies have shown that direct TLR-mediated activation of B cells is also required for eliciting humoral immune responses (13), although this has been challenged (14). However, TLR9 expression in B cells and not other antigen-presenting cells has been shown to be critical for the regulation of IgG isotype patterns (15). Moreover, different B cell subsets—including follicular, marginal zone (MZ), B1, and Peyer patch B cells—express multiple TLRs and have the ability to proliferate and secrete polyclonal antibodies to a variety of TLR agonists, even in the absence of DC activation or T cell help (11). Thus, splenic MZ B cells and B1 cells, which are located mainly in the peritoneal and pleural cavities, bridge innate and adaptive immunity by producing a rapid T cell–independent antibody response (16).

There is increasing evidence that TLRs can also recognize self-antigens released from damaged or stressed host tissue and that such recognition can lead to the development of autoimmune disease, including SLE (17). Most interest has focused on the nucleic acid–sensing TLRs TLR3, TLR7, and TLR9 (18, 19). In humans, the etiology of SLE is unknown, but inherited genes, viruses, ultraviolet light, and certain medications may all play some role. SLE patients experience kidney dysfunction leading to renal failure and a wide and variable range of symptoms, including arthritis, skin rashes, fever, and brain inflammation. The association of the nucleic acid–sensing TLRs with lupus originates mainly from mouse models, where activation of any of these receptors in vivo...
of MZ and peritoneal cavity B1 B cells and increased levels of circu-
stantigens, whereas shown that the Yaa locus in part mediates the autoimmune phe-

segue several murine models of lupus. Interestingly, recent studies have

delves an impressive acceleration of autoimmunity when bred to

–/– mice. We conclude that murine TLR8 plays an important

elevated levels of IgG autoantibodies to ribonucleoprotein (RNP),

–/– mice. To investigate the functional role of TLR8, we analyzed the

responses of Tlr8 –/– cells to various TLR ligands. Tlr8 –/– and WT

BMDCs, BM-derived macrophages (BMMs), and total splenocytes

were stimulated with various doses of TLR7 ligand R848, TLR3

t uptake

were assessed by ELISA. The production of IL-6, IL-12p40, and

were collected, and the protein levels of IL-6, IL-12p40, and TNF

and TLR7 ligand polyA:U, TLR9 ligand CpG, TLR3 ligand polyIC,

showed no obvious developmental or behavioral abnormalities. To study the natural expression of Tlr8, the expression of the lacZ reporter gene that was introduced under the TLR8 promoter in Tlr8 –/– mice was determined by β-galactosidase assay on cryostat organ sections. In the spleen, the site of TLR8 expression was con-

fined in few cells in the T cell area of the white pulp, consistent with the location of DCs, while there was widespread expression in the red pulp, which is rich in macrophages (Figure 1D). Within the mes-

enteric lymph nodes, the site of TLR8 expression was confined to the perifollicular regions, where DCs are also located (Figure 1D).

To determine the functional role of TLR8, we analyzed the responses of Tlr8 –/– cells to various TLR ligands. Tlr8 –/– and WT

BMDCs, BM-derived macrophages (BMMs), and total splenocytes were similar between WT and Tlr8 –/– mice (Figure 1A) were generated by a

high-throughput, automated approach (see Methods and ref. 28).

BMDCs, BM-derived macrophages (BMMs), and TLR8-deficient Tlr8 –/– mice were born at the expect-
ed Mendelian ratio; had normal growth, size, fertility, and life span; and

showed no obvious developmental or behavioral abnormalities. To study the natural expression of Tlr8, the expression of the lacZ reporter gene that was introduced under the TLR8 promoter in Tlr8 –/– mice was determined by β-galactosidase assay on cryostat organ sections. In the spleen, the site of TLR8 expression was con-

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BMDCs, BM-derived macrophages (BMMs), and total splenocytes were similar between WT and Tlr8 –/– mice (Figure 1A).

However, WT and Tlr8 –/– BMMs and total splenocytes showed

similar cytokine responses to R848, CpG, and LPS (Supplemental

Figure 1; supplemental material available online with this article; doi:10.1172/JCI42081DS1, and data not shown). Moreover, upon R848 stimulation, Tlr8 –/– BMDCs showed higher production of

Ifnb at the mRNA level than did WT cells (Figure 2B).

To further study whether the defect in the Tlr8 –/– BMDCs is restricted to the TLR7 ligands R848 and poly(AU), we compared the responses of WT and Tlr8 –/– BMDCs to a panel of known TLR7

ligands, such as TLR7/8 ligand CL075, which acts as a stronger ligand for human TLR8 than for human TLR7; TLR7/8 ligand CL097; and TLR7/8 ligand ssRNA-DR/LyoVec, composed of

ssRNA complexed with cationic lipid LyoVec to facilitate its uptake by the cells. We found that Tlr8 –/– BMDCs produced markedly higher amounts of IL-12p40 compared with WT cells in response to all the TLR7 ligands tested (Supplemental Figure 2). Moreover, we assessed the effect of delivering R848 or poly(AU) intracellularly using lipofectamine, a reagent for nucleic acid transfer. Although the responses of WT and Tlr8 –/– BMDCs were higher compared with non–lipofectamine-treated cells, the Tlr8 –/– cells were again higher producers than WT controls (data not shown). Next, we evaluated the activation of Tlr8 –/– and WT BMDCs based on the

Results

Generation and characterization of Tlr8 –/– mice. To investigate the func-
tion of TLR8 in vivo, Tlr8 –/– mice (Figure 1A) were generated by a

high-throughput, automated approach (see Methods and ref. 28).
The expression of Tlr8 mRNA was abrogated in Tlr8 –/–, but not WT, BM-derived DCs (BMDCs), as assessed by RT-PCR and Northern

Figure 1

Targeted disruption of the mouse Tlr8 gene. (A) PCR analysis of mouse genomic DNA with specific primers gave a 240-bp band for Tlr8 +/+, a 589-bp band for Tlr8 –/–, and both bands for Tlr8 +/–. M, molecular weight marker (100-bp DNA ladder; Invitrogen). (B) Expression of Tlr8 and Hprt mRNA by WT and Tlr8 –/– BMDCs either untreated or stimulated with 50 nM R848 or 1 ng/ml LPS for 4 hours, as determined by RT-PCR. (C) Northern blot analysis of RNA from untreated, 1 ng/ml LPS–, or 50 nM R848–stimulated WT and Tlr8 –/– BMDCs. Ethidium bromide staining after RNA transfer to the membrane is included as control (bottom). (D) Reporter gene expression (lacz reporter, blue) in spleen and mesenteric lymph node sections from Tlr8 –/– mice. (A–D) Data are representative of 2–4 independent experiments.

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expression of CD86 and MHC class II (MHCII). Untreated WT and Tlr8–/– cells had similar levels of CD86 and MHCII; however, upon stimulation with 25 or 50 nM R848, Tlr8–/– BMDCs showed higher augmentation of the expression of these surface molecules compared with WT cells (Figure 2C). Moreover, we compared the responses of WT and Tlr8–/– splenic DCs and found that TLR8 deficiency led to higher IL-12p40 production upon stimulation with R848, but not with LPS or CpG (Figure 2D). Next, Tlr8–/– and

Figure 2
Enhanced responses to TLR7 ligands by Tlr8–/– DCs. (A) BMDCs from WT and Tlr8–/– mice were stimulated with the indicated amounts of R848, polyA:U, CpG, polyI:C, and LPS. After 20 hours, the concentrations of IL-6, IL-12p40, and TNF in the culture supernatants were assessed by ELISA. (B) WT and Tlr8–/– BMDCs were left untreated or stimulated for the indicated times with 50 nM R848. Total RNA was extracted from the cells, and expression of Ifnb was assessed by Q-PCR. (C) WT and Tlr8–/– BMDCs were left untreated or stimulated with the indicated amounts of R848 for 16 hours, and cell surface expression of MHCII and CD86 was analyzed by flow cytometry on gated CD11c+ cells. (D) WT and Tlr8–/– CD11c+ splenic cells were isolated by magnetic-activated cell sorting, then left unstimulated or stimulated for 16 hours with 30 nM R848, 10 ng/ml LPS, or 30 nM CpG. The production of IL-12p40 in the culture supernatants was assessed by ELISA. * P < 0.05. (E) WT and Tlr8–/– mice (8 weeks old) were injected i.p. with 100 μl of 10 μM R848. Sera were collected 2 or 6 hours later, and serum levels of IL-6 and IL-12p40 were determined by ELISA. (A, D, and E) Data are mean ± SD of 3–4 (A and D) or 8–10 (E) mice per group and are representative of 2–5 independent experiments. (B) Data are mean ± SD of duplicates and are representative of 2 independent experiments.
WT mice were injected i.p. with R848, and their sera were collected 2 and 6 hours later. Th8−/− mice exhibited IL-6 and IL-12p40 production in their sera similar to that of WT controls (Figure 2E). Thus, TLR8 deficiency in DCs, but not in macrophages or total splenocytes, led to increased response to TLR7 ligands.

To further confirm that the increased response of Th8−/− DCs to R848 stimulation is caused by the absence of TLR8 and not by off-target effects, since TLR7 is located on the same chromosome as TLR8 and only 70 kb apart, we tested whether the addition of a functional Tlr8 gene restores the phenotype in Th8−/− BMDCs. Indeed, Tlr8 expression in Th8−/− BMDCs substantially reduced their hyperresponsiveness to R848 stimulation, but the response to the TLR9 ligand CpG was unaltered (Supplemental Figure 3).

Increased TLR7 expression and NF-κB activation in Th8−/− BMDCs in the absence of TLR8. Quantitative real-time PCR (Q-PCR) analysis revealed expression of murine TLR8 in WT BMDCs; upon stimulation with R848, Tlr8 mRNA levels were dramatically downregulated at 2 hours, and expression returned to normal levels after 6 hours (Figure 3A), suggestive of transient negative regulation of TLR8 expression. To further explore this phenomenon, we stimulated BMDCs with IFN-γ and TNF-α and found that Tlr8 mRNA expression was affected (Supplemental Figure 4, A and B), which suggests that regulation of TLR8 expression depends on the activation status of the cell. We next examined whether the higher cytokine production in response to TLR7 ligands in Th8−/− BMDCs correlates with altered TLR7 expression.

Th8−/− BMDCs were transfected with 50 pmol Tlr8-FLAG or GFP mRNA. 5 hours later, cells were harvested, total protein lysates were prepared, and the expression of Tlr7, Tlr8-FLAG, and p-actin were assessed by Western blot. The Tlr7/p-actin ratio is shown at right. (A, B, D, and E) Data are mean ± SD of triplicates and are representative of 3–5 independent experiments. (C, F, G, and H) Data are representative of 3–5 (C, F, and H) or 2 (G) independent experiments.
were expressed in WT BMMs; upon stimulation with R848, expression of both was downregulated and almost totally abolished by 16 hours (Figure 3, D and E). Untreated Tlr8−/− BMMs expressed higher levels of Tlr7 mRNA than did WT cells, but upon stimulation with R848, this difference was dramatically diminished: by 4 hours after stimulation, both Tlr8−/− and WT BMMs expressed similar levels of Tlr7 mRNA (Figure 3E). At the protein level, untreated WT and Tlr8−/− BMMs expressed similar levels of TLR7, and although Tlr8−/− cells showed higher expression of TLR7 at 4 hours after R848 stimulation, this difference was reduced by the 8-hour time point (Figure 3F). Thus, in untreated DCs, but not macrophages, TLR8 deficiency correlated with higher expression of TLR7 protein, and this difference in TLR7 expression further increased upon activation with R848. To further confirm the dose-response relationship between TLR8 and TLR7 expression, we evaluated the levels of TLR7 in Tlr8−/−/Tlr7−/− (WT), Tlr8−/−/Tlr7−/+ (Tlr8−/−), Tlr8−/+Tlr7−/−, and Tlr8+/+Tlr7−/− (Tlr7−/−) BMDCs. We found that TLR7 expression in Tlr8−−/Tlr7−/− DCs, at both the RNA and the protein levels, was lower than in Tlr8−/− cells and higher than in Tlr7−/− cells (Supplemental Figure 5, A and B), suggestive of TLR8-mediated regulation of TLR7 expression in DCs.

In addition, we investigated whether expression of TLR8 in Tlr8−/− BMDCs affects TLR7 protein levels. Tlr8−/− BMDCs were transfected with Tlr8-FLAG or GFP mRNA, and the expression of TLR7 and TLR8 was detected using antibodies against TLR7 and FLAG, respectively. Expression of TLR8 in Tlr8−/− BMDCs led to a dramatic reduction in the expression of TLR7 compared with cells transfected with GFP (Figure 3G), which suggests that TLR8 expression leads to reduced expression of the TLR7 protein.

Signaling via TLRs occurs through the recruitment of the adapter molecule MyD88 and/or Toll/IL-1 receptor domain–containing adaptor inducing IFN-β (TRIF) and leads to activation of NF-κB and MAPKs (2). We therefore analyzed R848-induced NF-κB and MAPK activation by Western blot analysis in Tlr8−/− cells. R848 stimulation of Tlr8−/− BMDCs showed substantially faster and stronger phosphorylation of NF-κBp65 compared with WT cells (Figure 3H). However, degradation of IκBα and phosphorylation of JNK, ERK1/2, and p38 were similar in WT and Tlr8−/− cells (Supplemental Figure 6).

Defect of MZ B cells in Tlr8−/− mice. We noticed that Tlr8−/− mice had exacerbated splenomegaly compared with sex- and age-matched WT controls (Figure 4A and Table 1). Flow cytometric analysis of various
The MZ defect was further confirmed by the reduction of assessed the MZ B cell population in with the flow cytometric results, the characteristic rim of B220 by FACS analysis (Supplemental Figure 7, B and C). In agreement in WT and to determine whether the reduced MZ B cell phenotype we observed ous activation was observed on B and T lymphocytes, as evaluated by CD69 and CD86 expression (Supplemental Figure 7A and data not shown). Furthermore, the myeloid progenitor fractions of the splenic cell compartments showed that BM and thymic development was found to be normal, as assessed and the CD11c

Next, we examined the expression of TLR7 and TLR8 in MZ and follicular B cells by Q-PCR. Both MZ and follicular B cells from WT mice were expressing Tlr8 mRNA, in accordance with previ-ously published studies (10, 11), and TLR7 expression was at least 2 times higher in Tlr8−/− cells (Supplemental Figure 8A). When we tested the expression of TLR8 by Q-PCR in WT MZ B cells, we were unable to detect any Tlr8 mRNA, in accordance with previ-ously published data (10, 11). However, by classical PCR, we found that, indeed, both MZ and follicular B cells of WT mice expressed TLR8, as was the case for WT BMDCs and BMMs, whereas TLR8 expression was absent in Tlr8−/− BMDCs (Supplemental Figure 8B). We further compared the ability of WT and Tlr8−/− MZ B cells to respond to R848, and found that both genotypes produced similar levels of IL-6 upon R848 and CpG stimulation (Supplemental Figure 8C). Moreover, they showed similar expression of the activation markers CD86 and CD69 and the chemokine receptors CXCR5 and CXCR4 (Supplemental Figure 8D and data not shown). Thus, although MZ B cells expressed both TLR7 and TLR8, and we observed higher expression of TLR7 in Tlr8−/− than WT cells, Tlr8−/− MZ B cells were not hyperresponsive to R848 stimulation.

**Table 1**

<table>
<thead>
<tr>
<th>Frequency of splenic cell subsets in WT and Tlr8−/− mice</th>
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<tbody>
<tr>
<td><strong>WT</strong></td>
</tr>
<tr>
<td>CD11b+</td>
</tr>
<tr>
<td>Resident monocyte (CD11b−GR-1+)</td>
</tr>
<tr>
<td>Inflammatory monocyte (CD11b−GR-1+)</td>
</tr>
<tr>
<td>Neutrophil (CD11b−GR-1+)</td>
</tr>
<tr>
<td>CD11c+</td>
</tr>
<tr>
<td>Plasma cell (B220+CD11c−Ly6C+)</td>
</tr>
<tr>
<td>DC (B220+CD11c−Ly6C−)</td>
</tr>
<tr>
<td>CD3+</td>
</tr>
<tr>
<td>CD3−CD4+CD8−</td>
</tr>
<tr>
<td>CD3−CD4−CD8+</td>
</tr>
<tr>
<td>CD19−</td>
</tr>
<tr>
<td>Follicular B (CD19−CD23−CD21−)</td>
</tr>
<tr>
<td>MZ B (CD19+CD23−CD21+)</td>
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<tr>
<td>Immature (CD19+CD23+CD21+)</td>
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Data are from 4.5-month-old female WT and Tlr8−/− mice. All values denote percentages (mean ± SD). Total spleen cell counts were 152 ± 10 × 10^6 ± 16.4 × 10^6 for WT and 264 ± 10 ± 26.1 × 10^6 for Tlr8−/− (P < 0.01). *P < 0.05 versus WT, **P < 0.01 versus WT.
shown to promote autoantibody production and pathological disease associated with lupus (24–26). To determine whether the augmented TLR7 expression we observed in Tlr8−/− mice correlates with autoimmunity, serum levels of IgM and IgG isotypes were assessed in 3-month-old WT, Tlr8−/−, and Tlr7−/− mice by ELISA. Levels of IgM and IgG2a were significantly higher, whereas levels of total IgG, IgG2b, and IgG3 were similar, in Tlr8−/− compared with WT mice (Figure 6). In contrast, Tlr7−/− mice showed significantly lower levels of IgM, total IgG, IgG1, and IgG2a, but similar levels of IgG2b and IgG3, compared with WT mice (Figure 6). Tlr8−/− mice also had significantly lower levels of IgG1 compared with control mice (Figure 6). Next, we assessed the levels of IgG autoantibodies against smRNP, RNP, and dsDNA and found that the titers of all 3 autoantibodies were significantly increased in Tlr8−/− versus WT sera (Figure 7A). To further study the role of TLR8 in the generation of antinuclear Abs (ANAs), we used the fluorescent ANA assay on Hep-2 cells as a sensitive detection method for Abs to both RNA- and DNA-containing autoantigens. Strikingly, Tlr8−/− sera were strongly positive for ANAs, whereas WT mice had weak ANA staining and both Tlr7−/− and Tlr8−/− Tlr8−/− mice were negative (Figure 7B). Moreover, Tlr8−/−/Tlr7−/− sera showed ANA titers lower than those in Tlr8−/− samples (Supplemental Figure 5D). Since we detected increased autoantibody production in Tlr8−/− sera, we analyzed the B cells in these mice and found that the B220lo CD138+ population, most likely representing Ab-producing cells, was increased (Supplemental Figure 8E). Immunostaining and fluorescence microscopy of WT, Tlr8−/−, and Tlr7−/− kidneys revealed that glomerular deposition of IgM, IgG, and complement component C3 were substantially greater in Tlr8−/− mice than in WT controls at 4 months of age, whereas Tlr7−/− mice showed diminished deposition of IgM, IgG, and C3 compared with WT mice (Figure 7C). In addition, Tlr8−/− Tlr7−/− mice showed glomerular deposition of IgM and IgG that was reduced compared with Tlr8−/− mice, but greater than that in Tlr8−/− Tlr7−/− mice (Supplemental Figure 5E). Taken together,
these results demonstrate that TLR8 deficiency leads to the production of autoantibodies to nucleic acid–containing material and disease pathogenesis as a result of TLR7 overexpression.

Discussion

Murine TLR8 has been previously suggested to be nonfunctional, based on the observation that ligand stimulation of human, but not mouse, TLR8 induces NF-κB activation in transfected HEK293 cells and the fact that TLR7-deficient mice do not respond to R848, even though TLR8 is present (29, 30). We showed in the present study that TLR8 deficiency in DCs led to hyperresponsiveness to TLR7 ligands and faster and stronger NF-κB activation as a result of TLR7 overexpression (Figures 2 and 3). Thus, TLR8 can modify the expression and signaling of TLR7 and plays an important biological role, since Tlr8−/− mice, but not Tlr8−/−Tlr7−/− mice, developed features of lupus-like syndrome.

Why does the absence of TLR8 lead to TLR7 overexpression? We speculate that TLR8 recognizes an endogenous, yet uniden-
tified, ligand; in WT cells, constant detection of this ligand and signaling through TLR8 leads to a certain steady-state activation of the cell. However, the absence of TLR8 signaling in Tlr8−/− DCs leads to lower steady-state cellular activation and, as a result, to higher TLR7 expression. In accordance with our speculation, we found that TLR7 expression was downregulated in activated DCs. Indeed, activation of DCs with either IFN-γ or TNF-α led to lower TLR7 expression compared with untreated cells (Supplemental Figure 4, A and B). How might a cell benefit from the ability to alter the expression of its TLR7 receptor depending on its activation status? TLR7 is a major regulator of DC activation — through the production of cytokines resulting from detection of viral RNA or endogenous RNA ligands — that fine-tunes DC responses to achieve clearance of viruses while limiting the amount of inflammation in order to avoid toxicity and tissue damage.

Although both DCs and macrophages expressed Tlr7 and Tlr8 (Figure 3, A, B, D, and E), we observed that TLR8 deficiency led to increased cytokine production by Tlr8−/− DCs (Figure 2, A, B, and D), but not macrophages (Supplemental Figure 1A), which suggests that the effect of TLR8 to TLR7 is cell type specific. This discrepancy between these 2 cell types can be attributed to the different kinetic pattern of TLR7 expression. Indeed, we found that upon R848 stimulation, Tlr8−/− DCs sustained constant higher TLR7 expression than did WT cells (Figure 3, B and C), whereas Tlr8−/− macrophages showed early transient TLR7 overexpression compared with control cells that disappeared by 4 hours at the mRNA level (Figure 3E) and was dramatically diminished by 8 hours at the protein level (Figure 3F). Thus, TLR7 expression is distinct for different cell types and might be correlated with expression of TLR8 and/or variability of the gene expression profiles induced in various cell types after stimulation with TLR agonists or pathogens.

In vivo treatment with LPS promotes MZ B cell migration from the MZ into the splenic follicles; this feature is not unique to TLR4, since agonists to TLR2, TLR3, and TLR7 also stimulate MZ B cells to become activated and leave the MZ (31, 32). In the present study, we demonstrated that TLR8 deficiency led to a marked reduction in the MZ B cell compartment (Figure 4, B and C). Since murine MZ B cells expressed both Tlr7 and Tlr8 (Supplemental Figure 8, A and B) and can proliferate in response to TLR7 agonists (11, 33), it is tempting to speculate that the low number of MZ B cells in Tlr8−/− mice may be caused by increased activation of autoreactive B cells in the MZ (as a result of TLR7 overexpression) and their exit from this compartment. Although freshly isolated Tlr8−/− MZ B cells had higher expression of TLR7 compared with WT cells (Supplemental Figure 8A), upon stimulation with R848, both genotypes produced similar levels of IL-6 (Supplemental Figure 8C). Since the expression pattern of different TLRs in immune and nonimmune cells appears to be highly regulated and complex, we cannot exclude the possibility that MZ B cells are activated indirectly by another cell type found in the MZ, for example, MZ DCs. Interestingly, a recent study showed that transgenic mice that overexpress TLR7 have a markedly reduced MZ B cell compartment compared with WT mice (24). Moreover, male C57BL/6 mice that carry the Yaa locus have 2 copies of Tlr7 and display impaired development of MZ B cells, whereas Tlr7-deficient male C57BL/6 mice that carry the Yaa locus have 1 Tlr7 allele and exhibit normal numbers of MZ B cells (24, 34). In line with our speculation that TLR8 is important for MZ B cell development as a result of TLR7 overexpression was our observation of a marked increase in the MZ B cell compartment of Tlr8−/−/Th7−/− compared with WT mice (Figure 4B). In addition, a similar increase in the number of MZ B cells was also observed in MyD88/TRIF double-deficient mice (data not shown). Furthermore, we showed that in irradiated chimERIC mice, TLR8 expression on radiosensitive hematopoietic cells, but not on radioresistant structural cells, was necessary and sufficient for the development of normal numbers of MZ B cells (Figure 4D). Thus, we conclude that tight regulation of TLR7 expression and signaling by BM-derived cells are critical for the development and/or maintenance of a normal MZ B cell compartment, and TLR8 plays a pivotal role in this process.

The reduction in MZ B cells in Tlr8−/− mice was also associated with a severe reduction in B1 B cells compared with WT mice (Figure 5A). B1 B cells are enriched in the peritoneal and pleural cavities, but are found at low frequency in the spleen and are known to participate in a very early T-cell–independent phase of immune responses against bacteria, viruses, and certain parasites (35, 36). Despite the importance of B1 B cells in protection from infections, little is known about how these cells are retained in the body cavities and the molecular signals required for their migration out of their compartment for antigen clearance. However, a recent study has shown that B1 B cells express extremely high levels of integrins and that direct signals through TLRs induce a massive egress of B1 B cells from the peritoneal cavity, which is associated with coordinated downregulation of integrins and CD9 (37). Moreover, the study revealed that germ-free mice accumulate significantly greater numbers of B1 B cells in the peritoneal cavity compared with mice kept under specific pathogen–free conditions (37), which suggests that TLR signaling is also important in steady-state conditions for the maintenance of immune system homeostasis. Since B1 B cells express various TLRs, including TLR7 and TLR8 (11, 33), the low numbers of B1 B cells in Tlr8−/− mice could be caused by increased TLR7 signaling in B1 B cells that leads to rapid mobilization of these cells out of the peritoneal cavity and participation in immune responses. It is also possible that TLR8 deficiency leads to defects in the production (by cells other than B1 B cells) of cytokines and chemokines that are critical for the migration and development of B1 B cell precursors and B1 B cells in the peritoneal cavity.

B cells in Tlr8−/− mice appeared to exhibit a hyperreactive phenotype, as demonstrated by increased spontaneous secretion of IgM and IgG2a, whereas Tlr7−/− mice showed decreased levels of IgGs compared with WT mice (Figure 6). It may be that in the absence of TLR8, B cells are more readily activated by an endogenous natural ligand as a consequence of TLR7 overexpression (either directly or indirectly by DCs), thereby contributing to enhanced maturation into follicular B cells and block of MZ B cell generation. Indeed, we observed bigger spleens in Tlr8−/− mice (Figure 4A) and a 2-fold increase in the absolute number of follicular B cells in Tlr8−/− compared with WT mice (data not shown).

Dysregulated activation of DCs by nucleic acid–containing immune complexes is implicated in the pathogenesis of SLE, and activation of the type I IFN system is a prominent feature of early and active disease (38). In vitro experiments have shown that FcγR-dependent activation of murine DCs and plasmacytoid DCs by immune complexes promotes the secretion of type I IFNs and IL-6 in a TLR7-dependent fashion (39). In the present study, we found that TLR8 deletion was accompanied by expansion of the CD11c+ population and overexpression of TLR7 that led to higher production of IL-6 and IFN-β by DCs upon stimulation...
with TLR7 ligands. Thus, we hypothesize that in Tlr8−/− mice, self nucleic acids or nucleoproteins bound to autoantibodies are internalized via FcγR receptors on DCs and are delivered to TLR7-containing vesicles, which leads to the production of type I IFNs. Furthermore, these immune complexes bind to B cell antigen receptors and are internalized for the activation of TLR7, which contributes to the activation of autoreactive B cells. So the autoimmune phenotype that we observed in Tlr8−/− mice might be the outcome of overexpression of TLR7 by DCs and cooperative activation of DCs and B cells, since both are important for the development of lupus disease.

The Yaa locus produces a striking acceleration of autoimmunity when bred to models of lupus, such as MRL/lpr or NZB/W mouse strains, and recent studies revealed that duplication of Tlr7 accounts for most aspects of the autoimmune phenotype associated with Yaa translocation (24, 25, 27). We demonstrated here that TLR8 deficiency in the C57BL/6 background, which in general is not prone to lupus, led to increased anti-smRNP, and anti-dsDNA autoantibody titers, elevated ANAs, and incidence of lupus nephritis as a result of increased TLR7 expression, while these features were absent in Tlr8−/− Tlr7+/− mice (Figure 7B and data not shown). In accordance with our findings, a previous study has shown that in transgenic mice on the C57BL/6 background, TLR7 overexpression is sufficient to trigger and accelerate autoimmunity to the point of fetal death in the absence of additional SLE susceptibility genes in a dose-dependent manner (24).

Furthermore, we found here that TLR8 deficiency also induced significant expansion of the CD11c+ population (Table 1), similar to what was found previously in TLR7-overexpressing transgenic mice or aging Yaa mice (24, 34). However, we did not observe spontaneous overproduction of the inflammatory cytokine TNF in the sera of 3- to 6-month-old Tlr8−/− mice (Supplemental Figure 9) or lethality in Tlr8−/− mice up to 12 months of age, in contrast to what has been previously described for TLR7 transgenic mice (24). These similarities and differences between Tlr8−/− and TLR7-transgenic mice can be attributed to differences in TLR7 overexpression levels. Interestingly, a recent study revealed that accelerated development of SLE in TLR9-deficient lupus-prone mice also correlated with upregulated expression of TLR7 (40).

Taken together, our data provide what we believe to be the first demonstration that murine TLR8 plays a pivotal role in the regulation of myeloid cells and prevention of autoimmunity by controlling TLR7 expression. It is important to investigate whether TLR8 deficiency in humans has a similar effect on TLR7 expression and whether cases of SLE in humans can be attributed to TLR8 deficiency.

Methods

Mice and BM chimeras. Tlr7−/−, Tlr8−/−, and Tlr7−/− Tlr8−/− mice were generated by the high-throughput and automated VelociGene approach, which uses targeting vectors based on bacterial artificial chromosomes, as previously described (28, 41). Briefly, the TLR8 targeting construct was generated using bacterial homologous recombination to delete 2.3 kb of the TLR8 coding sequence (amino acids 103–881) while inserting a lacZ/neom reporter/selection cassette into BAC 317g19 from Incyte Genomics release 2 (28, 41). The linearized targeting construct, which contains homology arms of 7 and 130 kb, was electroporated into C57 ES cells, and correctly targeted ES cells were identified using LONA assay (28, 41). Chimeric mice were generated by blastocyst injection. Male chimera mice were mated to C57BL/6 female mice, and heterozygous TLR8 mice were backcrossed to the C57BL/6 background for 10 generations. Tlr8−/− mice were intercrossed to generate Tlr8−/− mice. Tlr7−/−, Tlr8−/−, and Tlr7−/− Tlr8−/− mice on the C57BL/6 background (10 generations) and WT C57BL/6 mice were used for all the experiments and maintained in pathogen-free conditions.

Female Tlr8−/− Tlr7+/− mice were generated by crossing male Tlr8−/− Tlr7+/− mice with female Tlr8−/− (i.e., Tlr7−/−) mice. BM chimeras were generated as previously described (42). Mice were housed under specific pathogen-free conditions, and mouse experimental protocols were approved by the Comité consultatif d’éthique pour les sciences de la vie et de la santé (UnivMed, INSERM, CNRS, Marseille, France).

Reagents. Poly I:C, R848, LPS from E. coli 0111-B4, CL097, CL075, and ssRNA-DR/LyvEco were purchased from Invitrogen. Poly A.U was a gift from Innate Pharma. CpG oligonucleotide (5′-CCATGACGTTCCGCGGT-3′) was synthesized by Sigma-Aldrich. RNP 68K and smRNP antigens were from Arotec Diagnostics Limited.

BMDCs and BMMs. BM cells from WT and Tlr8−/− mice were cultured in RPMI medium supplemented with 5% FBS and 1% supernatant derived from a GM-CSF–producing cell line. At day 6, immature DCs were collected and plated at 1×10^6 cells/ml in RPMI plus 5% FBS in the presence or absence of stimuli. BMMs were generated as previously described (43).

RT-PCR and Q-PCR. Total RNA from DCs was isolated with TRIzol reagent (Gibco; Invitrogen) or RNAeasy kit (Qiagen). Total RNA (2–5 μg) was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions with oligo dT primers. Primer pairs specific for Tlr8 (5′-TTGCGACAAGTCGCTCTCTG-3′ and 5′-CATTGGGTTGCTGTTGTGTT-3′) or hypoxanthine phosphoribosyltransferase (Hprt; 5′-TGGTGATTACAGCAGAAGACTTTGTTGTTG-3′ and 5′-AGGGTGGAGGGCTGGCTATAGGCT-3′) and Taq polymerase (Invitrogen) were used for PCR, and PCR products were separated by agarose gel electrophoresis. For Q-PCR, cDNA was amplified with PCR Master Mix (Applied Biosystems) and the primers Tlr7 (5′-TGGCTCTCTCCCCCTAGGATGA-3′ and 5′-CCTGTGCCACATCCGAAAACA-3′), Tlr8 (5′-AGTTGGATGTTAAGAGAGAAACG-3′ and 5′-ATGGCAGTGTCCAGAGGA-3′), Hprt (5′-CGTCTGCTGCTGCTCTCCC-3′ and 5′-TCTGCTGCTGCTGCTCTG-3′) and β-actin (5′-CCGACATTCAAGGCAACC-3′ and 5′-GACAGCACACGCCGTTAGG-3′) in a final volume of 25 μl. Q-PCR was performed on an Applied Biosystems PRISM 7700 Sequence Detection System, and the amount of target was calculated relative to the calibrator by 2−ΔΔCt, resulting in data expressing a target copy number ratio (Tlr7/β-actin or Tlr8/β-actin).

Measurement of cytokines. Concentrations of IL-6 and TNF were measured by ELISA (ebioscience) or with cytometric Bead Array (CBA) Flex Set bead-based immunoassay (BD Biosciences) according to the manufacturers’ instructions with oligo dT primers. Primer pairs specific for Tlr8 (5′-TTGCGACAAGTCGCTCTCTG-3′ and 5′-CATTGGGTTGCTGTTGTGTT-3′) or hypoxanthine phosphoribosyltransferase (Hprt; 5′-TGGTGATTACAGCAGAAGACTTTGTTGTTG-3′ and 5′-AGGGTGGAGGGCTGGCTATAGGCT-3′) and Taq polymerase (Invitrogen) were used for PCR, and PCR products were separated by agarose gel electrophoresis. For Q-PCR, cDNA was amplified with PCR Master Mix (Applied Biosystems) and the primers Tlr7 (5′-TGGCTCTCTCCCCCTAGGATGA-3′ and 5′-CCTGTGCCACATCCGAAAACA-3′), Tlr8 (5′-AGTTGGATGTTAAGAGAGAAACG-3′ and 5′-ATGGCAGTGTCCAGAGGA-3′), Hprt (5′-CGTCTGCTGCTGCTCTCCC-3′ and 5′-TCTGCTGCTGCTGCTCTG-3′) and β-actin (5′-CCGACATTCAAGGCAACC-3′ and 5′-GACAGCACACGCCGTTAGG-3′) in a final volume of 25 μl. Q-PCR was performed on an Applied Biosystems PRISM 7700 Sequence Detection System, and the amount of target was calculated relative to the calibrator by 2−ΔΔCt, resulting in data expressing a target copy number ratio (Tlr7/β-actin or Tlr8/β-actin).

Flow cytometry. Before staining, Fc receptors were blocked for 15 minutes at 4°C with 24G2 hybridoma supernatant. Cell suspensions were stained with antibodies to the following molecules (all from BD Biosciences) — CD45-PTER, CD5-PECy5 (catalog no. 53-7.3); IgM-PerCP, -PE (catalog no. H1.2F3); CD86-PE (catalog no. B7-2); and MHCII-PE (catalog no. 7G6); CD69-FITC (catalog no. 145-2C11); CD5-PECy5 (catalog no. 53-7.3); IgM-PerCP, -PE (catalog no. R6-60.2), or -biotin (catalog no. 1/1); CD11c-FITC or -APC (catalog no. HL3); CD21-APC (catalog no. 7G6); CD23-PE (catalog no. B34); CD69-PE (catalog no. H1.2F3); CD86-PE (catalog no. B7-2); and MHCII-PE (catalog no. AF6-120.1). Stained cells were analyzed on a FACSCalibur or FACSLSRII (BD) flow cytometry machine and were later analyzed further with Flowjo software (Tree Star).

Western blot analysis. Total protein (20 μg) from BMDCs was resolved on 10% SDS-PAGE gels and transferred to Immobilon P membrane (Millipore). Blotting was performed with the following antibodies: ikBα, phos-
dsDNA were determined by specific ELISA. Plates were precoated with Biotech. Serum levels of IgG autoantibodies against RNP, smRNP, and

5

μ

μ

μ

μ

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μ

μ

were cut on a cryostat, thawed-mounted on Superfrost plus slides, air dried, and stored at −20°C for further use. For immunofluorescence staining, sections were rehydrated in PBS, incubated with blocking solution (PBS plus 2% BSA) for 2 hours at room temperature, and stained with B220-PE (catalog no. RA3-6B2; ebiosciences), CD3-APC (catalog no. 145-2C11; ebiosciences), MOMA1 (Serotec), or F(ab')2 IgG (H+L)-FITC (Beckman Coulter). Fluorescent images were acquired using a Zeiss LSM 510 laser scanning confocal microscope and analyzed by LSM Image Browser. For β-galactosidase activity assessment, sections were fixed in 4% paraformaldehyde for 10 minutes and incubated overnight at 4°C in X-Gal staining solution (5 mM potassium ferricyanide crystalline, 5 mM potassium ferricyanide trihydrate, 2 mM magnesium chloride, and 0.1% X-Gal).

Statistics. Significance of differences was calculated by nonparametric Mann-Whitney U test. A P-value less than 0.05 was considered significant.

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Supplemental Figure 1
Similar IL-6 production by wild-type and TLR8<sup>+</sup> macrophages and splenocytes. Wild-type or TLR8<sup>+</sup> (A) BMMs or (B) splenocytes were left untreated or stimulated with the indicated amounts of R848 or CpG for 20h. The levels of IL-6 in the culture supernatants were determined by ELISA. Data indicate the mean ± SD of four mice per group. Figures are representative of three independent experiments.
Supplemental Figure 2
Enhanced IL-12p40 production by TLR8⁻⁻ BMDCs in response to various TLR7 ligands. BMDCs from wild-type or TLR8⁻⁻ mice were stimulated with the indicated amounts of CL075 (TLR7/8 ligand), CL097 (TLR7/8 ligand), ssRNA-DR/LyoVec (TLR7/8 ligand) or CpG for 20 h. The concentration of IL-12p40 in the culture supernatants were assessed by ELISA. Data indicate the mean ± SD of three to four mice per group. *, p<0.05; **, p<0.01.
Supplemental Figure 3

Rescue of the phenotype TLR8⁻ phenotype by the addition of a functional TLR8 gene. Wild-type or TLR8⁻ BMDCs were transfected in triplicates with 1μg of TLR8 expression vector (pMAX-TLR8) or empty vector (pMAX) via the AMAXA kit (according to manufacturers instructions). Two hours later, cells were left untreated or stimulated with 50 nM R848 or 50 nM CpG. After 16 h, culture supernatants were collected and the concentration of IL-12p40 was assessed by ELISA. Data indicate the mean ± SD of triplicates.
Supplemental Figure 4
TLR7 and TLR8 mRNA expression in IFN-γ or TNFα activated BMDCs. Wild-type BMDCs were left untreated or stimulated with (A) 2 ng/ml IFN-γ or (B) 10 ng/ml TNFα for the indicated time points. Total RNA was extracted from the cells and the expression of TLR7 or TLR8 was assessed by quantitative PCR. Data indicate the mean ± SD of duplicates.
Supplemental Figure 5
Comparison of the responses and phenotype of TLR8⁺, TLR8⁺TLR7⁺ and TLR8⁺TLR7⁻ BMDCs and mice. Wild-type, TLR8⁺, TLR8⁺TLR7⁻ and TLR8⁺TLR7⁻ BMDCs were (A and B) left untreated or (B) stimulated with 100 nM R848 for the indicated time points. Total RNA or protein was extracted from the cells and the expression of TLR7 was assessed by (A) quantitative PCR or (B) Western blot and β-actin was used as control. (C) Erythrocyte-depleted splenocytes from age and sex matched wild-type, TLR8⁺, TLR8⁺TLR7⁻ and TLR8⁺TLR7⁻ mice were analyzed by flow cytometry for the expression of CD19, CD21 and CD23. The numbers denote the percentage of MZ B cells (CD21<sup>high</sup>CD23<sup>low/neg</sup>) on CD19<sup>+</sup> gated cells. (D) ANA staining patterns on Hep2 human epithelial cells for serum derived from TLR8⁺TLR7⁺, TLR8⁺TLR7⁻ and TLR8⁺TLR7⁻ mice at 1:160 dilution. (E) IgM and IgG immunofluorescence staining of kidney sections from TLR8⁺TLR7⁺ (9 weeks old), TLR8⁺TLR7⁻ (11 weeks old) and TLR8⁺TLR7⁻ (17 weeks old) mice. In panel A, data indicate the mean ± SD of duplicates. In panels C to E, data are representative of three mice per group.
Supplemental Figure 6
Activation of Iκ-Bα and MAP kinases in wild-type and TLR8<sup>−/−</sup> BMDCs upon R848 stimulation. Wild-type or TLR8<sup>−/−</sup> BMDCs were stimulated with 100 nM R848 and at the indicated time points, cells were lysed and phosphorylation of Iκ-Bα, JNK, p38 and ERK and degradation of Iκ-Bα were determined by Western blot. Actin, β-tubulin and total ERK and p38 were used as loading controls. Figures are representative of three independent experiments.
Supplemental Figure 7
No spontaneous activation on TLR8−/− B cells and normal myeloid progenitor fractions of the BM and thymic development in TLR8−/− mice. (A) The expression of activation markers CD69 and CD86 was assessed by flow cytometric analysis on B220+ gated cells from wild-type and TLR8−/− mice. (B) Bone marrow cells from 12 weeks old wild-type and TLR8−/− mice were analyzed by flow cytometric analysis using antibodies specific for CD31, Ly6C, CD19 and B220. With anti-CD31 and anti-Ly6C, the cell subsets were defined as follows: blast cells, CD31high/Ly6Clow; lymphoid cells CD31med/Ly6Clow; erythroid cells CD31low/Ly6Clow; myeloid progenitors and plasmacytoid cells CD31high/Ly6Cmed; granulocytes CD31low/Ly6Cmed; and monocytes, CD31low/Ly6Cmed. With anti-CD19 and anti-B220, the cell subsets were defined as B220high/CD19−, recirculating B cells and B220−/CD19−, pro-, pre- and immature-B cells. (C) Thymic cells from wild-type and TLR8−/− mice stained with anti-CD4 and anti-CD8 gated on live cells and analyzed by flow cytometric analysis. The percentage of cells in each quadrant is indicated.
Supplemental Figure 8
Analysis of wild-type and TLR8−/− MZ B cells, follicular B cells and plasmocytes. MZ and follicular (FO) B cells were isolated from wild-type and TLR8−/− spleens by Flow cytometry. (A) Expression of TLR7 mRNA by quantitative PCR of wild-type and TLR8−/− MZ and FO B cells. (B) Expression of TLR8 mRNA by PCR on cDNA derived from wild-type MZ and FO B cells, BMDCs and BMMs. cDNA from TLR8−/− BMDCs was included as negative control. (C and D) Wild-type or TLR8−/− MZ B cells were left untreated or stimulated with 100 nM R848 or 100 nM CpG overnight. (C) The levels of IL-6 production in culture supernatants were assessed by ELISA. Data are representative of three independent experiments. (D) The expression of activation markers CD86 and CD69 was assessed by flow cytometric analysis. (E) Flow cytometric analysis of splenocytes from 3 months old wild-type or TLR8−/− mice shows the B220−CD138+ population. Data are representative of three independent experiments.
Supplemental Figure 9
Similar spontaneous TNF production in wild-type, TLR8⁺, TLR7⁻ or TLR7/8⁻ mice sera. Sera were collected from wild-type, TLR8⁺, TLR7⁻ or TLR7/8⁻ mice aged (A) 3 or (B) 6 months old, and the production of TNF in their sera was assessed by ELISA. Data indicate the mean ± SD of each mouse group.
1-TLR8 inhibits TLR7 signaling

To test whether murine TLR8 can inhibit TLR7 signaling, HEK 293 cells were transfected with murine TLR7 and TLR8, together with an NF-κB-dependent reporter gene. In parallel transfection of TLR7 or TLR8 was used as control. When stimulated with R848, only those cells expressing TLR7, but not TLR8, showed a dose dependent response to R848 and cotransfection of TLR7 and TLR8 revealed that TLR8 inhibits TLR7 signaling in a dose dependent manner (Figure 17).

![Figure 17. Inhibition of TLR7 by TLR8.](image)

HEK 293 cells were transiently transfected with expression vectors corresponding to TLR7 or TLR8, alone or in a pairwise combination, together with NF-κB luciferase reporter (see Experimental procedures for details). Luciferase fold induction is expressed as the ratio of NF-κB activity of stimulated versus unstimulated cells. Each data point represents the mean ± SD of duplicates and each figure is representative of three independent experiments.

To investigate which part of TLR8 was responsible for this inhibitory effect, we generated two chimeric proteins between TLR7 and TLR8 that were composed of the extracellular region of TLR7 or TLR8 fused with the transmembrane and cytoplasmic region of TLR8 (TLR7-TLR8) or TLR7 (TLR8-TLR7), respectively. In transfection experiments and upon
Complementary results

stimulation with R848, the chimeric protein TLR7/TLR8 induced NF-κB activation, although not as strong as TLR7, suggesting that the TIR domain of TLR8 is functional (Figure 18A). However, the chimeric TLR8/TLR7 did not show any activation, indicating that the extracellular domain of TLR8 probably does not recognize R848 (Figure 18A). Furthermore, cotransfection of TLR7 and TLR7/TLR8 in HEK293 cells and stimulation with R848 revealed that TLR7/TLR8 inhibited TLR7 signaling in a dose dependent manner (Figure 18B). However, cotransfection of TLR7 and TLR8/TLR7 showed that the chimeric protein TLR8/TLR7 did not affect TLR7 signaling (Figure 18C). Thus, we showed that the intracellular domain of TLR8 is responsible for the inhibitory effect on TLR7 signaling.

**Figure 18. Effect of TLR7/8 and TLR8/7 chimeric proteins on TLR7 signaling.** HEK 293 cells were transiently transfected with expression vectors corresponding to (A) TLR7, chimeric TLR7/TLR8 (TLR7/8) or chimeric TLR8/TLR7 (TLR8/7), (B) TLR7 or chimeric TLR7/TLR8 (TLR7/8) and (C) TLR7 or chimeric TLR8/TLR7 (TLR8/7), alone or in a pairwise combination, together with NF-κB luciferase reporter (see Experimental procedures for details). Luciferase fold
induction is expressed as the ratio of NF-κB activity of stimulated versus unstimulated cells. Each data point represents the mean ± SD of duplicates and each figure is representative of three independent experiments.

2-The reduced MZ B cell population in TLR8\textsuperscript{-/-} mice is not B cell autonomous

We showed that TLR8-deficient mice have reduced numbers of marginal zone B cells (Demaria et al, 2010, Figure 4B) and that TLR8-deficient MZ B express more TLR7 than WT cells. However upon R848 stimulation TLR8\textsuperscript{-/-} MZ B cells produce the same amount of IL-6 and show similar expression of the activation markers CD86 and CD69 like WT cells (Demaria et al, 2010, Supplemental Figure 5). In order to investigate if TLR8\textsuperscript{-/-} MZ B cells develop and behave as WT cells in a normal environment, we irradiated WT mice and reconstituted them with either a mix of 50% WT BM cells (Ly5.1) and 50% TLR8-deficient BM cells (Ly5.2) or as control with a mix of 50% WT BM cells (Ly5.1) and 50% WT BM cells (Ly5.2). Eight weeks later, we analyzed by flow cytometry the proportion of WT and TLR8\textsuperscript{-/-} MZ B cells in the spleen of the chimeras.

Figure 19. Similar numbers of TLR8\textsuperscript{-/-} and WT MZ B cells in mixed chimera. WT Ly5.1 irradiated mice were reconstituted with either 50% WT Ly5.1 and 50% WT Ly5.2 or with 50% WT Ly5.1 and 50% TLR8\textsuperscript{-/-} Ly5.2 bone marrow cells. Eight weeks later, splenocytes were analyzed by flow cytometry for the presence of MZ B cells (CD19\textsuperscript{+}CD21\textsuperscript{high}CD23\textsuperscript{low}). Bars represent proportion of CD45.1 and CD45.2 MZ B population in chimeras. Data are representative of three mice per group.
We found the same proportion of WT and TLR8\(^{-/-}\) MZ B cells in chimeras containing TLR8\(^{-/-}\) and WT cells as well as in mixed-chimeras containing only WT cells (Figure 19). So we showed that TLR8\(^{-/-}\) MZ B cells have no developmental defect since in a normal environment they give rise to the same number of MZ B cells like WT cells.

**3-TLR9 is implicated in the control of TLR7 expression and signaling.**

TLR9\(^{-/-}\) mice have increased levels of RNA-related autoantibodies associated with an exacerbated kidney disease compared to WT mice (Christensen SR, Immunity, 2006). The characterization of the increased autoimmune pathology in the TLR9\(^{-/-}\) mice present similarities with what we found in TLR8\(^{-/-}\) animals. In order to investigate if the autoimmune phenotype present in the TLR9ko mice could be due to an overexpression of TLR7, we tested the response of TLR9\(^{-/-}\) BMDCs to diverse TLR ligands including R848. We found that upon R848 stimulation, TLR9\(^{-/-}\) BMDCs produce more IL-6 proinflammatory cytokine while upon LPS (a TLR4 ligand) or PAM\(_3\)CSK\(_4\) (a TLR2 ligand), they produce the same amount as WT cells (Figure 20).
Figure 20. Enhanced IL-6 production to R848 stimulation by TLR9⁻/⁻ BMDCs. BMDCs from WT and TLR9⁻/⁻ mice were stimulated with the indicated amounts of R848, LPS or PAM₃CSK₄. After 20 hours, the concentration of IL-6 in the culture supernatant was assessed by ELISA. Data are the mean ± SD of 3 mice. *P<0.05

In order to explore further the possibility that TLR9 could inhibit TLR7 signaling, we transfected HEK293 cells with murine TLR7, or TLR7 and TLR9, together with an NF-κB-dependent reporter gene. We found that upon stimulation with R848 TLR9 inhibits TLR7 signaling in a dose dependent manner (Figure 21). Thus, we found that like TLR8, also TLR9 has an inhibitory effect on TLR7 signaling.

Figure 21. Inhibition of TLR7 by TLR9. HEK 293 cells were transiently transfected with expression vectors corresponding to TLR7 or TLR9, alone or in a pairwise combination, together with NF-κB luciferase reporter (see Experimental procedures for details). Luciferase fold induction is expressed as
the ratio of NF-κB activity of stimulated versus unstimulated cells. Each data point represents the mean ± SD of duplicates and each figure is representative of three independent experiments.
Experimental procedures:

Luciferase reporter assay

HEK 293 cells were transiently transfected using lipofectamine 2000 reagent (Invitrogen), according to manufacturer’s instructions, with the indicated amounts of TLR7, TLR8 or TLR9 expression plasmids, 50 ng NF-κB luciferase reporter pBIIXLuc plasmid and 10 ng of Renilla luciferase (pRL-null, Promega). The total amount of transfected DNA was kept constant by adding empty vector. Eighteen hours post-transfection cells were stimulated with R848 for 6-7 h, lysed and luciferase activity was measured using Dual-Glo luciferase assay system (Promega) following manufacturer’s instructions. Luciferase induction was expressed as the ratio of NF-κB-dependent firefly luciferase activity divided by control Renilla luciferase activity. pUNO-mTLR7, pUNO-mTLR8 and pUNO-mTLR9 expression constructs were from InvivoGen.

Generation of TLR7/8 and TLR8/7 chimeric proteins

A single SalI restriction site was inserted at the ectodomain of murine TLR7 and murine TLR8 close to their transmembrane domain, using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen) to mutate Val→Leu at the position 3076 bp in pUNOmTLR7 (Invivogen) and Val→Ser at the position 3013 bp in pUNOmTLR8 (Invivogen). The region coding for the ectodomain of TLR7 was ligated to the transmembrane and TIR domain of TLR8 (TLR7/TLR8) and the ectodomain of TLR8 was ligated to the transmembrane and TIR domain of TLR7 (TLR8/TLR7).
DISCUSSION & PERSPECTIVES

Among the TLR family, endosomal TLRs (TLR3, 7, 8 and 9) are of particular interest since they face the challenge of discriminating against self and non-self nucleic acids. Indeed, TLR3 and TLR7 are able to detect viral dsRNA and ssRNA, respectively, while TLR9 can sense unmethylated CpG DNA motifs. Moreover, in nowadays there is considerable evidence that self recognition via TLR3, TLR7 or TLR9 can occur and contribute to autoimmunity since these TLRs are able to recognize DNA or RNA released by damaged or stressed cells. Among the endosomal TLRs, TLR8 remains the less studied. Murine TLR8 has been proposed to be not functional since it does not recognize the same ligand as human TLR8 (70) and no other specific ligand has been described. In this context the goal of my project was to elucidate the biological role of murine TLR8 in immunity.

In order to investigate the biological role of murine TLR8 we generated and analyzed TLR8-deficient mice (TLR8\(^{-/-}\)). We found that, upon R848 (TLR7 ligand) stimulation, TLR8\(^{-/-}\) BMDCs showed higher production of cytokines (IL-6, IL12p40, TNF), stronger activation (CD86, MHCII) and also stronger and faster NF-\(\kappa\)B activation compare to WT cells. TLR8\(^{-/-}\) BMDCs showed. However, BM-macrophages, total splenocytes and fibroblasts from TLR8\(^{-/-}\) mice responded normally to R848. Moreover, we tested the expression of TLR7 in TLR8\(^{-/-}\) DCs and macrophages. We showed that in unstimulated conditions both TLR8\(^{-/-}\) DCs and macrophages had higher TLR7 mRNA levels than WT cells (Demaria et al., 2010, Figure 3B). However, upon R848 stimulation, DCs sustained a constant TLR7 expression while TLR7 mRNA was fast down regulated and was almost abrogated by 16 hours in macrophages (Demaria et al., 2010, Figure 3E). Thus, the fact that TLR8\(^{-/-}\) DCs are hyperresponsive while
macrophages respond normally to TLR7 stimulation can be explained by a different regulation of TLR7 expression after cell activation.

The group of Dr Paul J. Utz showed that in B cells, TLR7 expression is upregulated upon IFNα treatment (192). In addition, TLR7 stimulation in DCs leads to a massive production of type I IFN (75) (193). So, we can imagine that in DCs R848 stimulation leads to type I IFN production that sustains a high TLR7 expression. To investigate this point, it would be interesting to compare kinetics of TLR7 expression in BMDCs stimulated with IFNα. If indeed, IFNα is important to sustain TLR7 expression, it would be worth to assess TLR7 expression in IFNR-deficient DCs stimulated with R848. If in these conditions TLR7 expression is abolished, R848-induced IFNα production is crucial for maintaining TLR7 expression. Something we should not forget when we study the regulation of TLR expression upon stimulation ex vivo in different cell types is that we do not take into account the effect brought by the surrounding cell types that could affect this regulation in vivo. In other words, cytokines released by DCs within the spleen could affect TLR7 expression on macrophages and lymphocytes.

Nevertheless, the question “why does the absence of TLR8 lead to TLR7 overexpression?” needs to be clarified. At the moment, we can hypothesize several scenarios: It has been demonstrated that TLR7 and TLR9 could be activated by endogenous ligands (194) and we showed that TLR7 expression is dependent of the activation status of the cells since it could be down regulated upon IFNγ or TNF stimulation (Demaria et al, 2010, Supplemental Figure 4). So we can speculate that TLR8 recognizes an endogenous, yet unidentified ligand that leads to a steady-state activation of the cells and keeps under control the expression of TLR7.
Discussion & perspectives

TLRs can form dimeric receptor complexes consisting of heterodimers or homodimers (44). We showed that upon cotransfection of murine TLR7 and TLR8 in HEK293 cells, TLR8 inhibits TLR7 in a dose dependant manner upon R848 stimulation (Figure 17). Previous studies have shown that TLR1 inhibits TLR4 signaling in endothelial cells (195). Moreover, cotransfection of mouse or human TLR7 and TLR8 in HEK293 cells showed that TLR8 inhibits TLR7 upon stimulation with the TLR7 ligand 3M-001, but not vice versa, and that the inhibitory effect of TLR8 result from direct or indirect physical interaction between TLR7 and TLR8 (93). In order to further investigate which part of TLR8 inhibits TLR7 we generated two chimeric proteins that were composed of the extracellular region of TLR7 or TLR8 fused with the transmembrane and cytoplasmic region of TLR8 and TLR7, respectively. We evaluated their ability to inhibit TLR7 signaling in cotransfection experiments in HEK293 cells. We found that the intracellular domain of TLR8 is functional since the chimeric TLR7/TLR8 protein could induce NF-κB activation (Figure 18 A). We showed also that the TIR domain of TLR8 could inhibit TLR7 signaling since cotransfection of TLR7 and TLR7/TLR8 leads to reduced NF-κB activation in a TLR7/TLR8 dose dependent manner (Figure 18B). However, the extracellular domain of TLR8 did not show any inhibitory effect on TLR7, since cotransfection of TLR7 and TLR8/TLR7 did not affect the ability of TLR7 to signal (Figure 18C). Thus, we showed that heterotypic TLR interactions can be inhibitory and play an important biological role.

SIGIRR (TIR8) is an orphan receptor of the Toll-like receptor/Interleukin-1 receptor family that inhibits TLR pathogen recognition (196) and SIGIRR-deficient dendritic cells show a higher TLR7 expression (197). Structure model prediction studies revealed that the BB loop of SIGIRR’s intracellular TIR domain interacts with TLR7 and prevents its homodimerisation and consequently its interaction with MyD88 (Figure 22). In addition, deletion of SIGIRR’s BB loop was sufficient to completely abrogate SIGIRR’s inhibitory effect on TLR7 signaling.
By aligning the sequences of mTLR8 and mSIGIRR proteins we found that mTLR8 carries the BB loop in its TIR domain (Figure 23). Based on these observations, it would be interesting to evaluate whether deletion of mTLR8 BB loop abrogates the ability of TLR8 to inhibit TLR7 signalling.

**Figure 22. Model of SIGIRR inhibiting the MyD88-dependent TLR7 signaling.** Complex B, TLR7 homodimers (dark blue), linked by BB loop (orange)-α-helix E (green) interaction, bind MyD88 homodimers (light blue), forming a T-shaped conformation able to signal. MyD88 complex are formed by BB loop interaction (orange). The TLR7-MyD88 complex may not form when SIGIRR recruits to this complex as follows: the predicted TLR7-SIGIRR interaction (complex A) should affect TLR7 homodimers formation, and SIGIRR-MyD88 interaction (complex C) may affect MyD88 homodimers formation (197).
UNC93B1 is a chaperone molecule involved in the trafficking of nucleotide sensing TLRs from the endoplasmic reticulum to the endosomes (99). Deficiency in UNC93B1 abrogates endosomal TLRs signaling in both human and mice (101) (99). TLR7 and TLR8 have been shown to interact within a complex with UNC93B1 (103). Thus, another possible scenario that we could consider for the inhibiting effect of TLR8 on TLR7 is that there is a competition between TLR7 and TLR8 for interaction with the UNC93B1 molecule. The lack of TLR8 in TLR8-deficient DCs leads to an advantage to TLR7 to be transported by UNC93B1 to the lysosomes and signal.

Deregulation of TLR signaling can be detrimental for the host since TLRs are pivotal for the detection not only of microbes but also of self molecules. In the current study we showed for the first time that TLR8 plays an important role in controlling TLR7 expression and that deletion of TLR8 leads to the development of autoimmune features due to TLR7 overexpression. The importance of TLR7 expression in the development of a lupus phenotype...
Discussion & perspectives

has been shown in various mouse models. A recent study have shown that TLR7 transgenic mice develop autoimmunity and that the severity of the disease is directly correlated with the copy number of TLR7 integrated in the genome of these mice (163). However, the first proof of TLR7 overexpression linked to autoimmune phenotype came from mice that carry a Y-linked autoimmune accelerator (Yaa) translocation. Yaa is a translocation of 17 genes, including TLR7, from the X to the Y chromosome, leading to two copies of TLR7 gene in Yaa male mice (160). Yaa mice develop spontaneous autoimmune features and deletion of one copy of TLR7 gene leads to less severe disease, suggesting that in Yaa mice most of the autoimmune features are due to TLR7 expression (163) (164). The role of TLR7 in the lupus pathology has also been documented using other mouse models of lupus. In the pristane induced model of lupus (a model of environmentally triggered SLE in the absence of genetic susceptibility to autoimmunity) (198), or in MRL/MP^lpr/lpr mice (a genetic background naturally prone to autoimmunity) (157) deletion of TLR7 is linked to less severe autoimmune phenotype.

MZ B cells are atypical B cells located at the border between white and red pulp in the spleen, in a zone where the blood is passing through before to reach the red pulp (199). Due to this location MZ B cells are readily exposed to blood-borne antigens and they can participate to a T-independent immune response by generating IgM antibody production at early stage of an immune response (200). Moreover, these cells perform a variety of effector functions including also antigen presentation (134). In the current study, we described a role of TLR8 in the maintenance of the MZ B population due to its control on TLR7 expression. The importance of TLRs including TLR7 in the control of the MZ B cell population has been documented. Indeed, mice bearing the Yaa translocation have two copies of TLR7 gene and reduced numbers of MZ B cells (201), while male TLR7-deficient bearing the Yaa translocation (one copie of TLR7 gene) have normal numbers of MZ B cells (163). In
addition, transgenic mice overexpressing TLR7 have a reduced MZ B cell population compare to normal mice, while TLR7<sup>−/+</sup> mice show increased number of B cells in the MZ B compartment (163).

The precise molecular basis of the reduced number of marginal zone B cells in mice overexpressing TLR7 is still unknown. In vivo treatment with LPS promotes MZ B cell activation and migration, and this is not a unique feature of TLR4, since agonists to TLR2, TLR3 and TLR7 also stimulate MZ B cells and promote their relocation from the MZ into either an adjacent follicle or possibly red pulp (202). The migration of the MZ B cells triggered by TLR stimulation can be due either to a direct effect on the MZ B cell population or indirect. To check the first possibility, we tested the responses of TLR8<sup>−/+</sup> MZ B cells to R848 stimulation and found that they did not show an overactivate phenotype (Demaria et al, 2010 Supplemental Figure 8C and 8D), suggesting that other cell types are responsible for the egress of the MZ B out of the MZ. The study of bone marrow chimera revealed that the MZ defect is present in WT mice reconstituted with TLR8<sup>−/+</sup> bone marrow cells, but not vice versa, suggesting that the cell types responsible for the MZ phenotype is of myeloid lineage (Demaria et al, 2010, Figure 4D). In addition, analysis of irradiated WT mice reconstituted with a mixed population of WT and TLR8<sup>−/+</sup> bone marrow cells revealed that the two genotypes contribute in the same degree to the MZ B cell compartment (Figure 13). Thus, the reduced MZ B cell population in TLR8<sup>−/+</sup> mice comes from myeloid cells other than the MZ B cells themselves. Blood dendritic cells have been shown to interact with splenic MZ B cells to initiate a T-independent immune responses (203), thus, having a role in the differentiation process of MZ B to plasma cells. The Flt3 ligand is a growth factor for hematopoietic progenitors and Flt3-deficient mice have markedly reduced numbers of dendritic cells (204). Previous studies regarding the contribution of DCs on the MZ B cell population revealed that in Yaa Flt-3<sup>−/+</sup> mice there is a higher proportion of MZ B cells compare to the Yaa mice,
suggesting that the decreased MZ B cell population in the Yaa mice is the result of a dendritic cell-mediated enhanced activation (205). We can easily speculate that the TLR7 over-expression in TLR8\(^{-/-}\) DCs leads to a hyperactivation due to self antigen detection that mediates a stronger migration of the MZ B cells out of the MZ.

Among all the endosomal TLRs that recognize nucleic acids and their implication in SLE there is one paradox concerning the case TLR9. Despite the lack of anti dsDNA autoantibodies, TLR9\(^{-/-}\) lupus prone mice develop a more severe autoimmune disease (157). Taking in consideration our data that the absence of TLR8 leads to overexpression of TLR7 and to autoimmunity, we can speculate that also TLR9 has an inhibitory effect on TLR7. Indeed, co-transfection of mTLR9 and mTLR7 in HEK293 cells revealed that TLR9 inhibits TLR7 signaling (Figure 15). Similar transfection experiments from the group of Reddy revealed that human TLR9 inhibits human TLR7 signaling (93). Moreover, we found that TLR9\(^{-/-}\) BMDCs stimulated with R848 produce more proinflammatory cytokines than WT cells (Figure 14). Thus, we generated data that support a role of TLR9 in TLR7-mediated signaling. Our original hypothesis regarding the control of TLR7 by TLR9 is now confirmed by two recent publications. The group of Izui revealed that enhanced TLR7 activity is crucial for the accelerated development of SLE in the TLR9-deficient lupus prone mice (118) and the group of Schlomchik showed that TLR9 regulates TLR7-dependent autoantibody production and disease in a murine model of lupus (117).

It becomes evident that TLR7 is crucial for establishment of autoimmunity and among different inhibition processes, there is an important regulating activity between endosomal TLRs until now not investigated. We showed that the interplay between TLR8 and TLR7 or TLR9 and TLR7 have pivotal roles in preventing lupus disease. In order to better understand the contribution of TLR8 and TLR9 to autoimmune processes, we consider to compare TLR8\(^{-/-}\)
/−, TLR9−/− and WT mice under the same experimental conditions. We also plan to investigate if both TLR8 and TLR9 have additive, redundant or divergent effects on controlling TLR7 expression and autoimmunity. To investigate this point, we are generating double TLR8−/−TLR9−/− mice in order to study how lupus phenotype is developed compared to single TLR8 or TLR9-deficient mice.

A recent study identified a functional polymorphism in humans that may confer elevated expression of TLR7 and predispose to the development of SLE suggesting an implication of TLR7 expression in human disease (206). Since human and mouse TLRs are phyllogenetically very close to each other (Figure 24), one of the main perspectives of our study is to verify if the interplay between TLR8 or TLR9 with TLR7 that we found in mice also exists in humans and could influence TLR7 expression and SLE incidence.

![Figure 24. Homology between human and mouse TLR family](image)

The use of dual TLR7 and TLR9 inhibitory molecules called immunoregulatory sequence (IRS) 954 can prevent the progression of the disease when injected in lupus prone mice (207).
So the idea of blocking TLR7 and TLR9 in B cells and/or plasmacytoid dendritic cells seems to be a promising approach for the treatment of SLE in humans (208). In addition, a recent article revealed that TLR7 and TLR9 stimulation made pDCs resistant to glucocorticoid treatment (used in SLE therapy) and suggested the use of TLR7 and TLR9 inhibitors to counteract this resistance (186). Thus, the strategy to inhibit TLR7 and TLR9 as a SLE treatment is already under investigation. However, a better understanding of the mechanism of regulation involved in the interplay between TLR8 and TLR7 or TLR9 and TLR7 that hamper TLR7 expression is pivotal for the development of novel approaches for SLE prevention and treatment.

In addition, we should not forget that endosomal TLRs including TLR7 have been firstly involved in viral recognition. Indeed, TLR7 participates in the recognition of many viruses, such as VSV, influenza and HIV (71) (70). Therefore, it could be interesting to evaluate the inhibitory effect of TLR8 or TLR9 on TLR7 in a context of viral infection. In addition, the role of murine TLR8 in viral detection is not clearly defined. Even it has been questioned (209) (210), a recent publication suggested a role for murine TLR8 in pDCs to recognize vaccina virus (VV) (95). So it could be interesting to use the model of TLR8-deficient mice in order to clarify if VV signals through TLR8 and to indentify a specific and potential viral ligand for this receptor. The discovery of such ligand will be important to better understand the biological role of TLR8 in viral recognition and might lead to novel approaches for the prevention and treatment of viral infection.
Contribution to other publications

During my PhD, I contributed to other projects in the field of TLRs that led to the following publications (See also annexes).

- **Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria.**


  **Objectives:** To assess the biological role of TLR5 signaling in antibacterial immune responses.

  **Main results:** Using TLR5-deficient mice and cells, we showed that TLR5 is essential for both in vivo and ex vivo recognition of bacterial flagellin. TLR5 contribution to antibacterial host response may be masked by the presence of functional TLR4 gene since TLR4/TLR5 double deficient-mice are more susceptible than TLR4<sup>-/-</sup> mice upon infection with *Salmonella typhimurium* or *Pseudomonas aeruginosa*.

- **Brucella control of dendritic cell maturation is dependent on the TIR-containing protein Btp1.**


  **Objective:** To decipher the role of the *Brucella* protein Btp-1.
Main results: *Brucella abortus* is an intracellular pathogen able to persist for a long periods within the host and establish a chronic disease. We showed that *Brucella* replicates within dendritic cells and we identified a new *Brucella* protein Btp-1, which inhibits DCs maturation by interfering with the TLR2 signaling pathway.

- **Contribution of TLR7 and TLR9 signaling to the susceptibility of MyD88-deficient mice to myocarditis.**


Objective: To elucidate the role of the intracellular TLRs, TLR3, TLR7, and TLR9 in autoimmune myocarditis.

Main results: Using single TLR3-, TLR7-, and TLR9-deficient mice, we studied the implication of intracellular TLRs in autoimmune myocarditis using two different experimental models. We found that TLR7 contributes to the development of myocarditis when the disease is induced upon immunization with cardiac myosin, while TLR9 signaling is implicated in heart inflammation in the model of mouse cytomegalovirus-induced myocarditis.

- **TLR3 and RIG-like receptor on myeloid dendritic cells and RIG-like receptor on human NK cells are both mandatory for production of IFN-γ in response to double-stranded RNA.**

Objectives: To decipher the involvement of accessory cells in dsRNA-mediated NK cell responses and to characterize the dsRNA receptors on DCs and NKs involved in this process.

Main results: Myeloid DCs are required for NK cell activation in response to dsRNA. Full activation of NK cells and production of IFN-γ in response to dsRNA requires the triggering of TLR3, Mda5 and RIG-I on myeloid DCs (mDCs) and only Mda5 and RIG-I on NK cells.

- Gender bias in susceptibility to MCMV infection correlates with TLR9 expression.

Traub S, Demaria O, Chasson L, Akira S, Alexopoulou L. (submitted)

Objective: To investigate the influence of gender on acute MCMV infection in laboratory mice.

Main results: WT males show higher resistance to MCMV infection compared to their female counterparts but this gender difference is absent in TLR9−/− mice. The male resistance correlates with increased number of splenic pDCs, stronger mobilization of the MZ B cells and increased neutrophil migration into the splenic white pulp. Interestingly, no survival differences were observed in MCMV-infected TLR9−/− male and female mice. We hypothesized that the protection of male mice to MCMV infection could be attributed to TLR9 since MCMV-infected male mice show higher TLR9 expression that leads to stronger immune response against the virus. Our results suggest that gender should be carefully considered when designing and interpreting studies of MCMV infection.
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ANNEXES

- Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria.

- Brucella control of dendritic cell maturation is dependent on the TIR-containing protein Btp1.

- Contribution of TLR7 and TLR9 signaling to the susceptibility of MyD88-deficient mice to myocarditis.

- TLR3 and Rig-Like Receptor on Myeloid Dendritic Cells and Rig-Like Receptor on Human NK Cells Are Both Mandatory for Production of IFN-γ in Response to Double-Stranded RNA.

- Gender bias in susceptibility differences to MCMV infection correlates with TLR9 expression.
Annexe 1

Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria
Involvement of Toll-like receptor 5 in the recognition of flagellated bacteria

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Contributed by Richard A. Flavell, June 27, 2006

TLR5 is a sensor for monomeric flagellin, which is a component of bacterial flagella known to be a virulence factor. In this study, we investigated the role of TLR5 signaling in the detection of flagellin and antibacterial immune responses to Salmonella typhimurium and Pseudomonas aeruginosa. We found that TLR5 is essential for the recognition of bacterial flagellin both in vivo and ex vivo. TLR5 contribution to antibacterial host response to i.p. infection with S. typhimurium or intranasal administration of P. aeruginosa may be masked by TLR4 or other sensing mechanisms. By using radiation bone marrow chimera, we showed that upon i.p. injection of flagellin immune responses are mediated by lymphoid cells, whereas resident cells are required for the initiation of response upon intranasal flagellin administration. These results suggest that flagellin recognition in different organs is mediated by distinct TLR5-expressing cells and provide insights into the cooperation of the TLR5 and TLR4 signaling pathways used by the innate immune system in the recognition of bacterial pathogens.

Recognition of microbial infection and initiation of immune response are controlled by multiple mechanisms. Toll-like receptors (TLRs) have recently emerged as key components of the innate immune system that recognize common molecular structures detected in certain groups of microorganisms and trigger the activation of adaptive immunity (1). Each TLR detects specific microbial components. For example, TLR4 recognizes LPS, TLR2 recognizes bacterial lipoproteins and lipoteichoic acid, and TLR3 recognizes viral double-stranded RNA. All TLRs share a common intracellular domain, the Toll-IL-1 receptor homology domain, and upon activation initiate signaling cascades that lead to common responses such as the induction of inflammatory cytokines and up-regulation of costimulatory molecules. Moreover, TLRs also have specific functions as exemplified by their different ability to induce type I IFN (1). Thus, TLRs activate multiple steps inLeukocytes from various organs were stained (stained for CD3, CD20, CD4, and CD8) and showed no obvious behavioral abnormalities. Flow cytometric analysis of leukocytes from the spleen, lymph nodes, and thymus revealed that the com-
position of lymphocytes was similar in WT and TLR5−/− mice (data not shown).

Next we analyzed the responses of TLR5−/− cells to flagellin. BM-derived dendritic cells (BMDCs) or macrophages from WT and TLR5−/− mice were stimulated with 1 or 3 μg/ml flagellin. After 16 h the culture supernatants were collected, and the protein levels of IL-6, IL-12, and TNF-α were measured by ELISA. Although no significant amounts of these cytokines were detectable (data not shown), the mRNA expression levels of IL-6 and IL-12 were significantly up-regulated in WT DCs stimulated with flagellin, but not in TLR5−/− cells, whereas they showed similar responses upon LPS stimulation (Fig. 1d). Similar results were obtained with WT and TLR5−/− macrophages (data not shown). To demonstrate the specificity of the ligand, we examined the responses of TLR5−/− BMDCs to various microbial molecules that are known to activate TLR5−/− cells. WT and TLR5−/− DCs were stimulated with 10 ng/ml PamCSDK, 1 μg/ml peptidoglycan, 10 μg/ml polycl IC, 1 ng/ml LPS, 100 nM R848, or 1 μg/ml CpG for 16 h, and concentrations of IL-6 and IL-12 in the culture supernatants were measured by ELISA.

TLR5 is Required for Responsiveness to Flagellin. To dissect the signaling pathways that are affected in the absence of a functional TLR5 we analyzed the activation of the signaling cascades in TLR5−/− DCs upon stimulation with flagellin. We found that flagellin-induced degradation of IκBα and phosphorylation of ERK, p38, and JNK were completely abolished in TLR5−/− mice were stimulated with 100 ng/ml flagellin. Sera were collected at 1, 2, 4, or 8 h after stimulation, and concentrations of IL-6 at 2 h and IL-12 at 2 and 4 h after flagellin stimulation were determined by ELISA. The data are means ± SE of three mice per group. We next tested the ability of TLR5−/− DCs to mature in vivo in response to flagellin. WT and TLR5−/− mice were injected i.p. with flagellin, LPS, or PBS, and 6 h later the expression levels of CD40, CD86, CD80, and MHC class II on CD11c+ gated cells were determined by flow cytometry. Histograms show expression levels of CD40, CD86, CD80, and MHC class II on CD11c+ gated cells. Gray area, untreated cells; dotted line, WT cells; continuous line, TLR5−/− cells. (c) WT and TLR5−/− mice were injected i.p. with 1 μg of flagellin. Sera were collected at the indicated time points, and serum levels of IL-6 and IL-12 were determined by ELISA. The data are means ± SE of three mice per group.

TLR5 was required for responsiveness to flagellin. CD80, CD86, and MHC class II on CD11c+ splenic cells were analyzed. A significant increase in the expression of these activation markers was observed in WT CD11c+ cells upon flagellin and LPS stimulation. In contrast, TLR5−/− cells did not show any response to flagellin despite exhibiting normal responses to LPS stimulation. We investigated whether signaling by TLR5 is required for the in vivo inflammatory response to flagellin. Mice were injected i.p. with flagellin, and the levels of IL-6 and IL-12 in their sera were assessed 2, 4, and 6 h later. WT mice showed a marked elevation of serum concentrations of IL-6 at 2 h and IL-12 at 2 and 4 h after flagellin challenge (Fig. 2c). In contrast, TLR5−/− mice were unresponsive to flagellin (Fig. 2c). These results demonstrate that TLR5 is
experiments. Flagellated strain (SB300) and an isogenic mutant, nonflagellated derivative (SB762). TLR5 gastroenteritis. We used two virulent strains of per 20 g of body weight of flagellated SB300 (and c) or nonflagellated SB762 (b and d). TLR5 murium. The group of mice were n = 22 (a), n = 19 (b), n = 8 (c), and n = 6 (d). Data in a and c are representative of three independent experiments.

Survival of TLR5−/− Mice to Challenge with S. typhimurium. To elucidate the role of TLR5 in host defense, we infected TLR5−/− mice with the Gram-negative bacterium S. typhimurium, a common human pathogen isolated from many cases of acute food-borne gastroenteritis. We used two virulent strains of S. typhimurium, a flagellated strain (SB300) and an isogenic mutant, nonflagellated derivative (SB762). TLR5−/− and WT mice were infected i.p. with S. typhimurium and monitored twice daily for morbidity and survival, and the time of death was recorded. Both groups of mice succumbed to infection with the WT and nonflagellated S. typhimurium strain in a similar time frame (Fig. 3 a and b). Furthermore, we infected WT and TLR5−/− mice i.p. with flagellated S. typhimurium, and the bacterial load in spleens and livers was examined 24 h after the infection. In both organs the number of the bacterial cfu of infected TLR5−/− mice was similar to WT controls (data not shown). Previous studies with C3H/HeJ mice carrying a natural dominant-negative mutation in the cytoplasmic domain of TLR4 and TLR4-deficient mice have uncovered the critical role of TLR4 in host resistance to Salmonella (22, 23). To tackle the possibility that TLR4 signaling compensates for the loss of functional TLR5 in TLR5−/− mice during S. typhimurium infection we performed the above infection studies using double TLR4−/−/TLR5−/− mice. As expected, TLR4−/− mice showed an increased susceptibility to S. typhimurium infection compared with WT or TLR5−/− mice (Fig. 3c). Interestingly, we also observed that double TLR4−/−/TLR5−/− mice were somewhat more susceptible than either single TLR4−/− or TLR5−/− mice (Fig. 3c). Next, we expanded our studies using the nonflagellated S. typhimurium, and with this strain we observed no difference in survival between double TLR4−/−/TLR5−/− and TLR4−/− mice (Fig. 3d). These findings suggest that the potential antibacterial effect of TLR5 in this infection model is compensated for by the presence of a functional TLR4 or other flagellin-sensing mechanisms.

Role of TLR5 in Inflammatory Lung Response Against Flagellin. Recombinant flagellin stimulates a transient innate immune response in the lung of several strains of mice characterized by the infiltration of neutrophils and rapid production of cytokines and chemokines (24, 25). To determine the role of TLR5 in innate immunity in the lung we challenged WT and TLR5−/− mice intranasally (i.n.) with flagellin. Ten and 24 h later the animals were killed, and the bronchoalveolar lavage fluids (BALFs) were collected and analyzed. The total cell count was significantly elevated in WT BALFs at both 10 and 24 h after challenge, whereas no cell infiltration was observed in TLR5−/− BALFs (Fig. 4a). The total neutrophil counts performed in the same BALFs showed a significant neutrophil accumulation in the lungs of flagellin-treated control mice at both 10 and 24 h after treatment (Fig. 4b). In contrast, neutrophils were absent in TLR5−/− BALFs (Fig. 4b). These results were confirmed by measuring myeloperoxidase (MPO) activity in cell pellets from WT and TLR5−/− BALFs (Fig. 4c). Next we examined the expression levels of several cytokines and chemokines that are important for neutrophil migration in vivo. We observed significant increases in TNF-α, IL-6, IL-1α, IL-10, and IFN-γ cytokines and macrophage inflammatory protein (MIP) 1α, MIP-2, and keratinocyte chemotactant (KC) chemokines at the mRNA level in WT lungs, at both 10 and 24 h upon flagellin instillation, whereas TLR5−/− lungs showed no or very slight expression of the same molecules (Fig. 4d). Moreover, flagellin administration induced a marked increase of IL-6 and KC at the protein level in the WT BALFs, whereas there was a very little production in TLR5−/− BALFs (Fig. 4e). These results suggest that the flagellin-induced pulmonary inflammatory response is TLR5-dependent.

Survival of TLR5−/− Mice upon P. aeruginosa Lung Infection. Previous studies have shown that the resistance of mice to P. aeruginosa acute lung infection is MyD88-dependent and does not seem to require
TLR2 or TLR4, because mice lacking both TLR2 and TLR4 did not show increased susceptibility (26). To explore the role of TLR5 in host defense against P. aeruginosa lung inflammation, WT, TLR5−/−, and double TLR4/5−/− mice were inoculated i.n. with 1 × 10⁶ cfu of P. aeruginosa strain PAK and monitored twice daily for morbidity and survival, and the time of death was recorded. Both WT and TLR5−/− mice showed similar rates of survival, whereas TLR4/5−/− mice showed higher susceptibility (Fig. 5 Left). Next we infected i.n. WT, TLR4−/−, TLR5−/−, and double TLR4/5−/− mice with a higher dose of P. aeruginosa (5 × 10⁶ cfu). Interestingly, WT, TLR4−/−, and TLR5−/− mice showed quite similar rates of survival, and all of them died by day 2, whereas TLR4/5−/− mice showed increased susceptibility and succumbed by day 12 after the infection (Fig. 5 Right). These data suggest that cooperation between TLR4 and TLR5 is important for effective innate response to P. aeruginosa lung infection.

**Role of TLR5 Expression in BM and Non-BM Cells upon in Vivo Flagellin Challenge.** In an attempt to clarify the relative contribution of BM and non-BM-derived TLR5-dependent signaling in the production of proinflammatory cytokines upon flagellin stimulation in vivo we constructed radiation BM chimeras between TLR5−/− (CD45.2) and WT congenic CD45.1 mice. Two groups of mice were compared: WT→TLR5−/− (WT BM into a TLR5−/− mouse) and TLR5−/−→WT (TLR5−/− BM into a WT mouse). Six weeks after irradiation and BM transfer, reconstitution of circulating leukocytes was evaluated by staining leukocytes for CD45.1 and CD45.2. We found that macrophages, DCs, B cells, and T cells were reconstituted at 98%, 88%, 99%, and 85%, respectively, with BM-derived cells in all mixed chimeras. At week 7 after reconstitution chimeras were injected i.p. with flagellin, and production of IL-6 and IL-12 in their sera was measured 2 h later. WT→TLR5−/− chimeras produced significant amounts of IL-6 (5.5 ± 1.9 ng/ml) and IL-12 (8.9 ± 1.9 ng/ml) in their sera, whereas TLR5−/−→WT chimeras showed a very slight response (0.6 ± 0.5 ng/ml for IL-6 and 0.9 ± 0.2 ng/ml for IL-12) (Fig. 6a). These data demonstrate that the major producers of serum IL-6 and IL-12 upon i.p. injection of flagellin are BM-derived cells.

Next we examined the contribution of BM- and non-BM–TLR5-expressing cells in the model of flagellin-induced lung inflammation. At week 12 after reconstitution BM chimeras were injected i.p. with flagellin, and pulmonary neutrophil infiltration and cytokine production in BALFs were evaluated. WT→TLR5−/− showed no significant neutrophil accumulation in airspaces or IL-6 production, whereas TLR5−/−→WT showed a considerable neutrophil infiltration that was accompanied by a slight production of IL-6 and TNF-α compared with WT treated mice (Fig. 6b and c). These data suggest that recognition of flagellin by non-BM–TLR5-expressing cells is required for the initiation of a lung inflammatory response.

**Discussion**

Although it is well established that TLR5 recognizes bacterial flagellin, recent studies have identified Ipaf as an essential sensor for cytoplasmic flagellin (27, 28). Thus, mammalian cells sense extracellular flagellin through TLR5 and intracellular flagellin through Ipaf. TLR5 activates NF-κB and MAPKs, leading to the secretion of many cytokines, including IL-6, IL-12, and TNF-α, whereas Ipaf permits the activation of caspase-1 and secretion of mature IL-1β. In the current study we describe the generation and characterization of TLR5-deficient mice and show that TLR5 is crucial for the in vivo recognition of flagellin but also may participate in the detection of systemic infection by S. typhimurium and lung infection by P. aeruginosa, although these phenomena can be masked by the presence of a functional TLR4 gene or other flagellin-sensing mechanisms.

In our studies, although in vitro stimulation of WT BMDCs by flagellin led to a strong early activation of IkBα and MAPKs and expression of IL-6 and IL-12 cytokines at the mRNA level, the protein levels of these cytokines were undetectable in the culture supernatants (Figs. 1d and 2a). In contrast, we observed that in vivo stimulation of control mice with flagellin led to strong secretion of these cytokines in sera and maturation of splenic DCs, responses that are undetectable in TLR5 mice (Fig. 2b and c). These results can be explained by the absence of cellular interactions and factors in our culturing conditions that block excessive flagellin-induced activation of TLR5 in vitro. Indeed, TLR5 signaling induces rapid activation of phosphoinositide 3-kinase that serves to limit MAPK signaling, thus limiting the potential negative consequences of excessive cytokine production (29).

To elucidate the role of TLR5 in the induction of antibacterial host defense, we tested the susceptibility of TLR5−/− mice upon i.p. infection with the bacterium S. typhimurium. We found no differ-

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**Fig. 5.** Survival curves of WT, TLR5−/−, TLR4−/−, and TLR4/5−/− mice upon i.n. P. aeruginosa infection. Survival of 7-week-old mice infected i.n. with 1 × 10⁶ cfu (Left) and 6 × 10⁶ cfu (Right) of P. aeruginosa was monitored every 12 h for 12 days.

**Fig. 6.** Role of TLR5 in BM and non-BM cells in response to flagellin stimulation in vivo. (a) BM WT→TLR5−/− and BM TLR5−/−→WT chimeras were injected i.p. with 1 μg of flagellin. Two hours later sera were collected, and production of IL-6 (Left) and IL-12p40 (Right) was assessed by ELISA. (b and c) WT and BM mice and BM chimeras were instilled i.n. with 1 μg of flagellin, and 10 h later BALFs were collected and used for the determination of cellular composition (b) and IL-6 and TNF-α production (c). The data are means ± SE. *P < 0.05, n = 5 WT→TLR5−/−, and n = 4 (a) and 3 (b and c) BM TLR5−/−→WT.
ence in survival between WT and TLR5−/− mice upon infection with flagellated or nonflagellated S. typhimurium. This finding is in accord with a recent report showing that the extreme susceptibility to infection of MOLF/Ei mice, a natural TLR5-deficient mouse, to S. typhimurium production in vivo requires TLR5 expression by BM. LPS is an important virulence factor of Salmonella, and the absence of a functional TLR4 leads to increased susceptibility upon S. typhimurium infection (22, 23). Because a general concept for TLR function is that multiple TLRs act in partnership in determining pathogen control, we speculated that the role of TLR5 in host resistance to S. typhimurium could be masked by the presence of a functional TLR4 gene. To test our hypothesis, we compared the survival rates of double TLR4/5−/− versus single TLR4−/− and TLR5−/− mice to S. typhimurium infection. Inflamed double TLR4/5−/− mice displayed enhanced susceptibility in direct contrast to TLR4−/− mice, which were slightly more susceptible than TLR5−/− or control animals (Fig. 3c). The rather modest effect of the absence of TLR5 in S. typhimurium infections may be due to the existence of redundant mechanisms to sense the bacteria and flagellin itself by the innate immune system. The fact that the nonflagellated mutant of Salmonella somewhat masked the increased susceptibility of the TLR4/5−/− mice (Fig. 3d) supports this hypothesis. Thus, although TLR5 may have a protective role in host resistance to S. typhimurium, it is clear that its action can be substituted by the action of TLR4 and probably other flagellin-sensing mechanisms.

It has been shown previously (24) and confirmed in this study that i.n. administration of purified flagellin in mice induces a transient innate immune response in the lung characterized by production of inflammatory mediators that affect neutrophil recruitment. Indeed, we showed here that all these responses were absent in the lungs of TLR5−/− mice that have been i.n. challenged with flagellin, demonstrating that the inflammatory lung response against flagellin is mediated by TLR5 (Fig. 4) and suggesting that TLR5 might play a critical role in the clearance of pulmonary infection that has been induced by flagellated bacteria. Although MDD88, the major adaptor molecule required for signaling events by most TLR/IL-1R family members, appears to play a major role in host resistance to P. aeruginosa, it has been difficult to attribute this requirement to the function of a single TLR. Thus, mice deficient in TLR2, TLR4, TLR2/TLR4, or IL-18, although in some cases displaying specific defects in antibacterial responses, exhibit no or only minor increases in susceptibility to i.n. challenge (26, 31). In contrast, absence or reduction in endogenous IL-1 activity improves host defense against Pseudomonas pneumonia (32). Our results showed that the lack of TLR5 alone cannot explain the increased susceptibility of MyD88−/− mice to P. aeruginosa lung infections. We also observed that TLR4/5−/− mice were more susceptible to P. aeruginosa lung infection than WT or TLR5−/− mice (Fig. 5). These results suggest that an efficient lung immune response against P. aeruginosa requires the simultaneous activation of TLR4, TLR5, and presumably other innate immune receptors and that the activation of one can compensate for the absence of activation of any of the others. Based on the data that we obtained from two infectious models, i.e., Salmonella and i.n. Pseudomonas, in the current study we could speculate that, depending on the bacterium that causes the infection, the route of infection, and the primary organ that is affected, the protective action of TLR5 can be substituted in a certain degree by another innate immune receptor (in our examples, TLR4).

We used radiation BM chimera between WT and TLR5−/− mice and found that, upon i.p. flagellin challenge, the early cytokine production in vivo required TLR5 expression by BM-derived cells. The complex mechanisms of inflammatory lung response presumably involve different cell types, including resident and hematopoietic cells. Indeed, we observed that the initiation of the inflammatory response in the lung, characterized by neutrophil infiltration, is mediated by TLR5 signaling by resident cells (Fig. 6b). We hypothesized that epithelial cells mediate this response, because they are the first non-BM-derived cells to directly contact flagellin. This finding is in agreement with the apical localization of TLR5 on the airway epithelium that correspond specifically to bacterial flagellin (33). This is not the case for other TLRs, because upon inhaled LPS the genotype of the donor BM cells seems to influence only in part the neutrophil recruitment, and both resident and myeloid cells are required for a full recruitment of neutrophils via MyD88 signaling (34). However, a recent study (where the requirement of MyD88 expression in stromal and hematopoietic cells was addressed in BM chimeras that have been challenged by P. aeruginosa) has shown that the early recruitment of polymorphonuclear cells required MyD88 expression by non-BM-derived cells and that early cytokine production was BM-dependent, whereas chemokine production was a non-BM-dependent phenomenon (35).

The host relies on two sensors for the detection of invading flagellated bacteria: TLR5 on the cell membrane and Ipaf in the cytoplasm. The relative importance of each one of these two molecules in the antibacterial host response likely depends on the type of the pathogen that is infecting the host, where extracellular bacteria might activate TLR5, whereas intracellular bacteria could trigger both TLR5 and Ipaf. Therefore, it would be interesting to address the host immune response to flagellated bacteria in the context of a system that both TLR5 and Ipaf signaling pathways are abrogated.

**Methods**

**Mice.** For the generation of the TLR5−/− mice, a genomic DNA fragment containing the TLR5 gene was screened from a 129/Sv mouse genomic library. The targeting vector was constructed by replacing a 2.6-kb fragment of the TLR5 exon, including the start codon of the TLR5 gene, with a neomycin-resistance gene cassette flanked by two loxP sites. A herpes simplex virus thymidine kinase gene, driven by an MCl promoter, allowed for negative selection. The targeting vector was transfected into embryonic W9.5 ES cells. Targeted ES cells were identified by Southern blotting and subsequently injected into C57BL/6 blastocysts. Male chimeric mice were then mated to C57BL/6 female mice. Heterozygous TLR5 mice were intercrossed to generate TLR5-deficient mice. TLR5−/− mice on the C57BL/6 background (10 generations) and WT C57BL/6 mice were used for all of the experiments. TLR4−/− mice (36) (C57BL/6) were a gift from S. Akira (Osaka University, Osaka, Japan). WT C57BL/6 congenic CD45.1 and CD45.2 mice were purchased from Charles River (L’Arbresle, France). Double TLR4/5−/− mice were obtained by intercrossing TLR5−/− with TLR4−/− mice (36).

**Reagents.** Endotoxin-free flagellin fliB from S. typhimurium was kindly provided by VaxInnate (New Haven, CT). LPS from E. coli was from Sigma (St. Louis, MO); peptidoglycan from Staphylococcus aureus was from Fluka (Buchs, Switzerland); polyI:C, R848, and Pam3CSK4 were from Invivogen (San Diego, CA); and CpG DNA was synthesized by Sigma-Genosys (St. Louis, MO).

**Salmonella and Pseudomonas Challenge of Mice in Vivo.** Age- and sex-matched groups of mice were infected i.p. with S. typhimurium or i.n. with P. aeruginosa. The P. aeruginosa PAK strain was a gift from Alain Filloux (Institut de Biologie Structurale et Microbiologie–Centre National de la Recherche Scientifique, Marseille, France). For i.n. challenge, mice were anesthetized by i.p. injection of ketamine–xylazine, and a 40-μl suspension of 1 μg of flagellin or P. aeruginosa was applied i.n. Lungs were collected at 10 or 24 h after challenge and homogenized, and RNA was extracted by TRIzol. BALFs were collected by five installations of 0.5 ml of normal saline.
Western Blotting. Cytosolic proteins extracted (20 µg) from BMDCs were resolved on 10% SDS/PAGE gels and transferred to Immobilon P membrane (Millipore, Billerica, MA). Blotting was performed with the indicated antibodies (Cell Signaling, Danvers, MA). Bands were visualized with secondary HRP-conjugated antibodies and the ECL System (Amersham Pharmacia, Piscataway, NJ).

**BMDCs and Macrophages.** BMDCs and BM macrophages were generated as previously described (37). Immature BMDCs and BM macrophages were collected at days 6 and 5, respectively, and plated at 1 x 10^5 cells per milliliter in the presence or absence of stimuli.

**RT-PCR.** Total RNA from BMDCs or mouse lungs was isolated with TRIzol reagent (Invitrogen), and contaminant DNA was removed by DNase I (Ambion, Huntingdon, U.K.) according to the manufacturer's instructions. Total RNA (5 µg) was reverse transcribed with using SuperScript II reverse transcriptase (Invitrogen). cDNAs were amplified with specific primers for cytokines, chemokines, or hypoxanthine phosphoribosyltransferase.

**Measurement of Cytokine, Chemokine, and MPO Production.** Concentrations of IL-6 (eBioscience, San Diego, CA), IL-12p40 (BD Bioscience, San Jose, CA), and KC (Biosource, Nivelles, Belgium) in culture supernatants, sera, or cell-free BALF were measured by ELISA. The MPO activity was determined in cell pellets derived from BALFs resuspended in 200 µl of substrate solution containing 0.5% hexadecyltrimethylammonium bromide, 0.17 mg/ml O-dianisidine dihydrochloride, and 0.05% H2O2. The enzymatic activity was determined by measuring the change in absorbance at 460 nm every minute.
Brucella control of dendritic cell maturation is dependent on the TIR-containing protein Btp1
Brucella Control of Dendritic Cell Maturation Is Dependent on the TIR-Containing Protein Btp1

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Brucella is an intracellular pathogen able to persist for long periods of time within the host and establish a chronic disease. We show that soon after Brucella inoculation in intestinal loops, dendritic cells from ileal Peyers’ patches become infected and constitute a cell target for this pathogen. In vitro, we found that Brucella replicates within dendritic cells and hinders their functional activation. In addition, we identified a new Brucella protein Btp1, which down-modulates maturation of infected dendritic cells by interfering with the TLR2 signaling pathway. These results show that intracellular Brucella is able to control dendritic cell function, which may have important consequences in the development of chronic brucellosis.

Introduction

The immune response to bacterial infection relies on the combined action of both the innate and adaptive immune systems. Dendritic cells (DCs) are known as mediators of pathogen recognition and are strategically located at the typical sites of bacterial entry and have the ability to migrate from peripheral tissues to secondary lymphoid organs to elicit primary T cell responses and initiate immunity.

DCs express several pathogen recognition receptors such as C-type lectins and Toll-like receptors (TLRs) that recognise molecular patterns expressed by pathogens and determine the type of immune response. Microbial stimuli induce significant morphological and biochemical changes, such as IL-12 secretion and increased surface expression of many co-stimulatory and MHC class II molecules. This activation of DCs, known as maturation, is required for efficient T-cell priming and pathogen elimination. However, several bacterial pathogens have developed mechanisms to subvert DC function and evade the immune system. Mycobacterium tuberculosi interferes with TLR signalling via the C-type lectin DC-SIGN, blocking DC maturation and IL-12 production [1]. The immune response is instead directed towards immune suppression with secretion of IL-10, which seems to contribute to the chronic carriage of this pathogen. Similarly, Bordetella pertussis-infected DCs secrete IL-10 and activate T regulatory cells that suppress a protective immune response and enhance colonisation of the lower respiratory tract [2]. In addition, several studies have shown that while Salmonella typhimurium induces normal maturation of DCs and secretion of pro-inflammatory cytokines [3,4] it is able to block MHC class II antigen presentation in bone marrow-derived DCs [5,6]. Another bacterial pathogen, Francisella tularensis, which also induces phenotypic maturation of DCs, has been shown to inhibit secretion of pro-inflammatory cytokines such as TNF-α while eliciting production of immunosuppressive cytokines that facilitate pulmonary infection [7]. F. tularensis has also been shown to replicate efficiently within DCs [7] in contrast to many other bacterial pathogens (including mycobacteria, Bordetella, Salmonella and Legionella pneumophila) for which DCs seem to restrict their intracellular growth.

Human brucellosis is a zoonotic disease that results from direct contact with infected animals or ingestion of contaminated food products. It is usually presented as a debilitating febrile illness that can progress into a chronic disease with severe complications such as infection of the heart and bones. It has been previously shown that B. abortus infection in calves using an intestinal loop model occurred via Peyers’ patches [8]. Several studies using animal models have established macrophages and placental trophoblasts as two key targets of Brucella infection [9] but a more recent study has shown that Brucella can replicate in vitro within human monocyte-derived DCs [10]. DCs may therefore constitute an important cellular niche to promote infection.

Brucella virulence is dependent on its ability to survive and replicate within host cells. Once internalised, Brucella is found within a compartment, the Brucella-containing vacuole (BCV) that transiently interacts with early endosomes [11]. However, BCVs do not fuse with lysosomes and instead fuse with membrane from the endoplasmatic reticulum (ER), to establish a vacuole suited for replication [11,12]. An important virulence factor of Brucella is its unconventional lipopolysaccharide (LPS) that is necessary for entry and early develop-

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Brucella Controls DC Immune Responses

Author Summary

A key determinant for intracellular pathogenic bacteria to induce infectious diseases is their ability to avoid recognition by the host immune system. Although most microorganisms internalized by host cells are efficiently cleared, Brucella behave as a Trojan horse causing a zoonosis called brucellosis that affects both humans and animals. Here we show that pathogenic Brucella are able to target host cell defense mechanisms by manipulating the function of the sentinel of the immune system, the dendritic cells. In particular, the Brucella TIR-containing protein (Btp1) targets the Toll-like receptor 2 activation pathway, which is a major host response system involved in bacterial recognition. Btp1 is involved in the inhibition of dendritic cell maturation. The direct consequence is a control of inflammatory cytokine secretion and antigen presentation to T lymphocytes. These bacterial proteins are not specific for Brucella and have been identified in other pathogens and may be part of a general virulence mechanism used by several intracellular pathogens to induce disease.

Infection of Intestinal DCs

We used a mouse intestinal loop model to characterise the intracellular fate of Brucella in the context of Brucella infection. We therefore analysed the recruitment of lysosomal associated membrane protein 1 (LAMP1) and of the ER-specific KDEL bearing molecules in DCs. Thus, in contrast to other cell types [15,19] the cyclic β-1,2-glucan synthesized by Brucella was not required for virulence during DC infection since envB mutant replicates as efficiently as the wild-type strain (Figure 2A). Loss of lysosomal and acquisition of ER markers on BCVs are hallmarks of Brucella infection. We therefore analysed the recruitment of lysosomal associated membrane protein 1 (LAMP1) and of the ER-specific KDEL bearing molecules in DCs. We therefore analysed the recruitment of lysosomal associated membrane protein 1 (LAMP1) and of the ER-specific KDEL bearing molecules in DCs. Thus, in contrast to other cell types [15,19] the cyclic β-1,2-glucan synthesized by Brucella was not required for virulence during DC infection since envB mutant replicates as efficiently as the wild-type strain (Figure 2A).

B. abortus Replicates within ER-derived Vacuoles in Murine BMDCs

Having established the relevance of DCs in the context of Brucella infection we used BMDCs as cellular model to characterise the intracellular fate of Brucella. We first analysed the survival of the wild-type B. abortus 2308 strain in murine BMDCs by enumerating the colony forming units (CFU) at different times after infection. We observed an increase in the number of intracellular bacteria up to 48 h after inoculation, showing that the wild-type B. abortus is able to survive within BMDCs (Figure 2A). Equivalent results were obtained in BMDCs-positive cells (Figure 2A, graph on the right). Intracellular replication was confirmed by microscopic examination of infected cells. At 2 h after infection cells contained on average 1.2 ± 0.1 bacteria per cell in contrast to 17.7 ± 3.4 bacteria per cell at 48 h. Although high intracellular bacterial loads were observed, viability of BMDCs was not affected up to 48 h after infection (Figure S1). At 24 and 48 h we found that only a small percentage of infected cells (approximately 12 and 25%, respectively) contained more than 10 bacteria, indicative of intracellular replication. This observation is similar to what was previously described for murine bone marrow-derived macrophages [12] and HeLa cells [11]. In agreement with previous reports, we did not observe replication with the wild-type Salmonella typhimurium [18]. In the case of a strain lacking the type IV secretion component envB, there was a continuous decrease in CFU numbers, suggesting that this system is necessary for the establishment of the Brucella replicative niche in DCs. Surprisingly, in contrast to other cell types [15,19] the cyclic β-1,2-glucan synthesized by Brucella was not required for virulence during DC infection since envB mutant replicates as efficiently as the wild-type strain (Figure 2A).

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Results

B. abortus Infection of Intestinal DCs

We used a mouse intestinal loop model to characterise the cellular targets of B. abortus by confocal microscopy during early stages of the infection. One hour after inoculation in a loop containing at least one Peyer’s patch, B. abortus mainly penetrated the epithelium through the FAE of Peyer’s patches and were either associated or internalized by cells presenting a “dendritic-like” morphology and identified as DCs on the basis of their positivity for CD11c (Figure 1). On more than one hundred cells associated with B. abortus we observed, 50% were CD11c+ cells, the others being either FAE cells (30%) or not determined. Few bacteria were also observed in the lamina propria of adjacent villi (Figure 1). These were always associated with CD11c+ cells. Thus, in the mouse model, DCs are a cellular target of B. abortus during enteric infection.
Brucella Controls DC Immune Responses
majority of BCVs were positive for this enzymatic activity at 24 h after infection. In cryosections of infected DCs we observed ER membranes directly fusing with BCVs (Figure 2F, blue arrows), which were also decorated with an antibody against the ER resident protein calnexin (Figure 2G). In addition to the ER cisternae, which were extensively labelled (data not shown), gold particles were present on the vacuolar membranes surrounding Brucella and on tubular-like ER membranes connecting with the vacuoles (blue arrows) as well as on vesicles in the vicinity of BCVs (red arrow). Together, these results confirm that Brucella is dependent on its VirB type IV secretion system to replicate within DCs in an ER-derived compartment that evades the lysosomal degradative pathway, similar to what has been observed for other cell types, in particular within bone marrow-derived macrophages [12].

**B. abortus Interferes with Phenotypic Maturation of DCs**

Most studies have shown that infection of DC is generally associated with their activation and a mature phenotype. This phenotype is characterized not solely by the up-regulation of co-stimulatory and MHC class II molecules at the cell surface, but also by the intense clustering of multivesicular bodies (MVB) around the microtubule organizing center (MTOC) [20], as well as the formation of large cytosolic dendritic cell aggresome-like induced structures (DALIS), which are made of defective newly synthesised ubiquitinated proteins [21].

This situation is well illustrated by *Salmonella*-infected DCs (Figure 3A), in which MHC class II molecules mostly localize on the cell surface and MVBs, labelled with LAMP1, are often tightly concentrated at the MTOC. Surprisingly, most of *Brucella*-infected DCs did not show any sign of maturation, since *Brucella*-bearing cells rarely displayed lysosomal clustering or MHC II surface accumulation (Figure 3A). Instead, MHC II molecules remained mostly intracellular co-localising with LAMP1 as observed in non-activated DCs. At 24 h after inoculation, only 14.3 ± 0.5% of *Brucella*-infected DCs (versus 31.9 ± 8.3% for *Salmonella*) displayed a mature phenotype in terms of MHC II molecules and LAMP1 distribution, suggesting that *Brucella* does not promote DC activation.

We then tested if DALIS formation was also impaired in infected cells. Although the function of DALIS is still unclear, these structures are easily detectable using the FK2 antibody that recognises both mono- and polyubiquitinated proteins, and therefore provide an effective way of monitoring DC maturation by microscopy. Thus, we compared the kinetics of DALIS formation in DCs infected with either *Brucella* or *Salmonella*, previously shown to induce normal maturation of DCs [3,6] (Figure 3B–3D). In the case of *Salmonella*, formation of DALIS began after 4 h (as reported with *E. coli* LPS [21]), with 90% of infected cells containing large and numerous DALIS 24 h after infection (Figure 3B and 3C), while only 20% of control cells contained DALIS, probably due to mechanical or spontaneous maturation. Although significantly higher than the control (p = 0.005), only 43% of *Brucella*-infected DCs contained DALIS 24 h after infection and this number remained stable a later time-points (Figure 3C). Furthermore, the average size of *Brucella*-induced DALIS was always considerably smaller than that of DALIS formed in the presence of *Salmonella* (Figure 3B). These observations suggest the *Brucella* replication in DC could be restricting the maturation process. A significantly higher proportion of DCs infected with *varB*<sup>−</sup>, a mutant that does not replicate in DCs, contained DALIS at 24 h after infection (p = 0.006, Figure 3D).

Similar results were obtained when analysing DCs activated with heat-killed *B. abortus* (p = 0.018, Figure 3D), consistent with the hypothesis that *B. abortus* is actively inhibiting the maturation of DCs.

The extent of this inhibition was monitored by flow cytometry of CD11c-positive DCs infected with GFP-expressing *Brucella* or *Salmonella*, for surface expression of classical maturation markers (eg CD80, CD86 and MHC molecules). Analysis of the median of fluorescence (Figure 4A) or the geometric mean (data not shown) in the CD11c<sup>+</sup>GFP<sup>+</sup> populations showed a consistently lower surface expression of all co-stimulatory and MHC class II molecules in cells infected with *B. abortus* than in *Salmonella*-infected cells (Figure 4A and 4B). However, statistical difference between *B. abortus* and *Salmonella* was only observed for CD86 expression (p = 0.011). This is probably due to the fact that in the case of *Brucella*-infected DCs two populations were observed (overlay histograms, Figure 4A and 4B), one with a moderately increased surface levels in comparison to control cells and the other with similar levels of expression to *Salmonella*-activated DCs. This is in agreement with microscopy observations where a high proportion of MHC class II molecules remained intracellular in *Brucella*-infected cells (Figure 2A). Surprisingly, we did not detect any significant difference in the surface expression of MHC class I molecules in cells infected by the two pathogens (Figure 4B), thus suggesting the expression of MHC class I molecules is controlled differently than MHC class II and co-stimulatory molecules. Together, these data confirm that maturation of *Brucella*-infected DCs is impaired.

In addition, we have found that *Brucella* can only establish its intracellular replicative niche in immature DCs (Figure S3). When *E. coli* LPS was added to cells (to induce maturation) early after infection (0.5 to 6 h), there was a significant inhibition of bacterial growth (p < 0.001, Figure...
significance was obtained at 24 and 48 h between wild-type and the virB mutant \( (p < 0.002) \) and also between wild-type *Brucella* and *Salmonella* at 24 h \( (p = 0.047) \) and 48 h \( (p < 0.002) \).

(B) Quantification of the percentage of wild-type or virB9\(^{-}\) mutant BCVs that contain LAMP1 by confocal immunofluorescence microscopy. The difference between wild-type and the virB9\(^{-}\) mutant were always statistically significant \( (p = 0.045 \) at 30 min and 4 h; \( p < 0.002 \) for other timepoints).

(C) Representative confocal images of DCs infected with 24 h with wild-type *B. abortus* or virB9\(^{-}\) mutant expressing GFP, labelled with antibodies against MHC II (blue) and LAMP1 (red).

(D) Confocal image of DCs infected with 24 h with wild-type *B. abortus* expressing GFP, labelled with antibodies against MHC II (blue) and KDEL (red). Samples were also processed for conventional electron microscopy (E), cytochemistry for glucose 6 phosphatase detection (F) or immunogold labelling with an anti-calnexin antibody (G). Bars: 5 \( \mu \)m (C and D); 0.5 \( \mu \)m (F and G). For (A) and (B) data are means \( \pm \) standard errors of four independent experiments.

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S3). However, bacterial CFU counts did not decrease when LPS was added at 12 h after infection, suggesting that *Brucella* survival is not affected by DC maturation at a time point that corresponds to its arrival in the ER \cite{11, 12}. In contrast, addition of *E. coli* LPS had no significant effect on the intracellular survival of *Salmonella* in DCs (data not shown), thus further underlining the need for *Brucella* to avoid DC maturation.

### Brucella Interferes with DC Function

DCs infected with *B. abortus* do not achieve full maturation. We therefore investigated the consequences of *Brucella* infection on the ability of DCs to secrete cytokines, by analysing the supernatants of infected cells. Levels of TNF-\( \alpha \), IL-12 (p40/p70), IL-6 and IFN-\( \beta \) were consistently and considerably lower in supernatants from *Brucella*- than from *Salmonella*-infected cells (Figure 5A). This effect was observed at 24 h as well as at 48 h after infection (data not shown) suggesting that *Brucella* infection inhibits the secretion of immuno-stimulating cytokines.

Intracellular IL-12 (p40/p70) expression was further analysed by flow cytometry. DCs incubated with either media alone or infected with GFP-expressing *Brucella* or *Salmonella* were labelled at 4 and 24 h post-inoculation with anti-CD11c and IL-12 (p40/p70) antibodies. Analysis of IL-12 expression was carried out on CD11c\(^{+}\) cells (Figure 5B). IL-12 expression in *Salmonella*-infected DCs peaked after 4 h, similarly to what observed with *E. coli* LPS (data not shown), whereas in *Brucella*-infected DCs, IL-12 expression was only detected after 24 h (Figure 5B). Similar results were obtained when analysing GFP-positive populations for each pathogen (data not shown).

These results indicate that *Brucella*, like *Francisella*, is capable of interfering with the ability of DCs to secrete cytokines. Interestingly, *Brucella* also reduced the capacity of DCs to present exogenous ovalbumin to specific T cells in the context of MHC class I and class II (Figure 5C) suggesting that *Brucella* is generally down-modulating the immuno-stimulatory properties of maturing DCs both at the cytokine production level and also at the antigen processing and presentation level.

### Identification of a Brucella Protein Involved in the Control of DC Maturation

The *Brucella* genome was searched for candidate proteins responsible for interfering with DC maturation. We identi-
Figure 3. B. abortus Interferes with DC Maturation

DCs were infected with different B. abortus strains or with Salmonella and labelled with the anti-MHC II and anti-LAMP1 antibodies (A) or FK2 antibody (B) to detect MVBs or DALIS, respectively.

(C) Comparison between the percentages of DCs infected with either wild-type B. abortus (black bars) or S. typhimurium (white bars) that contain DALIS. The negative control (grey bars) corresponds to mock-infected DCs with DALIS. The difference between Brucella and Salmonella infected cells at 24 h is statistically significant, p < 0.0001.

(D) Quantification of the percentage of DCs with DALIS at 24 h after incubation with media (negative), wild-type B. abortus (wt), virB9- mutant or heat-killed B. abortus (HK). The differences between wild-type wt and either virB9- or HK are statistically significant (p = 0.0059 and p = 0.018, respectively). For (C) and (D) data represent means ± standard errors of at least three independent experiments. Bars: 10 μm (A); 5 μm (B).

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Figure 4. *B. abortus*-infected DCs Have Reduced Expression of Co-stimulatory Molecules

(A and B) DCs were either incubated with media only (blue line) or infected with *B. abortus* (shaded grey) or *S. typhimurium* (red line) expressing GFP for 24 h. DCs were labelled with anti-CD11c conjugated with APC and anti-CD40, CD80, CD86, MHC II, and MHCI antibodies conjugated with PE. Representative histograms and values of median of the PE fluorescence (FL2) correspond to CD11c^+^ cells for the negative and CD11c^+^GFP^+^ cells for the *Brucella* and *Salmonella* infected cells. Values of the median fluorescence correspond to four independent experiments.

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Figure 5.  *B. abortus* Interferes with DC Function

(A) and (B) *B. abortus* blocks secretion of pro-inflammatory cytokines.

(A) Supernatants were obtained at 24 h from DCs inoculated with either media (negative), *B. abortus*, *S. typhimurium*, or heat-killed *B. abortus* (HKB) and the levels of cytokines were determined by ELISA. Values correspond to means ± standard errors of at least three independent experiments. A clear statistical difference was observed between *Brucella* and *Salmonella* for TNF (p = 0.014) and IL12 (p = 0.0098) and to a lesser extent for IL6 (p = 0.041) and INFβ (p = 0.040).

(B) DCs were infected for 4 or 24 h with either media (negative), *B. abortus* or *S. typhimurium* constitutively expressing GFP. DCs were then treated for 1 h with brefeldin A, permeabilised and labelled with anti-CD11c and anti-IL12 (p40/p70) conjugated with APC and PE, respectively. Representative dot blots are shown for CD11c+ populations.

(C) *B. abortus* reduces the capacity of DCs to induce T cell proliferation. DCs were infected with wild-type *B. abortus* and the model antigen ovalbumin (OVA) was added after 30 min at a final concentration of 50 μg/ml. Cells were stimulated with OVA for 2 h and then washed to remove the antigen. Splenic CD4+ T cells prepared from OTI and OTII transgenic mice were then added at a 1:1 ratio and co-cultured for 48 h. IL-2 production in culture supernatants was measured by 3H-thymidine incorporation on the IL-2-dependent CTLL-2 cell line. Data are the means ± standard errors from triplicates of a representative experiment. A small statistical difference was observed between Ova (Ova-Brucella) for both OT1 (p = 0.035) and OT2 (p = 0.045).

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*Brucella* Btp1 Protein Modulates TLR Signalling

TLR signal transduction pathways are essential for the induction of DC maturation and secretion of pro-inflammatory cytokines. The most important TLRs in the context of a bacterial infection are TLR2, TLR4, TLR5 and TLR9 that recognize lipoproteins, LPS, flagellin and Cpg DNA, respectively. However, recent work showed that DCs poorly respond to flagellin due to limited TLR3 expression in these cells [25] so we therefore excluded this pathway. To determine if Btp1 was interfering with a specific TLR in the context of an infection we used DALIS formation as a read-out, since the increased number of DCs with DALIS was the clearest phenotype for the *btp1* mutant when compared to wild-type. DCs from different TLR knockout mice were infected with *Brucella* wild-type or the *btp1* mutant and analysed by confocal immunofluorescence microscopy. We observed that DCs from wt mice infected with *btp1* contained a very high number of DALIS, contrasting with infected TLR2−/− DCs (Figure 7A), a phenotype reminiscent of DCs treated with the TLR2 ligand Pam. This difference was not noticeable in other TLR knockout mice used. Indeed, quantification of the number of infected DCs containing DALIS confirmed that DCs infected with the *btp1* mutant showed increased DALIS formation in wild-type, TRIF−/−, TLR4−/−, TLR7−/− and TLR9−/− DCs but not in TLR2−/− and to a lesser extent in MyD88−/− DCs (Figure 7B). These results suggest that inhibition of DC maturation by Btp1 is at least in part dependent on the TLR2 pathway. Appropriate ligands were used as positive controls for the assay (Figure S6).

In addition, we found that DC maturation induced by heat-killed *Brucella*, as measured by DALIS formation, was also dependent on TLR2 (Figure S7). This result is consistent with previous reports showing that TNF-α secretion induced by heat-killed *Brucella* in DCs is partially dependent on TLR2 and MyD88 pathways [26]. We then investigated if the ability of *Brucella* to reduce DC maturation was dependent on the TLR2 pathway. DCs were infected with wild-type *B. abortus* for 24 h to allow establishment of a replication niche and then incubated with either LPS or Pam for a further 24 h. We found that DCs infected with wild-type *Brucella* and treated with *E. coli* LPS were very activated and contained very large DALIS, similar to what has been observed in LPS-treated cells. In contrast, infected cells treated with Pam contained only small DALIS similar to untreated infected cells. However, non-infected cells treated with Pam contained...
large and numerous DALIS (Figure S8). These results confirm that Brucella is able to control TLR2-dependent DC matura-

tion.

To determine if Btp1 had the ability to specifically block TLR2 signalling we expressed increasing amounts of myc-
tagged Btp1 in HEK293T cells along with a constant amount of either TLR2 or TLR9 (as a negative control) and a luciferase NF-κB reporter plasmid. We then analysed lucifer-
ase activity in the presence of the appropriate ligands. A *Salmonella* effector protein, myc-PipB2, was also included as a negative control as it is known to affect kinesin recruitment rather than cell signalling [27]. We found that Btp1 efficiently inhibited TLR2 signalling, in a dose dependent manner but not TLR9 (Figure 7C).

In conclusion, Btp1 is able to interfere with the TLR2 to reduce the progression of maturation in infected DCs.

**Discussion**

*Brucella* enteric infection has been found to occur in calves’ Peyer’s patches [8]. Using a murine intestinal loop model, we found that after penetrating the epithelium, *B. abortus* localized in DCs just below the FAE of Peyer’s patches. Interestingly, a few bacteria were also observed in the interfollicular region of Peyer’s patches or in the lamina propria of adjacent villi, were they were always associated with DCs. Unlike most pathogens, *Brucella* was able to establish a replication niche within DCs cultured *in vitro* in a very similar manner to what has been described in macrophages and non-phagocytic cells. DCs therefore constitute a potentially important cellular target for *Brucella* infection.

Interestingly, we have found that although the VirB type IV secretion system is required for virulence in DCs, the *Brucella* cyclic β-1,2-glucan is dispensable for early events of BCV biogenesis. The *Brucella* cyclic β-1,2-glucan, which modulates lipid microdomain organisation [15], is essential for preventing fusion between BCVs and lysosomes in macrophages and non-phagocytic cells. It is possible that within DCs early BCVs have a different membrane composition than in macrophages. In the case of human monocyte-derived DCs, *Brucella* strongly induces the formation of veils on the plasma membrane during internalisation, a phenomenon not observed in macrophages [10]. Alternatively, the proteolytic and bactericide activity of DC endosomes and phagosomes, which has been shown to be considerably limited when compared to macrophages [28,29], potentially eliminates the need for cyclic β-1,2-glucan during infection. Further work will be required to characterise in detail the molecular mechanisms of *Brucella* internalisation by DCs.

An important aspect of *Brucella* pathogenesis is its ability to evade the immune system and persist within the host. *In vitro*
studies have illustrated that *Brucella* is efficient at remaining unnoticed by host cells, notably by having an atypical LPS [30], which is several hundred times less toxic than *Salmonella* or *E. coli* LPS. Although, shown to signal through the TLR4 pathway, *Brucella* LPS is not a potent inducer of pro-inflammatory cytokines and anti-microbial proteins such as the IFN-γ inducible p-47 GTPases [31]. DCs treated with up to 10 μg/ml of purified *Brucella* LPS, did not induce phenotypic maturation nor significant secretion of pro-inflammatory cytokines (Figure S4A and data not shown). Conversely, DCs incubated with heat-killed *B. abortus* showed significant maturation indicating that host cells can detect *Brucella* pathogen associated molecular patterns (PAMPs), other than LPS. A recent study has shown that a lumazine synthase from *Brucella* can activate DCs via TLR4 [32]. In addition, heat-killed *Brucella* can induce TLR9 and promote Th1-mediated responses in both DCs and macrophages [33] probably after bacterial degradation in lysosomes.

Immunity against *Brucella* requires cell-mediated mechanisms that result in the production of cytokines such as IL-12 and IFN-γ. In this study, we have shown that *Brucella* inhibits or delays the process of DC maturation to establish a replicative niche, which results in reduced cytokine secretion and disabled antigen presentation. It is likely that impairment of DC function and cytokine secretion by *Brucella* favours infection and/or promotes the establishment of the chronic phase of the disease. Although, we did not detect any significant secretion of IL-10 in infected DCs it is possible that, by preventing their full activation, *Brucella* uses the tolerogenic properties of DCs to subvert the immune response. There is growing evidence that induction of tolerance is not restricted to immature DCs. Recent studies have shown that migrating DCs, which are mature or in the process of maturing (bearing considerable surface expression of MHC class II molecules) are capable of inducing tolerance [34–36]. *Brucella*-infected DCs, which show an intermediate level of maturation could therefore contribute to tolerance induction in tissues and establishment of chronic infection. Consistent with this hypothesis, reduction of IL-10 levels in mice has been shown to improve host resistance to *Brucella* infection [37]. In addition, a new *Brucella* virulence protein (PrpA) was recently identified as a potent B-cell mitogen and IL-10 inducer [38]. Indeed, this protein is necessary for the early immuno-supression observed in *Brucella*-infected mice and the establishment of chronic disease. Brucellosis development is a complex process that involves many virulence factors some of them, such as the VirB type IV secretion system, are required for virulence in different cell types, whereas others may function in specific cell types, such as the *Brucella* cyclic β-1,2-glucan in macrophages and PrpA in B-cells.

A recent study has shown that *Brucella*-infected human myeloid DCs do not mature extensively and instead secrete low levels of IL-12 and TNF-α and have an impaired capacity to present antigens to naïve T cells [39]. These results are consistent with our data in murine DCs. However, this study implicates the *Brucella* outer membrane protein Omp25 and the two-component regulatory system BvrRS in the control of DC maturation through blockage of TNF-α secretion. This is not surprising as both these mutants have significant modifications of their outer membrane and LPS structure, which are an essential feature of *Brucella* virulence enabling bacteria to escape pathogen recognition [40,41]. Therefore the effects observed are more likely to be pleiotropic than specific to DC maturation. Furthermore, in the case of the *bvrR* mutant, which they show induces higher maturation than the *omp25* mutant, the increased maturation is most likely due to its degradation in lysosomal compartments (the equivalent results can be obtained with heat-killed *Brucella*) since this mutant was previously shown to be unable to replicate within human DCs by the same group [10].

In this study, we have identified a new *Brucella* protein, Btp1, which contributes to modulation of TLR signalling within host cells. A mutant lacking this protein showed increased levels of DALIS formation and also an increase in secretion of pro-inflammatory cytokines. However, the level of surface expression of some co-stimulatory molecules was not significantly increased in cells infected with the mutant strain. Thus the existence of other bacterial molecules, capable of further inhibiting the antigen presentation capability of DCs, is highly probable and additional studies are required to identify and characterize these putative new factors.

By ectopically expressing Btp1, we found that it can inhibit TLR signalling, particularly TLR2, in a manner consistent with observations in DCs obtained from different knock-out mice infected with the *btp1* mutant strain. Interestingly, we did not observe any inhibition of human TLR2 when using the same concentrations of Btp1. It is possible that higher amounts of Btp1 are required to block human TLR2 in this system or it may be that Btp1 specifically recognizes the mouse TLR2 TIR domain. Further work is now required to identify the cellular target of Btp1 and to analyse its role during infection *in vivo*. Previous studies have highlighted the importance of TLR signalling in the control of *Brucella* infection, namely the role of the MyD88 adaptor protein in the clearance of the S19 *Brucella* vaccine strain in macrophages at late stages of the infection [42]. It is interesting that *Brucella* produces at least one protein that can act as negative regulator of TLR signalling, which constitutes an essential link between the innate and adaptive immune systems. Along with the *Salmonella* TIR-containing protein TlpA, they may constitute a novel class of intracellular bacterial virulence factors that interfere with TLR signalling and control specific steps of the host immune response. We hypothesize that Btp1 is secreted into the host cytosol were it interacts with either the TLR2 directly and/or with its adaptor proteins resulting in reduction of TLR2 signalling.

In the case of *Salmonella*, TlpA is required for virulence in macrophages and in the mouse model of infection. This is not the case for Btp1, since a *btp1* mutant is not significantly attenuated in the mouse model of brucellosis (Marchesini, Comerci and Ugalde, unpublished results). Interestingly, *Salmonella* infection results in phenotypic activation of DCs and high secretion of pro-inflammatory cytokines probably due to its LPS (since addition of *Salmonella* LPS at 100 ng/ml to DCs results in DC activation; data not shown). Nonetheless, live *Salmonella* is able to restrict MHC class II-dependent antigen presentation [6], however no study has yet been carried out with the *tlpA* mutant in this context. It is possible that the TIR-containing protein TlpA is involved in the control of DC function but no mechanism has yet been proposed and it remains unclear how *Salmonella* interferes with antigen presentation in DCs. When comparing *Salmonella*
and Brucella, it is important to consider that these two pathogens cause very distinct diseases in susceptible mice; S. typhimurium infection is characterised by fast systemic spread of the bacteria whereas B. abortus establishes a chronic-like non-fatal disease. It is therefore likely that DCs play a very distinct role in the pathogenesis of these two bacteria and that these pathogens have developed specific mechanisms to control the immune response. For example, in the case of Salmonella, activation of DCs does not affect its intracellular survival (data not shown) whereas Brucella cannot reach its ER-derived replication niche in fully matured DCs, which impact on its survival. In the case of Brucella, infected DCs remain in an intermediate maturation stage. Although its atypical LPS enables bacteria to remain less noticeable by the host, it is interesting that Brucella expresses at least one protein that interferes to a certain level with DC maturation and particularly with secretion of pro-inflammatory cytokines by DCs. Therefore, it is possible that Btp1 contributes to the establishment of chronic brucellosis by controlling in the host immune response within specific tissues. Overall, Brucella infection of DCs may therefore be required to control the anti-bacterial immune response, in addition to providing a productive replication niche. Alternatively, the migration properties of DCs could facilitate infection spreading, as suggested by the rapid interaction of DCs with FAE penetrating Brucella.

Materials and Methods

Bacterial strains. The bacterial strains used in this study were S. enterica serovar Typhimurium strain 12023, smooth virulent B. abortus strain 2308 [11] and the isogenic mutants onB9 [43], gsp C1129 [15] and btp1 (this study, see below). In the case of Brucella, green fluorescent protein (GFP)-expressing derivatives contain a pBBR1MCS-2-derivative expressing the gfp-mut3 gene under the control of the lac promotor. The plasmid pVP25.1 carrying the gfp-mut3 under the control of a constitutive promoter was used for Salmonella. Brucella strains were grown in tryptic soy broth (TSB; Sigma-Aldrich) and Salmonella in Luria Bertani (LB) medium. For infection, we inoculated 2 ml of media for 16 h at 37 °C up to an optical density (OD600nm) of approximately 2.0 [12]. Salmonella strains were cultured 16 h at 37 °C with aeration to obtain stationary phase cultures.

Construction of btp1 complemented strain. A 1.257 Kbp EcoRI fragment containing the btp1 gene (BABL-09279) was amplified using primers 5′-cgaattcttcgatccgcg-3′ and 5′-acctaggggaatctggcctt-3′ and ligated to pGem-T-Easy vector (Promega) to generate pGem-T-btp1. The plasmid was linearized with EcoRI and ligated to a 0.7 Kbp Smal fragment containing a gentamicin resistance cassette to generate pGem-T-btp1::Gm. Plasmids were electroporated into R. abortus S2508 where they are incapable of autonomous replication. Homologous recombination events were selected using gentamicin and carbenicillin sensitivity. PCR analyses showed that the btp1 wild-type gene was replaced by the disrupted one. The mutant strain obtained was called R. abortus btp1::Gm (btp1').

Construction of the btp1 complemented strain. A 1.257 Kbp EcoRI fragment containing the btp1 gene was excised from pGem-T-btp1 and ligated to the EcoRI site of pBBR1-MCS4 [44]. The resulting plasmid was conjugated into btp1 mutant strain by bacterial mating.

Intestinal loop model of infection. C57BL/6 mice were starved 24 h before anaesthesia. After a small incision through the abdominal wall was done, a loop starting from the ileoocaecal junction and containing 2 to 3 Peyers patches was formed taking care to maintain blood supply. Before closing the loop, 280 µl of a 10% CFU/ml culture of R. abortus expressing GFP was injected. The intestine was then returned to the abdominal cavity for one hour before the mice were killed and the intestinal loops were removed, opened flat and washed extensively with PBS. Peyer's patches were then fixed with 3.2% paraformaldehyde for 60 minutes, rinsed in PBS, infused overnight in 35% sucrose and frozen in OCT compound. Immunofluorescence labelling was performed on 8 to 10 µm thick cryostat tissue sections overnight at 4°C using hamster anti CD11c (N418) and rabbit anti Brucella abortus LPS followed by incubation with goat anti-rabbit Alexa Fluor 546 and goat anti-hamster Cy5 for 1 h at room temperature.

Bacterial infection and replication assays. BMDCs were prepared from 7-8 week-old female C57BL/6 mice or TLR2+/− [45], TLR4−/− [46], TLR9−/− [47], TLR3−/− [48], TRIF−/− [49] and RIG-I−/− mice, as previously described [51]. Infections were performed at a multiplicity of infection of 50:1 for flow cytometry experiments and 20:1 for all remaining experiments. Bacteria were heat-killed on a shaking water bath at 400 g for 10 min at 37°C and then incubated for 30 min at 37°C with 5% CO2 atmosphere. Cells were gently washed twice with medium and then incubated for 1 h in medium supplemented with 100 ug/ml streptomycin to kill extracellular bacteria (or gentamicin for Salmonella). Thereafter, the antibiotic concentration was decreased to 20 ug/ml. Control samples were always performed by incubating cells with media only and following the exact same procedure for infection. To monitor bacterial intracellular survival, infected cells were lysed with 0.1% Triton X-100 in H2O and serial dilutions plated onto TSB agar to enumerated CFUs. When stated cells were previously selected based on CD11c-labeling using CD11c MicroBeads (MACS, Miltenyi Biotec) following the manufacturer’s instructions.

Antibodies and reagents. The primary antibodies used for immunofluorescence microscopy were: cow anti-B. abortus polyclonal antibody; hamster anti-CD11c (N418; Biologend); affinity purified rabbit Kivoli antibody against murine TLR-1 [29] and anti-mouse TLR4 (BD Biosciences); anti-gentamicin conjugated anti-CD11c antibody (HL3) was used in all experiments along with either phycocerythrin-conjugated CD40, CD80, CD86, IL-12 p40, or mouse anti-F4/80 (MinBio). For flow cytometry allophycocyanin conjugated-anti-CD11c antibody (HL3) was used in all experiments along with either phycocerythrin-conjugated IL-12 (p40/p70) monoclonal from Pharmingen was used. The following TLR ligands from InvivoGen were used: ODN1826, Poly(1,3C), PamsCSK4 and E. coli K12 LPS.

Immunofluorescence microscopy and flow cytometry. Cells were fixed in 3% paraformaldehyde, pH 7.4, 37°C for 15 min and then processed for immunofluorescence labelling as previously described [11]. Samples were either examined on a Leica DMRE epifluorescence microscope or a Zeiss LSM 510 laser scanning confocal microscope for image acquisition. Images of 1024 × 1024 pixels were then assembled using Adobe Photoshop 7.0. In all experiments we used an antibody against a conserved cytoplasmic epitope found on MHC-II. Before labelling, infected cells were washed in MitoXpress (Figure S2). All BMDCs significantly expressing MHC II were also labelled with an anti-CD11c antibody confirming that they are indeed DCs (Figure S2). Quantification was always done by counting more than 100 cells in 4 independent experiments, for a total of at least 400 host cells analysed.

For flow cytometry, infected DCs were collected and stained immediately before fixation. Isotype controls were included as well as DCs infected with non-gfp B. abortus as control for autofluorescence. Cells were always gated on CD11c for analysis and at least 100,000 events were collected to obtained a minimum of 10,000 CD11c-positive and GFP-positive events for analysis. A FACScalibur cytometer (Becton Dickinson) was used and data was analysed using FlowJo software (Tree Star).

Electron microscopy. The detection of glucose-6-phosphatase activity was performed by electron microscopy cytochemistry as previously described [52] with minor modifications. Cells were pre-fixed in 1.25% (vol/vol) glutaraldehyde in 0.1 M Pipes, pH 7.0, containing 5% (wt/vol) sucrose for 30 min on ice, washed three times for 5 min in 0.1 M Pipes, pH 7.0, containing 10% (vol/vol) sucrose and then briefly in 0.08 M Tris-maleate buffer, pH 7.2, containing 1% (vol/vol) sucrose. Cells were then incubated in 0.06 M glucose-6-phosphate (Sigma-Aldrich), 0.1% (vol/vol) nitrate (wt/vol) in 0.08 M Tris-maleate buffer, pH 6.5, for 2 h at 37°C. After 3 washes in 0.08 M Tris-maleate buffer and 3 washes in 0.1 M Pipes containing 0.08 M Tris-maleate buffer, pH 7.0, cells were then incubated in 5 mM MgCl2 and 5 mM MgSO4, cells were post-fixed in 1.25% (vol/vol) glutaraldehyde in the same buffer for 1 h at 4°C and, and with, 1% OsO4 in the same buffer devoid of sucrose for 1 h at room temperature. Samples were processed as previously described [52].

For immunoelectron microscopy samples were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 0.1 M sucrose, 5 mM CaCl2 and 5 mM MgCl2, for 1 h at room temperature.
temperature followed by 8% paraformaldehyde in the same buffer overnight. Sections were dehydrated, passed through bovine skin gelatin in phosphate Sorensen 0.1 M. Fragments of the pellet were infiltrated overnight with 2.3 M sucrose in PBS at 4 °C, mounted on aluminium studs and frozen in liquid nitrogen. Sectioning was done at −110 °C in an Ultracut cryo-microtome (Leica). The 60 nm thick sections were collected in 1:1 mixture of 2.3 M sucrose and 2% methyl cellulose, transferred onto Formvar-carbon-coated nickel grids and incubated for 2 min with PBS-glycine 50 mM at RT. Sections were then incubated for 50 min with primary antibodies in PBS-1% BSA. Sections were then incubated for 50 min with primary antibody in PBS-1% BSA, washed 5 times and then labelled with 10 nm protein A-gold particles in PBS-1% BSA for 20 min. Sections were finally washed 10 times in PBS, fixed for 5 min with 1% gluteraldehyde in PBS, rinsed in distilled water and incubated for 5 min with uranyl acetate solution. Grids were then rinsed in distilled water, incubated with 2% methyl cellulose (Sigma-Aldrich) and 0.4% uranyl acetate for 5 min on ice and then dried at room temperature. Samples were analysed with a Zeiss 912 electron microscope and images were then processed using Adobe Photoshop 7.0.

Cytotoxicity and cell viability assays. Measurement of lactate dehydrogenase (LDH) release in the supernatant of cells infected with different strains was carried out using the Detection Kit (Roche) as indicated by the manufacturer. The percentage of cytotoxicity corresponds to the ratio between the experimental value subtracted by the negative control (spontaneous LDH release) and the maximum LDH release (triton lysed cells) subtracted by the negative control. For detection of 7-AAD, cells were infected with GFP-expressing strains as described above and collected at 24 h in cold PBS. Cells were then labelled with FITC-conjugated anti-CD11c-APC, washed several times in cold PBS and then incubated with 7-AAD following the manufacturer's instructions (BD Pharmingen). The flow cytometric analysis were performed on fixed cells within 20 min.

CD11c+Cells were stained with Sandwich enzyme-linked immunosorbutent assays (ELISA) from Abcys were used to detect IL-12 (p40/p70), TNFα and IL-6 from supernatants of BMDCs infected with different Brucella strains. The ELISA kit for IFNβ measurement was obtained from PBL Biomedical Laboratories.

Antigen presentation assay. DCs were seeded at 1×105 cells per well in 96-well flat bottom plates and infected for 30 min as described above. Cells were then washed and ovalbumin (Sigma-Aldrich) was added at a final concentration of 50 μg/ml for 2 h in media containing 100 μg/ml of streptomycin to kill extracellular bacteria. Antigen was then removed by changing media and DCs were co-cultured with splenic CD4+ T cells prepared from OTI and OTII transgenic mice as described earlier. All experiments were done in triplicates and the data presented corresponds to a representative experiment of three independent replicates as the standard errors were always ≤ 5%.

Luciferase activity assay. HEK 295T cells were transiently transfected using FuGENE (Roche) for 24 h, according to manufac- turer's instructions, for a total of 0.4 μg of DNA consisting of 50 ng TLR plasmids, 200 ng of pBIXLac reporter plasmid, 5 ng of control Renilla luciferase (pRL-null, Promega) and indicated amounts of Bp expression vectors. The total amount of DNA was kept constant by adding empty vector. Were indicated, cells were treated with E. coli LPS (1 μg/ml), Pam3CSK4 (1 ng/ml) or Cpg ODN1826 (10 μM) for 6 h and then cells were lysed and luciferase activity measured using Dual-Glo Luciferase Assay System (Promega). The TLR2 construct was obtained by PCR amplification from cDNA of bone marrow-derived macrophages and subcloning in the pcDNA3.1 expression vector (Promega).

Statistical analysis. All experiments were carried out at least 3 independent times and all the results correspond to the means ± standard errors. Statistical analysis was done using two-tailed unpaired Student's t-test and p < 0.05 were not considered significant.

Supporting Information

Figure S1. Analysis of DC Viability during Infection
(A) BMDCs were infected with either S. typhimurium or B. abortus wild-type, or btp1 mutant strains and the supernatants collected at 24 or 48 h for detection of lactate dehydrogenase (LDH) activity. Stauroporin was used as a positive control at a final concentration of 200 nM. Results correspond to percentage cytotoxicity in relation to the negative and maximum values of LDH release and were obtained from three independent experiments. (B and C) DCs were infected with either S. typhimurium or B. abortus wild-type strains expressing GFP and analysed by flow cytometry for labelling with the viral dye 7-Amino-actinomycin (7-AAD). Results correspond to CD11c-positive (B) or CD11c GFP-positive (C) cells. The percentage of cells that are 7AAD-positive are indicated in each histogram.

Figure S2. Confocal Microscopy Analysis of MHC II Labelling in Bone Marrow-derived DCs and Macrophages
(A) Cells were labelled with anti-MHC II (rivoli, red) and anti-CD11c (blue) antibodies and, in the case of Brucella (wt) with anti-LPS antibody (green). All cells showing significant MHC II labelling were CD11c positive. (B) Control labelling of bone marrow-derived macrophages infected with B. abortus stained with the anti-MHC II antibody (red). No significant labelling was observed in macrophages whereas some small DALIS (labelled with the FK2 antibody, blue) were occasionally observed.

Figure S3. B. abortus Fails to Replicate in Mature DCs
Intracellular CFUs were enumerated by lysing cells at 24, or 48 h after infection of DCs with wild-type B. abortus, in absence (black) or presence of E. coli LPS (100 ng/ml). E. coli LPS was added at either 30 min (red), 1% BSA, washed several times in cold PBS and then incubated with 7-AAD following the manufacturer's instructions (BD Pharmingen). The flow cytometric analysis were performed on fixed cells within 20 min.

Figure S4. The btp1 Mutant Does Not Differ from Wild-type Brucella with regard to Expression of Co-stimulatory Molecules
DCs were either incubated with media only (blue line) or infected with B. abortus wild-type (shaded grey) or btp1 mutant (red line) expressing GFP for 24 h. DCs were labelled with anti-CD11c conjugated with APC and anti-CD40, CD86, and MHC II antibodies conjugated with PE. Representative histograms showing the PE fluorescence (FL2) correspond to CD11c+ cells for the negative and CD11c- GFP+ cells for the Brucella infected cells.

Figure S5. Analysis of the Effect of LPS Extracts on DC Maturation and Complementation of btp1 Mutant
(A and B) DCs were incubated for 24 h with crude LPS extracts from Brucella with or without the btp1 mean central muta- tion, or S. typhimurium. Samples were then labelled with the FK2 antibody (red), anti-CD11c (blue), and either anti-Brucella LPS or Salmonella-LPS antibodies. DALIS are indicated by arrows. (A) Corresponds to means ± standard errors of 3 independent experiments. (C) Analysis of TNFα and IL-12 (p40/p70) secretion measured by ELISA from the supernatant of DCs 24 and 48 h after inoculation with wild-type (wt), btp1-, or btp1/plus btp1. The results correspond to a representative of two independent experiments.

Figure S6. Analysis of the Formation of DALIS in Different Knockout Mice
BMDCs obtained from wild-type mice or different knockout mice were treated with the appropriate ligand for 24 h. The percentage of cells containing DALIS was then quantified by immunofluorescence microscopy. A representative from three independent experiments is shown.

Figure S7. Heat-Killed Brucella Induction of DALIS is Dependent on Myd88 and TLR2
DCs were incubated for 24 h with either the TLR2 ligand Pam (500 μg/ml) or heat-killed Brucella (HK). Samples were then labelled with the FK2 antibody (red) and anti-MHC II (green).

(A) Quantification corresponds to means ± standard deviations of two independent experiments.
Figure S8. Analysis of the Formation of DALIS in the Presence of LPS and PAM

DCs were infected for 24 h with wild-type B. abortus and either incubated for 24 h with or without E. coli LPS or PAM. Samples were then labelled with the FK2 antibody (red) and antibody against CD80 (green).

(A) Non-infected cells treated with E. coli LPS for 24 h.
(B) Wild-type Brucella gfp-infected cells at 48 h post-infection.
(C) Wild-type Brucella gfp-infected cells (24 h post-infection) further treated with E. coli LPS for another 24 h.
(D) Wild-type Brucella gfp-infected cells (24 h post-infection) further treated with PAM for another 24 h (see on the same coverslip the upper infected cell displaying a much less maturation pattern than the lower cell that has not been infected).

Accession Numbers

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Author contributions.
Suzana P. Salcedo conceived of and designed the experiments and carried out the majority of the experimental work and writing of the manuscript; Maria Ines Marcondes constructed Btp1 mutants and complementation; Hugues Lelouard conducted mouse intestinal loop experiments, Emilie Fugier constructed Btp1 expression vector; Gilles Joly carried out ELISA and antigen presentation assays; Stephanie Balor was responsible for electron microscopy; Alexandre Muller provided technical assistance in preparation of cells; Nicolas Lapaque participated in scientific discussions; Olivier Demaría maintained TLR knockout mice; Lena Alexopoulos contributed to the concept and provided reagents; Diego J. Comerci and Rodolfo A. Ugarte contributed to the concept of the study; Philippe Pierre contributed to the concept and design of the experiments and the writing of the manuscript; Jean-Pierre Gorvel contributed to the concept and design of the experiments and project coordination, confocal microscopy of LPS treated cells, and writing of the manuscript.

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Competing interests.
The authors have declared that no competing interests exist.

References
Annexe 3

Contribution of TLR7 and TLR9 signaling to the susceptibility of MyD88-deficient mice to autoimmune myocarditis
Contribution of TLR7 and TLR9 signaling to the susceptibility of MyD88-deficient mice to myocarditis

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Abstract

Toll-like receptors (TLRs) are evolutionary conserved molecules that recognize various microbial components and host-derived agonists from damaged cells and play a central role in innate and adaptive immunity. It has been reported that MyD88, the adaptor molecule downstream of all TLRs, except TLR3, is essential for initiation of experimental autoimmune myocarditis (EAM). To determine the role of the intracellular TLRs in EAM, TLR3−/−, TLR7−/−, and TLR9−/− mice were immunized with cardiac α-myosin heavy chain peptide (MyHC-α) in Complete Freund’s Adjuvant (CFA) and their EAM scores and associated immunological responses were compared to wild-type (WT) and MyD88−/− mice. MyD88−/− mice were completely resistant to EAM and had a profound defect in all the parameters we tested. Myocardial cellular infiltration and in vitro proliferation of MyHC-α-restimulated splenocytes were markedly reduced in TLR7−/− mice, while TLR3−/− and TLR9−/− mice showed similar inflammatory cell infiltration in the heart-like WT mice. Thus, the resistance of MyD88−/− mice to EAM can be attributed to a certain degree to TLR7 signaling. Moreover, upon murine cytomegalovirus-induced myocarditis, we found that the severity of myocardial inflammation was higher in TLR9−/− and MyD88−/− mice compared with WT, TLR3−/−, or TLR7−/− mice and paralleled the ability of the mice to fight the viral infection.

Keywords: Toll-like receptors, autoimmunity, myocarditis, inflammation, MCMV

Introduction

Myocarditis (from the Greek μυκαρδίτες) is a collection of diseases of infectious, autoimmune, and toxic etiologies characterized by inflammation of the myocardium. Subsequent myocardial destruction can lead to dilated cardiomyopathy and heart failure [1]. The signs and symptoms associated with myocarditis vary and relate either to the actual inflammation of the myocardium or to the weakness of the heart muscle that is secondary to the inflammation. Although myocarditis may be initiated by viral infection, several lines of evidence suggest that chronic stages of disease may be mediated by an autoimmune reaction against heart muscle myosin [2,3]. Experimental autoimmune myocarditis (EAM) can be induced in susceptible BALB/c mice by immunization with cardiac myosin or α-myosin heavy chain-derived peptides (MyHC-α) in CFA [4,5], by injection of myosin-presenting dendritic cells [6] or through the adoptive transfer of myosin-specific CD4+ T cells [7].

Proinflammatory cytokines such as TNF-α, IL-6, IL-17, and IFN-γ play an important role in the pathogenesis of autoimmune myocarditis. Whereas TNF-α, IL-6, and IL-17 are essential for the development of disease, IFN-γ deficiency leads to increased pericardial inflammation and fibrosis, suggesting that different cytokines can have opposite roles in EAM [8–11]. Many viruses have been implicated as causes of myocarditis. These most commonly include adenovirus, enterovirus, and cytomegalovirus (CMV) [12]. The mouse model of CMV, murine CMV (MCMV), has been useful

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in providing knowledge of the pathogenesis of CMV-induced myocarditis, since sublethal MCMV infections of adult BALB/c mice induce inflammation in murine cardiac tissue, ranging from focal lymphocytic infiltration to intense diffuse infiltration and necrosis [13,14].

Toll-like receptors (TLRs) form a large family of pattern-recognition receptors that recognize a wide variety of microbial components and potential host-derived agonists [15,16]. Activation of TLRs by ligand binding initiates signal transduction pathways, which triggers expression of various genes that control innate immune responses and further instruct development of antigen-specific adaptive immunity. TLR signaling pathways are finely regulated by Toll/interleukin-1 receptor (TIR) domain-containing adaptors, such as MyD88, TIRAP, TRIF, and TRAM [17]. To date, 10 and 13 members of TLRs have been identified in humans and mice, respectively. TLRs are expressed at the cell surface or in intracellular compartments such as endosomes, lysosomes, or the endoplasmic reticulum [18,19]. The intracellular TLRs, represented by TLR3, TLR7, TLR8, and TLR9, predominantly recognize microbial nucleic acids and trigger innate immune responses by producing type I IFN and inflammatory cytokines. TLR3 recognizes mainly viral double-stranded RNA and polyinosinic-polycytidylic acid (poly I:C), a synthetic analog of dsRNA [20]. TLR7 senses viral single-stranded RNA, imidazoquinolone derivatives, and guanine analogs [21–24]. TLR9 detects unmethylated CpG DNA motifs that are frequently present in bacteria and viruses and synthetic oligodeoxynucleotides [25–27].

Inappropriate activation of TLRs by self-components can result in sterile inflammation or autoimmunity [15]. Moreover, TLR-mediated signaling is an important link between innate immunity and a diverse panel of clinical disorders, including cardiovascular disorders such as heart failure, viral myocarditis, and atherosclerosis. Indeed, it has been shown that in the hearts of patients with myocarditis, the increased expression of TLR4 is associated with enteroviral replication [28]. Furthermore, TLR4-deficient mice develop significantly reduced levels of myocarditis upon infection with the enterovirus coxsackievirus B3 (CBV3) [29]. Interestingly, human cardiac cells express minimal levels of TLR1–TLR8, while infection with CBV3 virions increases the expression of TLR7 and TLR8 that are able to recognize viral single-stranded RNA [30]. In the EAM model, activation of TLRs on dendritic cells loaded with myosin, as self-peptide, is essential for the induction of myocarditis and heart failure [6]. Moreover, mice lacking the adaptor molecule MyD88 are protected from myocarditis after immunization with MyHC-α peptide in CFA due to impaired expansion of heart-specific CD4+ T cells [31].

In this study, we aimed to elucidate the role of the intracellular TLRs, TLR3, TLR7, and TLR9 in autoimmune myocarditis using TLR-deficient mice and the model of myocarditis induction upon immunization with MyHC-α in CFA. Moreover, we evaluated heart inflammation upon induction of myocarditis with MCMV infection in TLR3−, TLR7−, TLR9−, and MyD88-deficient mice.

Materials and methods

Mice and induction of EAM

TLR3−/−, TLR7−/−, TLR9−/−, and MyD88−/− mice were generated as previously described [20,24,26,32] and were backcrossed into the BALB/c background for 10 generations. Wild-type (WT) BALB/c mice were purchased from Charles River (L’Arbresle, France). For the induction of EAM, a murine heart muscle-specific peptide derived from α-myosin heavy chain (amino acids 614–629, Ac-RSLLKLMATLFSTYASADR) was used as antigen. The peptide purchased from NeoMPS (Strasbourg, France) was acetylated and flanked with arginines to increase its solubility in physiological water.

On days 0 and 7, female mice aged 6–8 weeks were injected subcutaneously with a 1:1 emulsion of 150 μg MyHC-α in CFA supplemented with 1 mg/ml of Mycobacterium tuberculosis strain H37Ra (Difco, Detroit, MI, USA). On day 21, mice were sacrificed, and blood, spleen, and heart samples were collected. Mice were housed under specific pathogen-free conditions and handled in accordance with approved institutional guidelines and French directives.

Histopathology

The severity of myocarditis was assessed on heart sections after hematoxylin and eosin coloration using a semi-quantitative scoring system ranging from 0 to 4: 0, no inflammatory infiltrates; 1, small foci of inflammatory cells; 2, larger foci of >100 inflammatory cells; 3, >10% of a tissue section involved; and 4, >30% of a tissue section involved.

Immunofluorescence

Heart tissues were embedded in Optimal Cutting Temperature (OCT) compound (tissue teck) and frozen in liquid nitrogen. Six micrometer sections were cut on a cryostat, thawed and mounted on SuperFrost plus slides (Menzel-Glaser, Braunschweig, Germany), air-dried, and then stored at −20°C for further use. For immunofluorescence staining, sections were fixed with acetone and incubated with a blocking solution (Phosphate Buffered Saline (PBS) containing 2% Bovine Serum Albumin (BSA)).
Then the slides were stained with anti-CD11b-Alexa 488 (1/100; BD Pharmingen, San Diego, CA, USA), anti-Gr-1-Alexa 647 (1/100; Biolegend, San Diego, CA, USA), and anti-CD4 PE (1/100; BD Pharmingen) followed by a secondary donkey antibody anti-rabbit Alexa 555 (1/200; Invitrogen, Carlsbad, CA, USA). Fluorescent images were acquired using a Zeiss LSM 510 laser scanning confocal microscope (Welwyn Garden City, Hertfordshire, UK) and analyzed on LSM Image Browser.

Splenocyte proliferation and cytokine and cardiac troponin I production

On day 21, mouse spleens were processed to make a single cell suspension, red blood cells were lysed with red blood cell lysis buffer (Cliniscience, Montrouge, France), and splenocytes were resuspended in Royal Park Memorial Institute (RPMI) medium, 10% Fetal Calf Serum (FCS), and 50 μM β-mercaptoethanol at 5 x 10^6 cells/ml. For splenocyte proliferation, cells were plated in 96-well flat-bottomed plates (200 μl/well) and either left untreated or stimulated for 72 h with 0.1, 1, and 10 μg/ml MyHC-α peptide. Triplicates were pulsed with [3H]-methyl-thymidine for the last 12 h, and thymidine incorporation was measured in a beta scintillation counter (Wallac, Gaithersburg, MD, USA). For cytokine production, cells were plated in 24-well plates (1 ml/well) and either left untreated or stimulated for 48 h with 3–15 μg/ml MyHC-α peptide. IL-6 and IFN-γ were measured in culture supernatants by enzyme-linked immunosorbent assay (ELISA) (Cliniscience) according to the manufacturer's instructions. The protein levels of cardiac troponin I in mouse sera were measured by ELISA (Life Diagnostics, Inc., West Chester, PA, USA) according to the manufacturer’s instructions.

Cardiac myosin preparation

Heavy chains of the cardiac myosin protein were purified according to the method of Shiverick et al. [33] with modifications as described [34]. Briefly, hearts from a dozen mice were homogenized in 10 ml/g of ice-cold KCl buffer, pH 6.8 (0.3 M KCl, 0.15 M K₂HPO₄, 10 mM Na₂HPO₄, and 1 mM MgCl₂), and stirred for 90 min at 4°C. Myosin extracted from muscle homogenate was centrifuged for 1 h at 140,000 g at 4°C. Supernatants were collected, 20 volumes of water added, and incubated at 4°C overnight to precipitate the myosin. On day 2, the precipitate was spun at 12,000g for 30 min at 4°C and resuspended in 10 ml/g of cold imidazole buffer, pH 6.8 (0.5 M KCl, 10 mM imidazole, 5 mM MgCl₂, 5 mM Na₂ATP, and 2 mM dithiothreitol). The solution was spun at 43,000g for 30 min at 4°C (to precipitate actin) and supernatant was collected.

The myosin was precipitated in eight volumes of ice-cold water at 4°C overnight. On day 3, steps of day 2 were repeated (to remove residual actin) with the exception of adding 6.5 volumes of ice-cold water to precipitate myosin. On day 4, the myosin precipitate was spun at 12,000g for 30 min at 4°C and resuspended in 50 mM Na₃P₂O₇. Purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Autoantibody titration

Sera from immunized mice were assayed for IgG1, IgG2a, and IgG2b autoantibodies using myosin-specific ELISA. Immuno-maxisorp plates (Nunc, Roskilde, Denmark) were precoated overnight at 4°C with 10 μg/ml whole cardiac myosin protein in PBS. Plates were blocked with PBS containing 1% BSA for 1 h at room temperature and sera were applied in duplicate wells at a dilution of 1:6000 for IgG1, 1:20 for IgG2a, or 1:500 for IgG2b in PBS containing 1% BSA and incubated for 2 h with shaking at room temperature. For the determination of IgG1, IgG2a, and IgG2b autoantibodies, samples were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, or IgG2b (Southern Biotech, Birmingham, AL, USA).

Figure 1. Expression of TLR3, TLR7, and TLR9 mRNA in mouse hearts during autoimmune myocarditis. WT BALB/c mice were immunized with MyHC-α and CFA, and the expression of TLR3, TLR7, and TLR9 in mouse hearts was evaluated by quantitative PCR on days 6, 10, and 21. Each dot represents an individual mouse, and horizontal bars represent the mean ± SEM. *P < 0.05, **P < 0.01. Data are representative of two independent experiments.
respectively, for 1 h at room temperature. ELISAs were developed with a tetramethylbenzidine substrate reagent set (BD Pharmingen), and optical densities were read at 450 nm. Purified mouse IgG1, IgG2a, and IgG2b proteins (Southern Biotech) were used for the generation of standard curves.

RNA extraction, quantitative-PCR, and MCMV viral load

Total RNA was isolated from homogenized hearts with TRIZol (Invitrogen), contaminant DNA was removed using DNase I (Ambion, Austin, TX, USA) and first-strand cDNA was synthesized using SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. For real-time PCR, 5 μg total RNA was subjected to a reverse transcription reaction. Following reverse transcription, 1 μl of cDNA was amplified by real-time PCR on an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Warrington, UK). For the determination of MCMV viral load, total DNA was isolated from mouse heart samples by DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Parallel reactions were performed by quantitative PCR for the detection of DNA of immediate early gene 1 (IE-1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each experiment was performed in duplicate in optical 96-well reaction plates using SYBR Green Master Mix (Applied Biosystems) and 300 nM of sense and anti-sense primers in a final volume of 25 μl. The sequences of primers are provided in Supplementary Table I.

Figure 2. Autoimmune myocarditis in TLR3<sup>−/−</sup>, TLR7<sup>−/−</sup>, TLR9<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice. WT, TLR3<sup>−/−</sup>, TLR7<sup>−/−</sup>, TLR9<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice were left untreated (UNT) or immunized with MyHC-α and CFA. On day 21, myocarditis was assessed on heart sections as described in the “Materials and methods” section. (A) Disease severity scores of individual WT, TLR3<sup>−/−</sup>, TLR7<sup>−/−</sup>, TLR9<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice. Horizontal bars represent the median value for each group. *P < 0.05, **P < 0.01. (B) Hematoxylin and eosin staining of heart sections revealed inflammatory infiltrates in immunized mice (magnification ×20). Data shown represent independent experiments reproduced five times for TLR3<sup>−/−</sup> mice, three times for TLR7<sup>−/−</sup> and TLR9<sup>−/−</sup> mice, and once for MyD88<sup>−/−</sup> mice.
Amplifications were performed with the following protocol: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

**MCMV and infection**

MCMV strain K181 was a kind gift of H. Farrell (Centre for Preventive Medicine, Suffolk, UK) and maintained by salivary gland passage in CD1 mice. Viral titers were determined by plaque assay using mouse embryonic fibroblasts, as previously described [35]. Groups of male age-matched mice (6–8 weeks old) were infected intraperitoneally with 5 × 10⁴ (TLR3⁻/⁻ mice) or 2 × 10⁴ (TLR7⁻/⁻, TLR9⁻/⁻, or MyD88⁻/⁻ mice) plaque-forming units (PFU) of MCMV. On day 10, mice were sacrificed and sera were collected for the measurement of cardiac troponin I, while hearts were harvested and processed for histological scoring and measurement of MCMV viral load.

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Notes: Statistical significance for median severity grade was assessed using the Mann–Whitney U-test. n.d., not done; *P = 0.028; †P = 0.033; ‡P = 0.047; †P = 0.007.
The nonparametric Mann–Whitney U-test was used to evaluate myocarditis severity scores. Cytokine and autoantibody levels between WT and knockout mice were compared with the nonparametric Mann–Whitney U-test.

**Results**

**Expression of TLR3, TLR7, and TLR9 in mouse hearts during autoimmune myocarditis**

To determine if TLR3, TLR7, and TLR9 expression is modulated during the course of EAM, WT BALB/c mice were immunized subcutaneously with MyHC-α in CFA on days 0 and 7. On days 6, 10, and 21 after the first immunization, the expression of TLR3, TLR7, and TLR9 mRNA was assessed by quantitative PCR on total heart RNA. We found that the expression of TLR3, TLR7, and TLR9 was upregulated in mouse hearts on days 6 and 10 post immunization compared to untreated mice (Figure 1). By day 21 post immunization, TLR7 and TLR9 mRNA expression was still upregulated compared to unimmunized mice, while TLR3 mRNA expression had returned to normal levels (Figure 1). Thus, the expression of TLR3, TLR7, and TLR9 in the heart is modulated during the course of the autoimmune myocarditis.

**TLR7, but not TLR3 or TLR9, contributes to EAM**

Previous studies have shown that MyD88 signaling controls autoimmune myocarditis induction, since MyD88−/− mice are protected from myocarditis after immunization with MyHC-α in CFA [31]. However, since the adaptor molecule MyD88 is common in the signaling pathways downstream of IL-1 receptor type 1 (IL-1R1), which also contributed to the development of autoimmune myocarditis [36], IL-18 receptor and most of the TLRs, it is not easy to conclude what is the contribution of TLRs in the development of autoimmune myocarditis. In the current study, we attempted to determine the contribution of TLR3, TLR7, and TLR9 in autoimmune myocarditis.

To do so, WT, TLR3−/−, TLR7−/−, TLR9−/−, and MyD88−/− mice were immunized with MyHC-α in CFA, and the development of myocarditis was evaluated 21 days later. WT, TLR3−/−, and TLR9−/− mice developed severe myocarditis with inflammatory infiltrates (Figure 2(A), (B) and Table I), while TLR7−/− and MyD88−/− mice were significantly protected from disease and developed only minimal inflammation compared to WT immunized mice (Figure 2(A), (B) and Table I).

Next, heart-infiltrating cells in mice immunized with MyHC-α in CFA were characterized for their neutrophil, monocyte, or CD4+ T cell phenotype using immunofluorescence staining of heart sections (Figure 3). Infiltrated neutrophils, monocytes, or CD4+ T cells could be detected in the areas of the inflammation in WT mice, while these cells were markedly reduced in TLR7−/− hearts (Figure 3). However, immunized TLR3−/− and TLR9−/− mice showed similar recruitment of neutrophils, macrophages, and CD4 T cells in their hearts like WT control mice (Figure 3).

**Splenocyte proliferation and cytokine production upon restimulation with MyHC-α**

Autoimmune myocarditis is a CD4+ T cell-mediated disease [7,37,38]. In TLR7−/− mice, disease resistance paralleled impaired expansion of heart-specific...
T cells, as suggested by the impaired in vitro proliferation of MyHC-α-restimulated whole splenocytes compared to WT controls (Figure 4(A)). However, TLR3−/− splenocytes showed similar proliferation like WT cells, while TLR9−/− splenocytes had increased proliferation compared with WT controls (Figure 4(A)). Moreover, we assessed the production of IL-6, IFN-γ, TNF-α, and IL-12p40 in supernatants of in vitro restimulated whole splenocytes from WT, TLR3−/−, TLR7−/−, TLR9−/−, or MyD88−/− mice were left untreated (UNT) or immunized with MyHC-α and CFA. On day 21, total mouse splenocytes were isolated. (A) For proliferation assays, cells were restimulated in vitro for 72 h with the indicated doses of MyHC-α peptide, and proliferation was measured by [3H]-thymidine incorporation. (B) For cytokine production, cells were restimulated for 48 h with MyHC-α peptide (black bars) or left untreated (white bars), and the levels of IL-6 and IFN-γ in culture supernatants were determined by ELISA. Values indicate means ± SEM. Data are representative of two–three independent experiments.

Impaired production of cardiac myosin-specific IgG1, IgG2a, and IgG2b autoantibodies in MyD88−/− mice

Since mice with autoimmune myocarditis develop high titers of anti-myosin IgG autoantibodies [39], we tested the levels of myosin-reactive IgG1, IgG2a, and IgG2b antibodies on day 21 of EAM. TLR3−/−, TLR7−/−, and TLR9−/− mice developed similar levels of myosin-reactive IgG1, IgG2a, and IgG2b, compared with WT mice (Figure 5). In contrast, production of IgG1, IgG2a, and IgG2b autoantibodies did not occur in MyD88−/− mice (Figure 5). These data indicate that the absence of MyD88 signaling in MyD88−/− mice but not in TLR3−/−, TLR7−/−, or TLR9−/− mice abrogates the production of cardiac-specific autoantibodies and IgG class switching.
Recent studies have demonstrated a new lineage of CD4\(^+\) T cells, termed T helper 17 (Th17), which are characterized by the production of IL-17 (IL-17A) and can mediate inflammation associated with several organ-related autoimmune diseases, including EAM [10,40,41]. IL-17 production from heart-infiltrating T cells promotes the recruitment of CD11b\(^+\) monocytes, the major heart-infiltrating mononuclear cells at the peak of myocarditis, to the heart [42]. Therefore, using real-time quantitative RT-PCR analysis of RNA from freshly isolated hearts of mice untreated or immunized with MyHC-\(\alpha\) in CFA, we tested the expression of IL-17 on day 21.

We found that immunization increased the expression of IL-17 mRNA in the hearts of some WT, TLR3-/-, TLR7-/-, and TLR9-/- mice, while none of the MyD88-/- immunized mice showed such an increase (Figure 6(A)). In parallel, we also investigated the expression of IL-6 and found that in nontreated mice, the expression levels of IL-6 were minimal, while upon immunization, the expression of IL-6 increased significantly and in a similar degree in WT, TLR3-/-, TLR7-/-, and TLR9-/- hearts. Interestingly, IL-6 expression remained minimal in MyD88-/- hearts (Figure 6(B)). Thus, MyD88 signaling is essential for myocardial IL-17 and IL-6 production.
Heart inflammation in MyD88\textsuperscript{-/-} and TLR9\textsuperscript{-/-} mice upon MCMV infection

Infection of BALB/c mice with the K181 strain of MCMV induces acute myocarditis from days 7 to 10 post infection, with chronic disease emerging from day 25 and remaining until at least day 56 post infection [43]. In order to address the importance of endosomal TLRs in a viral model of myocarditis, WT, TLR3\textsuperscript{-/-}, TLR7\textsuperscript{-/-}, TLR7\textsuperscript{-/-}, TLR9\textsuperscript{-/-}, or MyD88\textsuperscript{-/-} mice were left untreated (UNT) or immunized with MyHC-\alpha and CFA. On day 21, mouse hearts were collected, and total RNA was isolated. Expression of (A) IL-17 and (B) IL-6 were determined by quantitative PCR. Horizontal bars represent mean \pm SEM. Data are representative of two–three independent experiments.

Discussion

The recent discovery of mammalian TLRs and their signaling pathways has provided new understanding regarding the association between innate and adaptive immunity and their impact on autoimmune diseases [15]. In the present study, we examined the dependence of EAM on TLR3, TLR7, or TLR9 signaling and compared it with that of the adaptor molecule MyD88. MyD88 was originally isolated as a myeloid differentiation primary response gene, which is rapidly induced upon IL-6-stimulated differentiation to macrophages in M1 myeloleukemic cells [46]. To date, we know that MyD88 is an adaptor molecule downstream of all TLRs, except TLR3, as well as IL-1R1 and IL-18 receptors [17,32]. Using histopathological analysis, we found that myocardial cellular infiltration in TLR7\textsuperscript{-/-} and MyD88\textsuperscript{-/-} mice, but not TLR3\textsuperscript{-/-} or TLR9\textsuperscript{-/-} mice, was markedly suppressed compared to WT controls (Figure 2).

Moreover, TLR7\textsuperscript{-/-} mice showed impaired \textit{in vitro} proliferation of MyHC-\alpha-restimulated whole splenocytes, while TLR3\textsuperscript{-/-} and TLR9\textsuperscript{-/-} mice showed similar and higher proliferation than control mice, respectively (Figure 4). Interestingly, it has been shown previously that in MyD88\textsuperscript{-/-} mice, disease resistance parallels impaired expansion of heart-specific T cells,
as suggested by impaired proliferation of splenocytes upon MyHC-α restimulation [31]. However, cytokine production by splenocytes upon restimulation with MyHC-α and serum autoantibody production against cardiac myosin revealed that these parameters were dramatically reduced or absent in MyD88<sup>−/−</sup> mice, while TLR3<sup>−/−</sup>, TLR7<sup>−/−</sup>, and TLR9<sup>−/−</sup> mice showed similar responses like WT mice (Figures 4 and 5). So the reduced heart inflammation that we observed in MyD88<sup>−/−</sup> mice could be attributed to a certain degree to TLR7, but not TLR9, signaling.

Previous studies have shown that indeed myocarditis induction in the EAM model requires MyD88 signaling, which is essential for the stimulation of self-antigen-presenting dendritic cells to induce heart-specific CD4<sup>+</sup> T cell responses in the peripheral compartments in vivo [31]. Similarly, IL-1R1, which signals through MyD88, has also been shown to be important for the activation of peripheral myeloid dendritic cells that induce the expansion of autoreactive CD4<sup>+</sup> T cells and subsequent induction of autoimmune heart disease [36]. So the overall protection from myocarditis, which we observed in MyD88<sup>−/−</sup> mice using the EAM model, is the outcome of cooperation of at least TLR7 and IL-1R1 signaling.

The fact that TLR9<sup>−/−</sup> mice developed similar myocarditis like WT mice in the EAM model was a bit
TLR4, TLR6, and TLR9 both "ized antigens that are potent stimulators of TLR2, and one possible explanation is that the other TLR
ligands in the CFA can compensate for the absence of TLR9 activation would lead to a weaker immunization effect needed to induce disease [49]. Similarly, several available evidence suggest that multiple rather than single TLRs are required for innate defense against most pathogens [50].

Recent findings have suggested that IL-17 is produced by Th17 cells plays a central role in organ-specific autoimmunity, including autoimmune myocarditis [10,40,41]. When we tested the myocardial expression of IL-17, we found that upon immunization with MyHC-α in CFA, the overall expression was increased in WT, TLR3−/−, TLR7−/−, and TLR9−/− mice, while MyD88−/− mice did not show any expression of IL-17 (Figure 6). Thus, MyD88 signaling is pivotal for myocardial IL-17 production, and the absence of myocarditis in MyD88−/− mice is partly due to the absence of myocardial IL-17. Indeed, in the EAM model, neutralization of IL-17 reduces myocarditis and heart autoantibody responses, suggesting that IL-17 is an effector cytokine responsible for myocarditis [10]. However, IL-17-deficient mice are not fully protected against EAM, since they still develop myocarditis that is only slightly reduced in severity compared with WT mice [51]. Similarly, genetic deficiency of IL-17 does not abrogate EAU susceptibility [52]. So the current view is that depending on the model, autoimmune pathology can be driven by either Th17 or Th1 cells.

The pathogenesis of MCMV-induced myocarditis appears to be primarily due to the immunological response of the murine host to the virus. Infection of mice with CVB3 or MCMV results in a disease similar to the clinical heart disease observed in humans, with the development of acute myocarditis on days 7–14 after infection that later progresses to a chronic autoimmune disease [53]. Indeed, it has been reported that upon infection with CVB3, MyD88−/− mice show a dramatic higher survival rate and significantly decreased cardiac inflammation compared with WT mice [54]. In order to test the role of TLR3, TLR7, TLR9, and MyD88 in the pathogenesis of viral myocarditis, we induced acute viral myocarditis in TLR3−/−, TLR7−/−, TLR9−/−, or MyD88−/− mice by infection with a sublethal dose of MCMV. All the mice, independent of their genotype, survived and developed myocarditis; however, TLR9−/− and MyD88−/− mice showed more severe heart inflammation than WT, TLR3−/−, or TLR7−/− mice (Figure 7(A),(B)).

Thus, the severity of myocardial inflammation paralleled the ability of the mice to fight the viral infection, since both TLR9−/− and MyD88−/− mice had significant higher viral load in their hearts compared with WT, TLR3−/−, or TLR7−/− infected mice (Figure 8(A)). Moreover, we have shown previously that mainly TLR9 and MyD88 and

![Figure 8. Cardiac viral load and circulating cardiac troponin I levels in MCMV-infected mice.](Image)
to a much lesser extent TLR3 are involved in the innate immune defense against MCMV infection [55]. In addition, the implication of TLR7 in the antiviral response to MCMV can be seen only when it is coupled with TLR9 deficiency [56].

In summary, although the intracellular TLRs have been implicated in a different degree and in various autoimmune phenomena, our data using single TLR3-/-, TLR7-/-, and TLR9-deficient mice have pointed out to a contribution of TLR7, but not of TLR3 or TLR9, in the induction of EAM. Moreover, using the model of MCMV-induced myocarditis, we found that in TLR9-/- and MyD88-/- mice, the severity of myocardial inflammation was higher than in WT, TLR3-/-, and TLR7-/- mice and paralleled the capacity of the mice to fight the viral infection. However, we cannot exclude the possibility that these TLRs have redundant roles in autoimmune myocarditis induction and that double or triple TLR-deficient mice should be studied in order to uncover their implication in autoimmune myocarditis. Thus, we showed that to a certain degree, the resistance of MyD88-/- mice to EAM could be attributed to TLR7 signaling, while the susceptibility of MyD88-/- mice to MCMV-induced myocarditis is mirrored in TLR9-/- mice.

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References

TLR3 and RIG-like receptor on myeloid dendritic cells and RIG-like receptor on human NK cells are both mandatory for production of IFN-γ in response to double-stranded RNA.
TLR3 and Rig-Like Receptor on Myeloid Dendritic Cells and Rig-Like Receptor on Human NK Cells Are Both Mandatory for Production of IFN-γ in Response to Double-Stranded RNA

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TLR3 and Rig-Like Receptor on Myeloid Dendritic Cells and Rig-Like Receptor on Human NK Cells Are Both Mandatory for Production of IFN-\(\gamma\) in Response to Double-Stranded RNA

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Cross-talk between NK cells and dendritic cells (DCs) is critical for the potent therapeutic response to dsRNA, but the receptors involved remained controversial. We show in this paper that two dsRNAs, polyadenylic-polyuridylic acid and polyinosinic-polycytidylic acid [poly(I:C)], similarly engaged human TLR3, whereas only poly(I:C) triggered human RIG-I and MDA5. Both dsRNA enhanced NK cell activation within PBMCs but only poly(I:C) induced IFN-\(\gamma\). Although myeloid DCs (mDCs) were required for NK cell activation, induction of cytolytic potential and IFN-\(\gamma\) production did not require contact with mDCs but was dependent on type I IFN and IL-12, respectively. Poly(I:C) but not polyadenylic-polyuridylic acid synergized with mDC-derived IL-12 for IFN-\(\gamma\) production by acting directly on NK cells. Finally, the requirement of both TLR3 and Rig-like receptor (RLR) on mDCs and RLRs but not TLR3 on NK cells for IFN-\(\gamma\) production was demonstrated using TLR3- and Cardif-deficient mice and human RIG-I-specific activator. Thus, we report the requirement of cotriggering TLR3 and RLRs on mDCs and RLRs on NK cells for a pathogen product to induce potent innate cell activation.

N
atural killer cells are potent cytotoxic cells capable of killing infected or tumor cells (1) and are also able of producing proinflammatory cytokines including IFN-\(\gamma\) and TNF. These cytokines are crucial in the enhancement of the innate resistance by activating macrophages and neutrophils but also regulate the adaptive immune response (2).

Although NK cells can directly sense infections and cellular transformation, in part by recognizing stress-associated surface markers, many studies have outlined the importance of accessory cells (ACs) in modulating their function (3). Activated NK cells induce dendritic cell (DC) maturation (4, 5) through both TNF/IFN-\(\gamma\) secretion and cell-cell contact involving NKp30 (5–7). In turn, cytokine production by activated DCs enhances NK cell IFN-\(\gamma\) production, proliferation, and cytotoxic potential (4, 5, 8–10). IL-12 is essential for IFN-\(\gamma\) production by innate lymphocytes (11) and the enhancement of antitumor and antiviral activity in vivo (12). This DC/NK cross-talk has been reported to be critical in Th1 and CTL responses (13–15).

DCs sense viral infection through motifs that are conserved between large classes of pathogens and bind to germline-encoded receptors (16). Among these receptors, the TLR3 (17) and more recently the cytosolic helicase MDA5 (18, 19) have been described to sense synthetic dsRNA or viral infection, leading to strong type I IFN production and contributing to inflammatory cytokine production (20). Furthermore, a mouse vaccination study showed that the production of type I IFN by DCs upon engagement of MDA5 and TLR3 by polyinosinic-polycytidylic acid [poly(I:C)] was required for Th1 driving CD4\(^+\) T cell immunity (21), illustrating the therapeutic potential of poly(I:C) in vaccination relative to other TLR agonists.

The role of ACs in dsRNA-mediated NK cells activation remains unclear. Both ACs-dependent (5, 22, 23) and -independent activation (24–27) of NK cells have been reported. The aim of the current study was to decipher the involvement of ACs in dsRNA-mediated NK cells response and to characterize the dsRNA receptors on DC and NK involved in this response. We used three synthetic ligands: poly(I:C) dsRNA that triggers both TLR3 and Rig-like receptor (RLR) pathways, polyadenylic-polyuridylic acid [poly(A:U)] dsRNA that displays specificity for TLR3, and 5’-triphosphate ssRNA (3pRNA) that engages only the RIG-I helicase. We demonstrated that mDCs are required for NK cells activation in response to dsRNA. Most importantly, the specificity of the three ligands and the use of TLR3\(^\text{−/−}\) and Cardif\(^\text{−/−}\) mice allowed us to demonstrate that the full activation of NK cells and especially the production of IFN-\(\gamma\), in response to dsRNA required the triggering of both TLR3 and RLR on myeloid DCs (mDCs) and RLRs on NK cells. Our study deciphers the mechanisms of dsRNA-induced NK cell physiological response and supports the novel concept that a single pathogen product can...
lead to potent innate cell activation, because it engages different classes of receptors expressed by different cell types.

Materials and Methods

Reagents
dsRNA or recombinant cytokines were used at indicated concentrations: 1300 kDa poly(IC) (InvivoGen, San Diego, CA), standardized high molecular mass poly(AU) (Innate-Pharma, Marseille, France), IFN-β (PBL Biomedical Laboratories, Piscataway, NJ), poly(I:C) (InvivoGen, Courtaboeuf, France) and poly(A:U) (Innate-Pharma, Marseille, France), IFN-α (R&D Systems, Minneapolis, MN). 3pRNA was synthesized and purified with the MEGAscript and MEGAClear kits, respectively (Ambion, Austin, TX). The following neutralizing mAbs were used: 20 µg/ml mouse anti-human IFN-α/βR2 (PBL Biomedical Laboratories) or 10 µg/ml mouse anti-human IL-12p70 (clone 20C2) and mouse anti-human IL-15 (R&D Systems). Twenty micrograms per milliliter mouse IgG was used as control (R&D Systems). All these mAbs were previously validated using the corresponding recombinant cytokine on relevant NK activation parameters (data not shown).

Cell purification

From human blood of healthy donors, NK cells were selected by depletion using the NK cells isolation kit, whereas mDCs and monocytes were positively selected with, respectively, anti-BDCA1 plus BDCA3 and -CD14 microbeads (Miltenyi Biotec, Auburn, CA), monocytes, and mDCs and were 98.5 ± 0.8, 99.1 ± 0.2, and 94.2 ± 2.6%, respectively. In some experiments, whole PBMCs or depleted in monocytes, mDCs, or plasmacytoid DCs (pDCs) with, respectively, anti-CD14, -BDCA1 plus BDCA3, and -BDCA4 microbeads (Miltenyi Biotec) were treated with dsRNA.

For real-time PCR, NK cells and mDCs were stained with anti-CD56PE plus -CD3FITC (for NK) or anti-CD3, -CD14, and -CD56, and 3D5FITC (lineage) plus -CD4PECy5.5 plus CD11cAPC mAbs (BD Biosciences, San Jose, CA) (for mDCs) and sorted with a FACSVantage cell sorter (BD Biosciences) by gating on CD56+CD14+CD56- (NK cells) or lineage-CD4+CD11c+ (mDCs) cells. Final purity of NK cells and mDCs were 99.5 ± 0.3 and 98.7 ± 0.5%.

From spleen of C57BL/6 mice. Spleens from wild-type, TLR3−/− (17), Cardif−/−, and Cardif−/− (28), and C57BL/6 mice were disinfected and B, T, and erythrocyte cells depleted with anti-CD19, -CD3 -Ter119 (BD Biosciences), and sheep anti-rat Ig Dynabeads (Dyna1, Oslo, Norway). DX5+ NK cells and CD11c+ DCs were then positively selected with anti-DX5 and anti-CD11c microbeads, respectively (Miltenyi Biotec). Purities of NK cells and DCs were 87.8 ± 2.4 and 86.4 ± 6.1%, respectively.

dsRNA-activated DC supernatant generation

MACS-sorted mDCs or in vitro-generated monocyte-derived DCs (MDmDCs) were incubated with 30 µg/ml poly(A:U) for 24 h (continuous treatment) or pulsed for 4 h, washed three times in PBS, and incubated for 20 supplementary h (pulse treatment). Supernatants (SNs) were harvested, filtered through a 0.22-µm membrane, and used to stimulate purified NK cells diluted as indicated, alone or in combination with 30 µg/ml poly(I:C) or poly(A:U).

Cell activation follow-up

CD107-based degranulation assay. Twenty-hour-activated effectors (PBMCs, NK cells alone, or with mDCs) were seeded in 96-well plates with HCC1806 breast cancer target cell line (American Type Culture Collection, Manassas, VA) at an E:T ratio of 10:1 for PBMCs and 1:1 for NK cells. Anti–CD107aPE-Cy5 mAb (1/20) and monensin (1/1500) (BD Biosciences) were added. After 4 h at 37°C, cells were stained with anti-CD56PE and -CD3FITC and analyzed by flow cytometry. Percentage of CD107a+ CD56+ CD3+ cells were then selected with anti-DX5 and anti-CD11c microbeads, respectively (Miltenyi Biotec). Purities of NK cells and DCs were 87.8 ± 2.4 and 86.4 ± 6.1%, respectively.

Cytokine quantification. SNs of 20-h-activated human PBMCs, mDCs, or NK cells ± mDCs were collected and production of IFN-γ, IL-12p40, IL-12p70, IL-15 (BD Biosciences), and IFN-β (BioSource International, Camarillo, CA) was determined by ELISA. Mouse IFN-γ (BD Biosciences) was also quantified by ELISA in SNs of 20-h-activated mouse NK cells ± conventional DC (cDC) cultures.

In some experiments, IFN-γ was quantified by cytokyme-based intracellular staining. A total of 2 × 10⁶ PBMCs were activated or not with poly(IC) for 2 h before addition of brefeldin A (BD Biosciences) for the last h of culture. Cells were then stained with anti-CD56PE, -CD14PE-Cy5.5, -CD4PE-Cy7 (BD Biosciences), and -Vp9FITC (Innate Pharma) mAbs. Cells were then fixed and permeabilized using Cytofix-Cytoperm kit, stained with anti–IFN-γ APC mAb (BD Biosciences), and analyzed by flow cytometry. Cells were gated on the IFN-γ-positive cells, and the percentage of γδ T cells, NK cells, NKT-activated T cells, and CD4+ and CD8+ T cells was determined.

Stable expression in HEK293T

HEK293T cell line (American Type Culture Collection) was transfected using FuGene 6 (Roche, Basel, Switzerland) with 5 µg pSIRE-TA-luciferase plasmid (BD Clontech, Palo Alto, CA) and 500 ng of the pTK-Hyg plasmid (Invitrogen, Carlsbad, CA) containing hygromycin resistance gene. After an overnight incubation, transfected cells were selected with 150 µg/ml hygromycin B (Invitrogen), cloned by limiting dilution, and analyzed for luciferase activity in response to IFN-α/β2. A responding clone was transfected as described above with 2 µg pUNO-hTLR3, pUNO-hMDA5, and pUNO-hRIG-I plasmids (InvivoGen) containing blasticidin resistance gene. Transfected cells were selected with 10 µg/ml blasticidin (Invitrogen), cloned, and analyzed for specific gene expression by real-time PCR.

Luciferase assay

A total of 4 × 10⁶ TLR3−, RIG-1−, and MDA5-expressing cells or control cells were seeded into 96-well plates (Micro-Well96; Greiner Bio One, Courtaboeuf, France) and incubated for 20 h at 37°C. Cells were then activated for 6 h at 37°C with poly(I:C), poly(A:U), 3pRNA, or IFN-αβ2 (100 U/ml). Luciferase activity was measured on a TopCount NXT apparatus (Packard Instrument, Meriden, CT) after addition of the Steady-Glo reporter assay reagent (Promega, Madison, WI). Results were expressed as a ratio between stimulated and nonstimulated cells. In some experiments, results were normalized to the IFN-αβ2 response to compare ligands efficacy.

Biocare assay

Biocare T100 apparatus (Biocare Life Sciences, GE Healthcare, Fairfield, CT) was used for these experiments. Recombinant human TLR3 (rhTLR3; R&D Systems) was immobilized onto the dextran layer of a Biocare CM5 Series S sensor chip by injecting rhTLR3 (10 µg/ml) until reaching the desired immobilization level (~3000 RU). A total of 10 µg/ml poly(A:U) or poly(I:C) were injected onto rhTLR3 surface at a flow rate of 10 µl/min. Injection was performed for 120 s, followed by a dissociation period of 180 s. Sensorgrams curves were analyzed using BioEvaluation software version 4.1.

Real-time PCR

Total mRNA was extracted from cells of interest and cDNA was synthesized using RNeasy Plus and QuantiTect RT Kits, respectively (Qiagen, Valencia, CA). GAPDH was used as a housekeeping gene. PCRs were performed with the QuantiTect SYBR Green PCR kit (Qiagen), primers, and 20 ng cDNA on a MX3000P thermocycler (Stratagene, La Jolla, CA). GAPDH, RIG-1, and MDA5 primers were purchased from Qiagen. TLR3 primer sequences were as follows: 5’-TGGTGTGCGCCACCTAGAAGTA-3’ (forward) and 5’-TCCTACTTTCTGGTGCTG-3’ (reverse). Cycling conditions were as follows: 1 cycle of 10 min at 95°C; 40 cycles of 30 s at 95°C, 1 min at 60°C and 30 s at 72°C; and 1 cycle of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C. Because of using primer pairs that amplified two or more exons from a unique gene, genomic contamination was excluded by melting curve analysis. Gene expression was calculated as the ratio between gene of interest and GAPDH using the MxPRO QPCR software (Stratagene).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.1 software. Student’s t test (two-tailed) was used in the study.

Results

Both poly(I:C) and poly(A:U) signal via TLR3 but only poly(I:C) triggers RIG-1 or MDA5

To determine the ability of dsRNA to trigger type I IFN production through TLR3 and RLR, HEK293T cells stably expressing the pSIRE-TA-luciferase plasmid were transfected with plasmids encoding human TLR3, RIG-1, or MDA5. The transfected or control cell lines were stimulated for 6 h with different doses of poly(I:C) and poly(A:U) dsRNA or with 100 U/ml IFN-α/β2 as internal calibrator and luciferase activity were measured. The activity of dsRNA was normalized to the response to IFN-α/β2. Both dsRNAs triggered TLR3 with the same efficacy, but a specific response in RIG-1 and MDA5-transfected cells was observed only with poly(I:C) (Fig. 1A). A high concentration (100 µg/ml) of poly(A:U) only
induced a weak response in RIG-I- and MDA5-expressing cell lines to that also detected on cells expressing ISRE-TA-luciferase, only suggesting activation of endogenous TLR3 that appeared to be expressed at low levels in 293T cell line, as detected by real-time PCR (Supplemental Fig. 1).

We then tested both dsRNA on an rhTLR3-coated sensor chip analyzed using a Biacore T100 apparatus. Poly(A:U) and poly(I:C) displayed an identical avidity for binding to TLR3 as indicated by the association/dissociation curves of the dsRNA (Fig. 1B), confirming that poly(A:U) and poly(I:C) triggered TLR3 with the same efficiency.

Altogether, these results demonstrate that poly(A:U) and poly(I:C) signal via TLR3 with the same efficiency, but only poly(I:C) triggers the RLR pathway via RIG-I and/or MDA5.

Within PBMCs, poly(I:C) and poly(A:U) lead to different qualitative NK cell activation

Human PBMCs were cultured with either dsRNA. After 20 h, IFN-γ was quantified in the SN, and following an additional 4-h coculture with HCC1806 breast cancer target cells, degranulating CD56+ CD3+ NK cells were identified by the expression of CD107. The two dsRNAs enhanced NK cell degranulation with the same efficiency (Fig. 2A, left panel), but poly(I:C) was much more efficient than poly(A:U) (p < 0.0001 between 1 and 100 μg/ml dsRNA) to induce IFN-γ production (Fig. 2A, right panel). As detected by intracellular staining, NK cells represented the majority of IFN-γ-producing cells in poly(I:C)-activated PBMCs with a minor participation of CD8+ CD3−CD56+, and γδ T cells (Fig. 2B).

This differential activity of dsRNA suggests that the induction of degranulation is mainly dependent on the TLR3 pathway activation shared by poly(I:C) and poly(A:U), whereas optimal IFN-γ induction required additional signaling through the RLR engaged by poly(I:C) exclusively.

mDCs are required for NK cell activation in response to dsRNA

To analyze the requirement of ACs in the human NK cell response to dsRNA, PBMCs were immunomagnetically depleted of various cell populations before culture with poly(I:C) or poly(A:U), and CD107 expression and IFN-γ production were analyzed. Depletion of mDCs (BDCA1+3) before activation with either dsRNA dramatically decreased the cytolytic activity and completely abrogated the production of IFN-γ (Fig. 3A), whereas depletion of monocytes (CD14) and pDCs (BDCA4) had no effect. To further confirm the role of mDCs in dsRNA-mediated activation of NK cells, both cell types were purified by immunomagnetic sorting and activated either separately or in coculture. Using purified NK cells, the two dsRNA did not induce IFN-γ production or CD107 (Fig. 3B); however, in the presence of mDCs, both dsRNA similarly enhanced the cytotoxic activity of purified NK cells, whereas poly(I:C) was much more efficient than poly(A:U) in triggering IFN-γ production.

To link these biological activities with the receptor selectivity of poly(I:C) and poly(A:U), we then determined the levels of dsRNA receptor expression by real-time PCR on nonactivated cell populations. The three studied mRNAs were detected on MDCs and mDCs but, except RIG-I, were much less expressed in NK cells (Fig. 3C).

These observations demonstrated that dsRNA-mediated activation of NK cells required the participation of mDCs that were likely activated by poly(I:C) and poly(A:U) through engagement of TLR3. However, the higher activity of poly(I:C) than poly(A:U) for IFN-γ induction suggested the contribution of another dsRNA receptor (likely an RLR) on mDCs and/or NK cells.

IL-12 and IFN-β produced by dsRNA-activated mDCs contribute to activate NK cells

We next addressed whether soluble mediators produced by human dsRNA-activated mDCs were involved in the NK cell response to dsRNA. dsRNA-activated mDC SNs generated by treating cells for 20 h were used to stimulate purified NK cells. SNs of mDCs activated for 20 h with the same dose of either dsRNA were sufficient to activate NK cell cytolytic activity (Fig. 4A), whereas SNs generated with poly(A:U) were much less efficient than those generated with poly(I:C) to induce IFN-γ.

NK cells were treated with dsRNA-activated DC SNs with or without neutralizing Abs. The blockade of type I IFN receptor significantly diminished NK cell degranulation [p = 0.048 for poly(I:C) and p = 0.0010 for poly(A:U)] but not the production of IFN-γ (Fig. 4B). IL-12 neutralization had no effect on the CD107 staining, whereas the production of IFN-γ was completely abrogated [p = 0.0005 for...
Poly(I:C) and poly(A:U) were equally effective in inducing type I IFNs production by purified mDCs (Fig. 4C) or whole PBMCs (Fig. 4D), whereas poly(I:C) was a stronger inducer of IL-12p70 than poly(A:U). Of note, when PBMCs were depleted of their mDC fraction (BDCA1+3), IFN-β production was totally abrogated and dramatically decreased in response to poly(A:U) and poly(I:C), respectively (Supplemental Fig. 2), suggesting that mDCs were the main producers of type I IFN within PBMCs in response to dsRNA.

Finally, upon activation with IFN-β or poly(I:C), but not with poly(A:U), the levels of RIG-I and MDA5 transcripts in highly purified FACS-sorted NK cells were increased 50- to 100-fold, whereas the level of TLR3 transcripts remained very low (Fig. 4E). In addition, as shown in Fig. 4F, poly(I:C) significantly increased the level of IFN-β mRNA in purified NK cells as compared with medium (p = 0.022) or poly(A:U) (p = 0.021), indicating that the regulation of an RLR was presumably dependent on type I IFN.

These observations demonstrated that soluble mediators produced by dsRNA-activated mDCs participated in NK response to dsRNA.
and that type I IFN mainly contributed to the enhancement of cytotoxic activity and IL-12 to IFN-\(\gamma\) production. Furthermore, poly(I:C) and poly(A:U) engaged at least TLR3 on mDCs, leading to IFN-\(\beta\) secretion that upregulated the expression of RLRs on NK cells.

**Requirement of direct effect of poly(I:C) on NK for IFN-\(\gamma\) production**

Because, in the experiments described above we could not rule out the presence of remaining dsRNA within the mDC SNs, the contribution of mDC-produced soluble factors was mimicked using rIL-12 and type I IFN to decipher a possible contribution of direct activation of NK cells through one of the dsRNA receptor. We treated MACS-sorted NK cells with different doses of rIL-12 or IFN-\(\beta\) alone or in the presence of poly(I:C) or poly(A:U). IFN-\(\beta\) alone triggered a strong cytolytic activity of NK cells but did not induce IFN-\(\gamma\) (Fig. 5A, upper panel). IL-12 alone was a weak NK cell activator in both readouts (Fig. 5A, lower panel). However, poly(I:C) but not poly(A:U) synergized with IL-12 to induce a strong activation of purified NK cells, suggesting that poly(I:C) directly engaged NK cells for the production of IFN-\(\gamma\) and that NK cells do not express receptors for poly(A:U) even after priming with IL-12 or IFN-\(\beta\).

We next produced SNs from mDCs pulsed with dsRNA for 4 h, extensively washed and cultured for an extra 20 h to avoid contaminating dsRNA in the SNs. Although SNs from pulsed mDCs induced CD69 expression, they were unable to induce IFN-\(\gamma\) production unlike SNs from mDCs continuously stimulated with dsRNA (Supplemental Fig. 3A).

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**FIGURE 4.** IL-12 and type I IFN secreted by dsRNA-activated mDCs mediate NK cells activation. A, SNs from MACS-sorted mDCs (BDCA1 and BDCA3) activated with p(IC) (open histograms) or p(AU) (filled histograms) were used in a dose range to activate \(1 \times 10^6\)/ml MACS-purified human NK cells. Degranulation and IFN-\(\gamma\) production are evaluated as described (Fig. 2). Data are from one of three representative experiments (mean of duplicate culture with <10% variation) performed with NK cell from three independent donors and two batches of mDC SNs. B, A total of \(1 \times 10^5\)/ml MACS-purified human NK cells were cultured with 10% p(IC)- or p(AU)-activated mDC SNs in the absence or presence of neutralizing mouse anti–IL-12p70, anti–IFN-\(\gamma\)R2, anti–IL-15, or mouse IgG as control (cont. mAb). Data obtained in the presence of the Abs are expressed as percentage of the activation observed in the absence of Abs and represent mean \(\pm\) SD from three independent NK cells donors activated with two different SN batches. C and D, IFN-\(\beta\) and IL-12p70 concentrations were measured by specific ELISA in SNs of MACS-purified human mDCs (\(5 \times 10^5\)/ml) stimulated 20 h with 50 \(\mu\)g/ml p(IC) or p(AU) (C) or of whole PBMCs (\(1 \times 10^6\)/ml) stimulated 20 h with a dose range of both dsRNA (D). Symbols represent individual results from four different donors and the horizontal bars the median (C), and curves represent one representative experiment from one of four donors (D). *\(p < 0.05\); **\(p < 0.001\). E and F, RIG-I, MDA5, TLR3, and IFN-\(\beta\) mRNA levels were determined by real-time PCR on FACS-sorted NK cells activated with IFN-\(\beta\) (1000 U/ml), IL-12 (10 ng/ml), p(IC), p(AU) (both 50 \(\mu\)g/ml) (E) or only p(IC) or p(AU) (F). Results are expressed as arbitrary units normalized to value for the housekeeping gene GAPDH. Data represent independent values of three to eight different donors and horizontal bars represent the median. *\(p < 0.05\); **\(p < 0.01\). p(AU), poly(A:U); p(IC), poly(I:C).
We next performed experiments with MdDC SNs that displayed almost the same efficiency than mDC SNs to activate NK cells (Supplemental Fig. 3B). Poly(I:C) synergized with poly(A:U)-pulsed MdDC SNs to trigger the production of IFN-γ by MACS-sorted NK cells (Fig. 5B), with an 18-fold increase compared with SN alone. Using poly(A:U) continuously treated MdDC SNs, we observed a similar synergy with poly(I:C) for IFN-γ production by NK cells. In contrast, the addition of poly(A:U) on NK cells did not synergize with any of the pulsed DC SNs (Fig. 5B), in line with the absence of poly(A:U)-interacting receptors on NK cells.

Collectively, our results suggest that dsRNA treatment of mDCs induced the production of IL-12 that synergized with TLR3-independent but TLR3 dependent and probably RLR-dependent direct effect of poly(I:C) on purified NK cells for IFN-γ production.

**Synergy between TLR3 and RLR engagement on mDCs and RLRs on NK cells for IFN-γ production**

As in humans, poly(I:C) synergized with IL-12 to induce the production of IFN-γ by purified mouse NK cells (Fig. 6A). With the aim of confirming that the direct effect of poly(I:C) on NK cells was TLR3 independent, we performed experiments with mouse DX5+ NK cells and CD11c+ DCs purified from TLR3 or Cardif-deficient and -sufficient mice. The synergy between poly(I:C) and IL-12 was observed with WT and TLR3<sup>−/−</sup> but not Cardif<sup>−/−</sup> NK cells, demonstrating that the direct effect of poly(I:C) on NK cells was RLR dependent but TLR3 independent. As a control, IL-2 used in combination with IL-12 showed that WT, TLR3<sup>−/−</sup>, and Cardif<sup>−/−</sup> NK cells produced almost the same amount of IFN-γ (Fig. 6Aa).

In coculture experiment using purified NK cells from either WT, TLR3<sup>−/−</sup>, or Cardif<sup>−/−</sup> and DCs from WT mouse spleen, IFN-γ production induced by poly(I:C) also required the RLR but not the TLR3 pathway on NK cells (Fig. 6B). Finally, IFN-γ production was completely abrogated and 70% reduced when WT NK cells were cocultured with DCs from TLR3<sup>−/−</sup> and Cardif<sup>−/−</sup> mice, respectively (Fig. 6C). When activated with LPS (TLR4), DCs from WT, TLR3<sup>−/−</sup>, or Cardif<sup>−/−</sup> mice induced IFN-γ production in NK cells. These observations demonstrated the crucial requirement of both TLR3 and RLR on DCs but of only the RLR on NK for production of IFN-γ in response to poly(I:C).

To further confirm that the triggering of the RLR pathway on human NK cells was involved in the production of IFN-γ, we stimulated MACS-sorted NK cells with 3pRNA, a newly described RIG-I-specific ligand (29, 30) (Supplemental Fig. 4). We then observed that 3pRNA synergized with IL-12 to enhance IFN-γ production by NK cells similarly to poly(I:C) but unlike poly(A:U) (Fig. 6D). In addition, the combination of 3pRNA and poly(A:U) induced IFN-γ production in NK-DC cocultures at levels comparable to those induced by poly(I:C) (Fig. 6E). Altogether, these results demonstrate that TLR3 and RLR engagement on DCs and RIG-I on NK are required for optimal IFN-γ production by NK. The mechanisms deciphered along the study concluding that the optimal production of IFN-γ by NK cells required the concomitant engagement of TLR3 and RLRs on NK cells by dsRNA are depicted in Fig. 7.

**Discussion**

In the current study, we report the first evidence that optimal NK cell response to synthetic dsRNA requires the triggering of both TLR3 and RLR on mDCs and RIG-I on NK cells (Fig. 7). In particular, we show that 1) NK cell activation in response to dsRNA required the presence of mDCs; 2) poly(I:C) dsRNA (an agonist for both TLR3 and RLR) and 3pRNA (an agonist for RIG-I) but not poly(A:U) dsRNA (an agonist for TLR3 only) directly activated NK cells leading to high IFN-γ production in synergy with mDC-released soluble mediators induced by either dsRNA; and 3) TLR3 and RLR expression were required on mDCs, whereas RLR was needed on NK cells for IFN-γ production in response to dsRNA.

In Biacore experiments and in the luciferase assay with TLR3-transfected cell line (Fig. 1A, 1B), poly(A:U) and poly(I:C) showed similar efficiency (identical avidity and potency) to bind to and trigger TLR3. In contrast, using RLR-overexpressing cell lines, only poly(I:C) activated the two RLRs, RIG-I and MDA5. Our results are in agreement with the study of Kato et al. (31), showing that dsRNA is recognized by either RIG-I or MDA5, although in a length-dependent way.

DC depletion and NK-DC coculture experiments established that mDCs were critical for dsRNA-mediated NK cell activation. These
representative experiments performed with three independent donors with or without p(A:U) or p(I:C).

Responses involved in the control of viral infection (33, 34) and to the establishment of the innate and adaptive immune.

Reports deciphered the interplay between NK cells and DCs (36) and to the development of high levels of IFN-γ in combination with IL-12. Cytotoxic potential of NK cells mostly dependent of the type I IFN produced by dsRNA-activated mDCs and not of a direct triggering by dsRNA was comparable with both poly(IC) and poly(A:U).

The fact that both poly(A:U) and poly(I:C) induced the secretion of soluble mediators from mDCs leading to NK cell activation clearly suggested that the dsRNA activity on mDCs was at least dependent on TLR3. This observation is in agreement with several studies demonstrating the involvement of TLR3 on mDC response to poly(I:C) (4, 5, 22). However, although both dsRNAs induce comparable levels of type I IFN, poly(I:C) induced significantly higher levels of IL-12p70 suggesting the engagement of a receptor distinct from TLR3 for the optimal IL-12p70 production by mDCs. Experiments performed with mouse CD11c+ DCs deficient for TLR3 or Cardif expression confirmed that TLR3 and also RLRs are involved in dsRNA-mediated DC-dependent NK cell activation, notably IFN-γ production. McCartney et al. (37) showed that production of IL-12p40 in response to an in vivo injection of poly(I:C) was totally abrogated in TLR3−/− mice but not in MDA−/− mice. This observation might appear in contradiction with our results showing an abrogation or a dramatic diminution of IFN-γ production by NK cells when cocultured with DC from TLR3−/− or Cardif−/− mice, respectively. However, the involvement of RIG-I was not explored in the study of McCartney et al. (37).

The synergistic activity between DC-derived soluble mediators and poly(I:C) acting on purified NK cells demonstrated a direct effect of dsRNA on NK cells. Furthermore, the biological responses observed with purified human NK cells and mouse TLR3- or Cardif-deficient NK cells unambiguously demonstrated that the direct effect of poly(I:C) on NK cells is RLR dependent and TLR3 independent. The fact that the RIG-I agonist 3pRNA mimicked the ability of poly(I:C) to activate NK cells further confirm the role of RLRs on human NK cells for induction of IFN-γ in synergy with IL-12. In addition, upon treatment of NK with type I IFN or

**FIGURE 6.** Engagement of TLR3 and RLR on cDCs and RLRs on NK cells is essential for IFN-γ production. A. A total of 5 × 10^5/ml MACS-sorted mouse NK cells from TLR3−/−, Cardif−/−, and WT mice were stimulated with 100 ng/ml IL-12 or 100 µg/ml p(IC) alone or in combination: 10 ng/ml IL-2 + 100 ng/ml IL-12 were used as positive control. B. A total of 5 × 10^5/ml MACS-sorted mouse NK cells from TLR3−/−, Cardif−/−, and WT mice were cultured in the presence of 10^7/ml MACS-sorted mouse CD11c+ cDCs from WT mice with or without 100 µg/ml p(IC). C. A total of 5 × 10^5/ml MACS-sorted mouse NK cells from WT mice were cultured with 10^7/ml MACS-sorted cDCs from either WT, TLR3−/−, or Cardif−/− in the presence of 100 µg/ml p(IC) or 100 ng/ml LPS. A–C. Data are from one of three representative experiments (mean ± SD of triplicate cultures). Each experiment was performed with the pool of spleens from at least five animals. D. A total of 1 × 10^7/ml MACS-sorted human NK cells were activated with the indicated concentrations of rIL-12 alone or in combination with p(IC), p(A:U), or 3pRNA complexed with Lipofectamine 2000. E. A total of 1 × 10^7/ml MACS-purified human NK cells were stimulated with 6 h with Lipofectamine 2000-associated 3pRNA. Cells were then washed twice with ice-cold PBS and incubated for an additional 20 h with 2 × 10^5/ml autologous MACS-purified human mDCs with or without p(A:U) or p(IC). D and E. Data are from one of three representative experiments performed with three independent donors (mean ± SD of culture triplicates). p(A:U), poly(A:U); p(IC), poly(I:C).

observations are in agreement with several studies both in humans (5) and in mouse models (22) showing the mandatory role of mDCs or conventional CD11c+ cDCs, respectively, for NK cells activation upon treatment with synthetic poly(I:C). Numerous reports deciphered the interplay between NK cells and DCs leading to the complete activation of both cell types (4, 6, 9, 10, 32) and to the establishment of the innate and adaptive immune responses involved in the control of viral infection (33, 34) or tumor growth (8). In particular, using an inducible and selective ablation system of CD11c+ DCs, Lucas et al. (35) have shown that naive NK cells do not acquire effector functions without prior priming by contact with cDCs in draining lymph nodes.

We show in our experimental model, that for DC/NK cooperation, cell-to-cell contact was not required. Type I IFN and IL-12 produced by either poly(I:C)- or poly(A:U)-activated mDCs were sufficient to enhance the cytolytic potential and IFN-γ production by NK cells, respectively. This is in agreement with previous reports that highlighted the respective role for IL-12 and type I IFN in the production of IFN-γ and the cytolytic potential of NK cells (5, 12, 36). In several other experimental settings, a direct contact between NK cells and DCs (membrane IL-15 and Nkp30) was shown to participate in efficient NK cells triggering (8, 32). To examine the role of dsRNA receptors on NK, our study focused on soluble mediators, but a role for membrane interaction between NK cells and DC was not excluded.

The fact that both poly(A:U) and poly(I:C) induced the secretion of soluble mediators from mDCs leading to NK cell activation clearly suggested that the dsRNA activity on mDCs was at least dependent on TLR3. This observation is in agreement with several studies demonstrating the involvement of TLR3 on mDC response to poly(I:C) (4, 5, 22). However, although both dsRNAs induce comparable levels of type I IFN, poly(I:C) induced significantly higher levels of IL-12p70 suggesting the engagement of a receptor distinct from TLR3 for the optimal IL-12p70 production by mDCs. Experiments performed with mouse CD11c+ DCs deficient for TLR3 or Cardif expression confirmed that TLR3 and also RLRs on DCs were required for the dsRNA-mediated DC-dependent NK cell activation, notably IFN-γ production. McCartney et al. (37) showed that production of IL-12p40 in response to an in vivo injection of poly(I:C) was totally abrogated in TLR3−/− mice but not in MDA−/− mice. This observation might appear in contradiction with our results showing an abrogation or a dramatic diminution of IFN-γ production by NK cells when cocultured with DC from TLR3−/− or Cardif−/− mice, respectively. However, the involvement of RIG-I was not explored in the study of McCartney et al. (37).
poly(I:C), the expression of both RIG-I and MDA5 was significantly enhanced, whereas TLR3 was still only marginally expressed (Figs. 3C, 4E). Similarly to our results, Tu et al. (23) showed that poly(I:C)-treated Kupffer cells allowed NK cells to produce IFN-γ after a direct sensing of poly(I:C), but these authors did not identify the dsRNA receptor involved on NK cells.

Our present conclusion that in NK cells poly(I:C)-mediated activation of RLRs and in particular RIG-1 but not TLR3 play an important role in enhancing the production of IFN-γ in response to DC-produced cytokines such as IL-12 is based on several lines of evidence both in human and mouse cells. However, this conclusion may appear in contradiction with previous studies suggesting that NK cells directly respond to dsRNA in a TLR3-dependent manner (25, 26, 38, 39). Sivori et al. (26), in particular, demonstrated a convincing correlation between poly(I:C)-mediated activation and TLR3 mRNA expression in in vitro-expanded clones of IL-2–activated NK cells. However, these results were obtained using in vitro-expanded NK cell clones that may have upregulated TLR3 upon activation and, thus, do not contradict our own results that have been obtained with freshly purified resting NK cells and that may more closely reproduce the physiological NK response to infection in naive organisms. Most other studies did not exclude the contribution of other dsRNA receptors expressed by NK cells. The discovery that viral dsRNA and poly(I:C) are capable of triggering receptors other than TLR3 and in particular the RLR family is recent (18, 31, 40, 41), and most other studies on the contribution of other dsRNA receptors expressed by NK cells have not directly tested the contribution of RLRs. However, our results demonstrating a critical role for RLRs on NK cells are in contradiction with Miyake et al. (42) and McCartney et al. (37) recent reports that failed to identify a role of either RLR or MDA5 on NK cells for IFN-γ production in DC-NK cultures in response to poly(I:C). The reason for this discrepancy is unclear but might be due to different experimental conditions that may mask the requirement for RLRs on NK cells.

Taken together with the recent work of Lucas et al. (35), our observations would suggest that in vivo mDCs activated through TLR3 migrate into the draining lymph node and produce cytokines, leading to NK cell priming. Then, upon recirculation and access to the inflamed tissues, primed NK cells would directly respond to viral invasion through lytic synapse and pathogens components transfer and sensing through cytosolic helicases. This secondary activation of the primed NK cells would further enhance their ability to kill infected or transformed cells and would induce the production of inflammatory cytokines contributing to the shaping of the adaptive immune response (39). These observations are consistent with the concept that, to trigger a productive innate immune response, viruses need to engage different classes of receptors either constitutively expressed or induced on different cell types. Other known examples that support this concept include imidazoquinolines (43) compounds that mimic microbial components by triggering TLR7/8 and cGAS/STING signaling that triggers several receptors including TLR2 (40) and NOD1 (41, 42). Thus, suggesting that this concept may also apply to some bacteria; however, in our experimental conditions, we could not detect such cooperation for LPS-induced IFN-γ by NK cells. A similar scenario involving synergy between different TLRs expressed within different cellular compartments on a unique DC type (45) or cooperation between different DC types (39) have been suggested as a requirement to mount an appropriate immune response against a single pathogen. Our model strengthens the notion that powerful but also potentially harmful immune effector mechanisms are elicited only in response to those invading pathogens that the immune system senses cooperatively through multiple cells, compartments, and receptors. Furthermore, Longhi et al. (21) showed that the production of type I IFN by DCs in response to poly(I:C) as adjuvant that triggered both TLR3 and RLR pathways leads to a strong adaptive immunity. This study clearly highlighted the clinical potential of a drug with specificity for TLR3 and RIG-I/MDA5 or of two drugs specific for each pathway. The combination of different products that act individually on each receptor will allow to tune very finely the immune response and to ensure the safety of the treatment.

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Disclosures

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Annexe 5

Gender bias in susceptibility to MCMV infection correlates with TLR9 expression
Gender bias in susceptibility to MCMV infection correlates with TLR9 expression

Running title: Gender differences upon MCMV infection: implication of TLR9

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Abstract

Toll-like receptor (TLR)-dependent pathways control the activation of various immune cells and the production of cytokines and chemokines that are important in innate immune control of viruses, including mouse cytomegalovirus (MCMV). Here we report that upon MCMV infection wild-type and TLR7\(^{-/-}\) male mice were more resistant than their female counterparts, while TLR9\(^{-/-}\) male and female mice showed similar susceptibility. Interestingly, 36h upon MCMV infection TLR9 mRNA expression was higher in male than in female mouse spleens. MCMV infection led to stronger reduction of marginal zone (MZ) B cells, and higher infiltration of plasmacytoid dendritic cells and neutrophils in wild-type male than female mice, while no such gender differences were observed in TLR9\(^{-/-}\) mice. In accordance, the serum levels of KC and MIP-2, major neutrophil chemoattractants, were higher in wild-type, but not in TLR9\(^{-/-}\), male versus female mice. Our data demonstrate gender differences in susceptibility to MCMV infection that are accompanied by a lower activation of the innate immune system in female mice, and can be attributed, at least in a certain degree, to the lower expression of TLR9 in female than male mice.
Introduction

Fundamental differences associated with the sex of an individual exist at every biological level, including the immune system. In general, females respond to infection, vaccination and trauma with increased antibody production, and suffer a higher incidence of autoimmune diseases, whereas inflammation is usually more severe in males, resulting in an increased mortality upon infection (1-3). Males are hypothesized to be more susceptible to infection than females not only because androgens can modulate immunocompetence, but because sex steroid hormones affect disease resistance genes and behaviors that make males more susceptible to infection (2). Nevertheless, the final outcome of an infection, susceptibility or resistance, does not depend only on the sex of the infected organism, but also on the infectious agent. For example, it has been reported that HIV infection in women is associated with significantly shorter survival than that for men, among those individuals that have not received antiretroviral therapy (4). Studies with rodents have also revealed gender differences in susceptibility to infection for many viruses, including herpes simplex virus, vesicular stomatitis virus, coxsackievirus B3, and Theiler’s murine encephalomyelitis virus (5-9).

Human CMV, a member of the herpes virus family prevalent in most human populations, is rarely symptomatic in immune-competent individuals, but can cause life-threatening disease in immunodeficient hosts, including brain damage and hearing loss in congenitally infected children (10). Since there is species tropism, murine cytomegalovirus (MCMV) has become an animal model for systemic human CMV infection, and has contributed greatly to our understanding of the molecular determinants of pathogenesis. MCMV is commonly administered in experimental animals via i.p. injection and can infect many different organs and cell types. In acute lethal MCMV infection the spleen and the liver are the principal sites
of early viral replication, and lethality is often associated with destruction of the liver (11). MCMV infection in the spleen follows a very distinctive pattern, beginning in the marginal zone and spreading into the red pulp by 17h, while by 48 h after infection there is a widespread infection both in the spleen and liver with generation of infected cells (12). Control of MCMV infection requires both innate and adaptive host immune responses, with type I IFN (IFN-α/β) signaling serving as a key component of innate immunity (13). Although many cell types can produce type I IFNs in response to viral infection, plasmacytoid dendritic cells (pDCs) are considered the major producers of IFN-α in response to infection with various viruses, including MCMV (14). Indeed, splenic pDCs are responsible for the high IFN-α/β production that is observed at 36 h upon MCMV infection in the sera of C57BL/6 mice (15). IFN-α and NK cells largely limit early viral replication in the mouse spleen, whereas T cells are required for eventual control of acute infection and reactivation from latency. In addition to type I IFNs, other cytokines that play an important role on the mounting of an efficient host response against MCMV include IL-12, TNF, lymphotoxin α/β, IL-6 and IFN-γ (16).

Although initially neutrophils were considered as the first line of defense mainly against bacterial infections by ingesting and killing invading microorganisms, further studies have uncovered a more general and important role of neutrophils in shaping the immune responses and contributing in the repair of tissue as well as its breakdown, upon bacterial or viral infections (17-19). The implication of neutrophils in CMV infection has also been explored in some studies. For example, coculture of murine neutrophils with MCMV induces reduction on the chemotactic and phagocytic activity of neutrophils, while infection of human neutrophils with human CMV by contact with human CMV-infected human pulmonary artery endothelial cells, make neutrophils more effective in performing their effector functions (20,
21). CMV-infected endothelial cells can recruit neutrophils by the secretion of IL-8 and GROα chemokines, and can transmit the virus to them by direct cell-to-cell contact (22). Rodents lack a direct homologue of IL-8, but the chemokines KC and MIP-2 are regarded as functional homologues of IL-8 and are the most critical for the recruitment of neutrophils at the site of infection or inflammation (23).

The study of wild-type and genetically modified mice upon MCMV infection has been particularly useful in elucidating the role of antiviral innate and adaptive immune response mechanisms, and in combination with the discovery of TLRs have advanced our comprehension of host defense against MCMV infection (14, 24). TLRs are a family of conserved transmembrane molecules that detect microbial components and play a pivotal role in shaping both innate and adaptive responses. From the 13 mammalian TLRs (10 in humans and 12 in mice), the ones that are located in the plasma membrane recognize mainly bacterial components, while the TLRs that are found within endosomal-lysosomal compartments are specialized on the detection of nucleic acid-based ligands. The endosomal TLRs include, TLR3 that detects viral double-stranded RNA, TLR7 and TLR8 that sense viral single-stranded RNA and TLR9 that respond to unmethylated CpG-containing DNA, which is found in the genomes of virus and bacteria (25-28). TLR7, TLR8 and TLR9 signal through the adaptor molecule MyD88 that leads to the production of inflammatory cytokines, while TLR3 signal through Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF) and mediates the induction of type I IFNs (24). The TLR9-MYD88 signaling pathway is critical for rapid MCMV clearance, since both TLR9<sup>-/-</sup> and MyD88<sup>-/-</sup> mice are highly susceptible to MCMV infection (29, 30). TLR3<sup>-/-</sup> and TRIF<sup>-/-</sup> mice show increased MCMV replication, however, the TLR3-TRIF pathway appears less important than TLR9 (30, 31). Upon MCMV infection, although TLR7<sup>-/-</sup> and wild-type mice show similar survival rates, double TLR7/TLR9<sup>-/-</sup> mice
show higher susceptibility and viral titers than TLR9⁻/⁻ mice, suggesting that TLR7 also participates in antiviral host defense to MCMV (32).

In the present study, we investigated the influence of gender on acute MCMV infection in laboratory mice. We found that C57BL/6 WT male mice show higher resistance to MCMV infection compared to female counterparts and this gender difference is absent in TLR9⁻/⁻, but not TLR7⁻/⁻, mice. MCMV-infection in male and female mice revealed higher expression of TLR9 in male spleens that was accompanied with stronger reduction of the MZ B cell compartment and higher infiltration of pDCs and neutrophils. Interestingly, the reduction of MZ B cells was TLR9-independent, while the mobilization of neutrophils in the spleen was TLR9-dependent. These results reveal gender differences in MCMV infection, with higher susceptibility and lower activation of the immune system in female mice, that in part can be attributed to the lower splenic expression of TLR9 in female than male mice.
Materials and Methods

Mice
C57BL/6 mice were purchased from Charles River (L’Arbesle, France). TLR7\(^{-/-}\) were generated as previously described (28). TLR9\(^{-/-}\) mice were provided by Mark Dalod (CIML) upon written approval of Shizuo Akira (27). Both, TLR7\(^{-/-}\) and TLR9\(^{-/-}\) mice were backcrossed for 10 generations in the C57BL/6 background. Mice were housed under specific pathogen-free conditions and handled in accordance with approved institutional guidelines and French directives.

MCMV infection
MCMV strain K181 was a kind gift of Helen Farrell (Centre for Preventive Medicine, Suffolk, UK). MCMV was prepared as a homogenate of salivary glands harvested from 6 weeks old CD1 mice that have been infected with MCMV two weeks earlier. Viral titers were determined by standard plaque assay on confluent monolayers of mouse embryonic fibroblast cells. Groups of age-matched mice (8 to 12 weeks old) were infected i.p. with 1 or 1.2 \(\times 10^5\) PFU of MCMV per 20 g body weight.

Quantitative PCR for TLR9 expression and MCMV viral load
Total RNA from mouse spleens was isolated with TRIzol reagent (Invitrogen, Auckland, NZ) and contaminant DNA was removed by DNase I (Ambion, Huntington, UK) according to the manufacturer’s instructions. Total RNA (5 µg) was reversed transcribed using SuperScript II reverse transcriptase (Invitrogen). cDNA was amplified by quantitative PCR using the following primers: TLR7 5’-TGGCTCCCTTCTCAGGATGA-3’ and 5’-CCGTGTCCACATCGAAAACA-3’; TLR9 5’-AGCCTGAGCCACACCAACAT-3’ and 5’-
GGACGCGCAGGCTGTATAGG-3’; and β-actin 5’-CCCTGAACCCTAAGGCCA-3’ and 5’-GACAGCACAGCCTGGATGG-3’. For the determination of MCMV viral load, total DNA was isolated from tissues by DNeasy tissue Kit (Qiagen, Hilden, Germany). Parallel reactions were performed for the detection of DNA of immediate early gene-1 (IE-1) and GAPDH. Amplification conditions were identical for all reactions: 10 min at 95 ºC, 40 cycles of 15 sec at 95 ºC and 60 sec at 60 ºC. Quantitative PCR was performed using Power SYBR Green PCR mastermix (Applied Biosystems) and the following primers: IE-1 5’-GGCTTCATGATCCACCCTGTT-3’ and 5’-TGCCATACTGCCAGCTGAGA-3’, GAPDH 5’-TTGCAGTGGCAAAGTGGAGA-3’ and 5’-GGCTCCCCGTTGATGACAAG-3’ in concentrations of 300 nM. Quantitative PCR was performed on an Applied Biosystems PRISM 7700 Sequence Detection System (Warrington, UK). The amount of target was calculated relative to the calibrator by 2^(-ΔCT), resulting in data expressing a target copy number ratio (TLR7/β-actin, TLR9/β-actin or IE-1/GAPDH).

Measurement of Cytokine and Chemokine production

The protein levels of IL-6, TNF, IL-12p70, IFN-γ, KC and MCP-1 in mouse sera were measured by CBA flex (BD Bioscience, San Diego, USA), while IL-12p40 (BD OptEIA, BD Bioscience), IFNα (PBL Biomedical Laboratories, New Jersey, USA) and MIP-2 (R&D Systems, Abington, UK) were measured by ELISA according to manufacturer’s instructions.

Flow cytometry

Mouse spleens were harvested, fine minced and digested with collagenase type II (Worthington, Lakewood, NJ) and DNase I from bovine pancreas (Sigma, St. Louis, USA) for 20 min at room temperature. The reaction was stopped by adding 0.1 M EDTA (Invitrogen, Carlsbad, USA) and the cell suspension was passed through a 70 µm cell strainer. Red blood
cells were lysed with Red Blood lysis buffer (eBioscience, San Diego, CA). Splenic cells were incubated with anti-Fc-receptor antibody (clone 2.4G2) for 15 minutes on ice, washed and stained for the surface markers B220, CD19, CD3, CD4, CD8, NK1.1, CD23, CD21, IgM, IgD, Ly6C (clone AL-21), MIPC (clone 120G8) and CD11c. All antibodies were from BD Bioscience, except MIPC (clone 120G8) was from AbCys (Paris, France). Cells were washed in PBS, fixed with Cytofix (BD Bioscience) and analyzed using a Calibur flow cytometer equipped with CellQuest (BD Bioscience). Data were analyzed by FlowJo software (Ashland, USA).

**Immunohistochemistry and Immunofluorescent staining**

Spleens were embedded in OCT-compound (Sakura Finetek, Torrance, USA) and frozen in liquid nitrogen. Sections were cut on a cryostat at 8 µm, thaw-mounted on gelatinized slides, air-dried and stored at -20°C. Immediately before use, sections were fixed in acetone containing 0.03 % H2O2, rehydrated in PBS and incubated with anti-mouse 7/4 antibody (AbD Serotec) over night, followed by incubation with biotin-conjugated goat anti-rat IgG antibody (Chemicon, Billerica, USA). Subsequently, sections were treated with Vectastain ABC kit (Vector, Burlingame, USA) and developed with DAB (Sigma). For immunofluorescent stainings, frozen sections were stained with the following antibodies: B220-PE (BD Bioscience), MOMA-1-FITC and 7/4-Alexa 647 (AbD Serotec) or ER-TR7 (BMA Biomedicals, Augst, Switzerland) followed by anti-rat IgG-Alexa 488 (Invitrogen). Confocal microscopy was performed with a Zeiss LSM510 microscope. For the staining ER-TR7 and 7/4 the color for 7/4-Alexa 647 was changed to red. Image processing was performed with Zeiss LSM software and Adobe Photoshop.
Statistical analysis

Statistical analysis was performed using the GraphPad Prism program (GraphPad Software, San Diego, USA). All data were mean ± SEM. Significance of differences was assessed by two-tailed unpaired t-test for two groups and by one-way ANOVA employing the Kruskel Wallis test for experiments with more than two groups.
Results

Gender differences in survival upon MCMV infection

To study gender differences in susceptibility to MCMV infection, age matched wild-type, TLR7\textsuperscript{−/−} and TLR9\textsuperscript{−/−} male and female mice, all in the C57BL/6 background, were infected i.p. with MCMV and monitored twice daily for morbidity and survival, and the time of death was recorded. During the course of the infection studies all mice showed obvious signs of sickness starting at day 4 after MCMV infection. Upon infection with $1 \times 10^5$ PFU of MCMV all wild-type (WT) and TLR7\textsuperscript{−/−} male mice survived, while female WT and TLR7\textsuperscript{−/−} mice were susceptible with survival rates, 58% and 45%, respectively (Fig. 1A). However, both TLR9\textsuperscript{−/−} male and female mice showed the same susceptibility to MCMV infection with a survival rate of 50% (Fig. 1A). Moreover, upon MCMV infection TLR3\textsuperscript{−/−} male mice were more resistant than female mice (data not shown). Next, WT and TLR9\textsuperscript{−/−} male and female mice were infected with a higher dose, $1.2 \times 10^5$ PFU, of MCMV. Upon this dose, WT male mice showed intermediate susceptibility (50% survival), while all WT female and TLR9\textsuperscript{−/−} mice, died by day 8 and 6, respectively (Fig. 1B). Our data confirmed previous studies regarding the importance of TLR9 in innate immune defense against MCMV infection (30), and that TLR7\textsuperscript{−/−} MCMV infected mice show similar survival rates like WT control mice (32). Moreover, we found that upon MCMV infection WT and TLR7\textsuperscript{−/−} male mice are more resistant than female counterparts, while TLR9\textsuperscript{−/−} male and female mice show similar survival rates. Taken together, these results suggest that there is a gender difference regarding susceptibility to MCMV infection, and this difference seems to be TLR9, but not TLR7, dependent.

Differences in TLR9 expression in MCMV-infected male and female mice
Next, we tested whether the differences in survival upon MCMV infection between wild-type male and female mice, could be explained by a differential expression of TLRs. To do so, WT male and female mice were left untreated or infected with MCMV and the expression of TLR7 and TLR9 in mouse spleens was determined by quantitative PCR at 36 hours and 4 days upon the infection. Spleens derived from uninfected mice showed low and similar levels of TLR7 and TLR9 mRNA expression between the two genders. Interestingly, 36 hours upon MCMV infection there was a dramatic increase in splenic TLR7 and TLR9 mRNA expression, whereas male mice showed an average of 5 times higher TLR9 expression than female mice, while the expression of TLR7 was similar in both genders (Fig. 2). However, 4 days after MCMV infection the levels of TLR7 and TLR9 expression were back to basal levels, both in male and female spleens (Fig. 2). Thus, MCMV infection leads to a dramatic up-regulation of TLR7 and TLR9 mRNA expression in mouse spleens 36 hours upon MCMV infection, and that TLR9 expression is significant higher in male than in female mice.

*Similar viral organ load and cytokine production in MCMV-infected male and female mice*

To determine whether the difference in MCMV susceptibility between male and female mice is due to difference in viral replication, we determined by quantitative PCR the viral load in spleen and liver of WT and TLR9−/− male and female mice four days after MCMV infection. WT or TLR9−/− male mice showed no significant difference in organ viral load compared with their female counterparts (Fig. 3). However, and in accordance with previously published studies (30, 32), TLR9−/− male and female mice showed increased viral load compared to their WT controls (Fig. 3). Next, we assessed the protein levels of IL-6, TNF, IL-12p40, IL-12p70, IFN-α and IFN-γ in mouse sera derived from WT or TLR9−/− male and female mice, that have been infected with 1x10⁵ PFU MCMV for 36 hours. No difference was observed between WT or TLR9−/− male and female mice, however, TLR9−/− mice produced much lower levels of all
cytokines measured compared to WT controls (Fig. 4). Thus, the difference in susceptibility between WT male and female mice upon MCMV infection cannot be attributed to altered viral replication or cytokine production between the two genders.

Differences in the number of pDCs and MZ B cells in MCMV-infected WT male and female mice

Since both, plasmacytoid dendritic cells (pDCs) and conventional dendritic cells (cDCs) play an important role in antiviral immunity, including MCMV infection (15, 33), we next examined the numbers of these two cell types. The percentage of the pDC population (B220+Ly6ChighCD11clow or B220+120G8+CD11clow) and cDCs (B220−Ly6C−CD11c+) in uninfected WT and TLR9−/− male and female mice were similar (Fig. 5). However, 36 h upon MCMV infection the percentage of cDCs was slightly decreased, but in a similar degree in all four groups of mice. In contrast, the percentage of pDCs was increased in MCMV-infected WT male and TLR9−/− male and female mice, while WT female mice showed much lower increase compared to uninfected mice (Fig. 5). Moreover, in WT MCMV infected mice the percentage of pDCs was statistically much higher in male than in female mice, while no such difference was observed between TLR9−/− male and female mice.

The micro architecture of secondary lymphoid organs, like the spleen, facilitates effective communication between antigen-presenting cells and T lymphocytes to mount protective immunity to pathogens. To examine if the differences in survival between male and female mice upon MCMV infection was due to differences in the number of certain cell populations, we assessed the percentage of various cell types in the spleens of WT or TLR9−/− uninfected or MCMV infected male and female mice. No obvious differences were found regarding the numbers of B, T (CD4 and CD8) and NKT cells, between male and female mice before or after infection (Table I). Regarding, NK cells we observed no gender differences in
uninfected mice, while upon MCMV infection the number of NK splenic cells was reduced but in the same degree in WT and TLR9−/− male and female mice (Table I). Since MZ B cells constitute the first line of defense against blood-borne microorganisms, viruses and toxins in the spleen and MCMV infection in the spleen begins in the MZ (12, 34), we next evaluated the MZ B cell compartment. We found that 36 h after MCMV infection the MZ B cell population (B220+ CD19+CD23lowCD21high) in WT male and TLR9−/− male and female mice was dramatically reduced compared to uninfected controls, while this reduction was mild in MCMV-infected WT female mice (Fig. 6A). This phenotypic defect was further confirmed by the reduction of CD21high cells among the IgMhighIgDlow B lymphocytes, a characteristic trait of MZ B cells (Fig. 6B). Thus, 36 h upon MCMV infection WT female mice show reduced numbers of splenic pDCs and mobilization of the MZ B cells compared to WT male mice, but these gender differences are absent in TLR9−/− mice.

Differences in neutrophil attraction in MCMV-infected WT male and female mice

Neutrophils are the first major population of leukocyte to infiltrate infected or injured tissues and are crucial for initiating host innate defense and adaptive immunity (18). Interestingly, several studies have identified altered function of neutrophils during animal cytomegalovirus infection (35, 36). In order to evaluate the splenic distribution of neutrophils upon MCMV infection, spleens from WT and TLR9−/− male and female mice that have been infected with 1x10⁵ PFU of MCMV or left untreated were analyzed by immunocytochemistry using antibodies against neutrophils (7/4), B cells (B220) and marginal metallophilic macrophages (MOMA-1). We found that 4 days after MCMV infection there was a dramatic increase in the number of neutrophils that were present in the splenic red pulp compared to uninfected mice (Fig. 7A). Moreover, MCMV infection led to an almost complete disappearance of the MZ metallophilic macrophages, in accordance with previous published data (37), and an increase
in the number of neutrophils in the white pulp areas compared to uninfected mice (Fig. 7A, upper panel). Indeed, counting the infiltrating neutrophils in the white pulp areas revealed that infected wild-type male mice had statistically significant higher number of infiltrating neutrophils compared to wild-type female or TLR9^−/−^ male and female mice (Fig. 7B). Furthermore, splenic sections were stained with 7/4 and ER-TR7 antibodies. ER-TR7 stains fibroblasts, connective tissue and endothelial cells of blood vessels and helps to discriminate the white and red pulp area of the spleens. Analysis of this staining showed that MCMV-infection induced a compact ring shape accumulation of neutrophils at the periphery of the white pulp areas in WT male mice, while this accumulation was dramatically reduced in wild-type female and TLR9^−/−^ male and female mice (Fig. 7A, lower panel).

Neutrophils are attracted to the infected tissue by chemokines such as KC or MIP-2, while monocytes are attracted by MCP-1 (38, 39). We assessed the protein levels of KC, MIP-2 and MCP-1 in the sera of uninfected or MCMV infected WT or TLR9^−/−^ male and female mice at 36 hours upon infection. We observed higher amounts of KC and MIP-2 in WT male than in WT female and TLR9^−/−^ male and female mice. However, no differences were observed regarding the release of MCP-1 between WT male and female mice, although the levels of MCP-1 in TLR9^−/−^ mice was decreased compared to WT controls (Fig. 8). Thus, we observed a decreased attraction of neutrophils in the spleens of MCMV-infected WT female and TLR9^−/−^ male and female mice compared to WT male mice. This reduction in the number of attracted neutrophils, was also accompanied by decreased sera levels of KC and MIP-2, the major chemo-attractants of neutrophils.
Discussion

Many laboratory studies investigating viral susceptibility in mice use only one gender or do not consider potential differences between genders if both are used, thus making comparisons among various studies difficult. However, some publications have addressed the gender differences using different viral infectious mouse models. For example, upon infection with herpes simplex virus type I (7), vesicular stomatitis virus (5), coxsackievirus B3 (40) or Theiler’s murine encephalomyelitis virus (6), male mice are significantly more susceptible to disease than female mice, while, upon lymphocytic choriomeningitis virus infection, female mice develop enhanced pathology (41).

The present study was undertaken to define if there is a gender difference in MCMV infection in C57BL/6 mice, an inbread strain commonly used as a background for knockout and transgenic mice, and to investigate the potential contribution of TLRs in this phenomenon. We found that upon MCMV infection C57BL/6 male mice are significantly more resistant to infection than female mice, and that TLR3−/− and TLR7−/− mice show similar gender bias. However, TLR9−/− female and male mice showed similar susceptibility upon MCMV infection. Based on these initial observations and the fact that TLR3, TLR7 and TLR9 have been implicated, although in a different degree, in the pathophysiology of MCMV infection (30, 32, 42), we thought that differences in TLR activation between the two genders might explain the differences in survival. We found that in naïve C57BL/6 mice the mRNA expression levels of TLR7 and TLR9 in mouse spleens were similar in male and female mice. However, 36 hours upon MCMV infection we noticed a dramatic upregulation of TLR7 and TLR9 mRNA expression, where infected male mice showed 7 times higher TLR9 expression than female counterparts, while no such gender differences were observed regarding TLR7
expression. At this point we do not know what is/are the factor/s that lead to the differential expression of TLR9 between male and female mice upon MCMV infection. We could speculate that sex hormones might regulate the expression of TLR9. Interestingly, previous studies have also identified gender differences in response to TLR stimulation that were or not attributed to sex hormones. For example, a study has reported that male mice have exaggerated airway inflammatory and functional responses to LPS compared to females, and that these differences are not due to differential expression of TLR4 before or after LPS exposure, but are mediated, at least in part, by effects of androgens (43). Moreover, another study on PBLs from healthy donors revealed that stimulation of TLR7, but not TLR9, promotes significantly higher IFN-α production in female than in male subjects (44). This sex difference was not due to estrogen signaling or putative X-inactivation escape of the TLR7 gene. Also, the TLR7 mRNA expression levels were found to be similar in female and male naïve pDCs and B cells, however the expression levels of TLR7 in TLR7-ligand stimulated cells was not tested.

To our surprise, the difference in survival between WT male and female mice upon MCMV infection could not be attributed to difference in viral replication since both genders showed similar viral load in spleen and liver (Fig. 3). Moreover, both male and female WT mice showed similar levels of the major cytokines that produced during MCMV infection, in their sera (Fig. 4). However, TLR9−/− mice produced statistical significant lower levels of IL-12p40, IL-12p70, TNF, IFN-α and IFN-γ than WT mice. These results are in accordance with previous reports, where it has been shown that TLR9 is an important component of the innate immune defense against MCMV infection (29, 30, 32), but could not explain the differences in MCMV susceptibility that we observed between the two genders in WT mice.
pDCs represent an essential immune cell type for the initiation of both innate and adaptive immune responses to viral infections, including MCMV, due to their ability to quickly secrete large amounts of type-I IFNs (14, 15, 29). pDCs can enter normal organs at very low levels, however, they are far more numerous in diseased or inflamed tissues. In the current study, we found that MCMV infection induces a 4 fold increase in the percentage of splenic pDCs in wild-type male mice compared to uninfected counterparts, while in female mice this increase was less than 2 fold (Fig. 5). Interestingly, this sex difference was not observed in TLR9−/− mice. So the decreased ability of wild-type female versus male mice to survive MCMV infection could be correlated with the decreased rate of pDCs migration to the spleen. Indeed, previous studies have shown that pathogen-associated molecules or inflammatory mediators exert a dramatic effect on pDC trafficking, causing pDC accumulation in the tissues from which these signals are released and in corresponding draining LNs. For example, in mice infected with respiratory syncytial virus pDCs accumulate in the lungs and mediastinal lymph nodes where they inhibit pulmonary immunopathology and viral replication (45).

MZ B cells are critical for antibody protection against bacterial and viral infections at relatively early stages of infection. MZ B cells have a partially activated phenotype, are nonrecirculatory and reside in the splenic MZ (34). However, in vivo treatment with either heat-killed E. coli or purified LPS acting on TLR4 has long been appreciated to promote re-localization of MZ B cells from the MZ into the B-cell follicles of the white pulp (46). In addition, agonists to TLR2, TLR3 and TLR7 similarly lead to the rapid release of the MZ B cells out of the MZ, suggesting this is a general feature of TLR signaling by MZ B cells (47). Our experiments show that 36h upon MCMV infection there is a dramatic reduction of the MZ B cell population in wild-type male mice, but this phenomenon is less evident in the spleens of infected female mice, suggesting that MZ B cells are less activated in female mice.
However, in TLR9\(^{-/-}\) the reduction of the MZ B cell population is similar in the two genders and with that in WT male mice, suggesting that the reduction of the MZ B cell compartment upon MCMV infection is not TLR9 dependant.

Access of the splenic white pulp, which play a major role in the development of immune responses, has been shown to be restricted to lymphocytes and dendritic cells, and the distribution of neutrophils is almost exclusively confined to the red pulp, which is involved in the clearance of bacteria and dead blood cells from the bloodstream (48). Nevertheless, we found that MCMV infection induced a substantial increase in the number of splenic neutrophils and their migration to the T cell area of the white pulp in WT male mice, and this increase was accompanied by elevated serum levels of the neutrophil chemoattractants MIP-2 and KC (Fig. 7 and 8). However, these phenomena were less obvious in WT female and TLR9\(^{-/-}\) male and female mice (Fig. 7 and 8), suggesting that the increased survival of WT male mice to MCMV infection might be in part attributed to neutrophil migration and function, a phenomenon that depends on TLR9. Interestingly, migration of splenic neutrophils to the T cell area of the white pulp can also be induced upon injection of LPS and this movement is strictly depend on MyD88 and CD14 (49).

In conclusion, upon MCMV infection, male mice show increased survival compared to females due to better immune responses, including increased numbers of splenic pDCs, stronger mobilization of the MZ B cells and increased neutrophil migration in the splenic white pulp. Part of the protection of the male mice may be attributed to the higher expression of TLR9 that leads to stronger immune response against the virus. However, it is not yet clear what underlying mechanisms are involved in gender-dependent activation of TLR9 upon MCMV infection. Protective effects of androgens in male mice, detrimental effects of
estrogens in female mice, or some combination thereof may contribute to the observed differences. Our results also suggest that gender should be carefully considered when designing and interpreting studies of MCMV infection.
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Disclosures:

The authors declare no conflict of interest or financial interest.
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Footnotes

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3 Abbreviations used in this paper: MCMV, mouse cytomegalovirus; MZ, marginal zone; pDC, plasmacytoid dendritic cell; TRIF, Toll/IL-1R domain-containing adapter inducing IFN-β; WT, wild-type.

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Figure 1. Survival curves of WT, TLR7"/" and TLR9"/" male and female mice upon MCMV infection. Mice were infected i.p with 1x10^5 PFU (A) or 1.2x10^5 PFU (B) of MCMV and monitored twice daily for mortality for 14 days. (A) WT males, WT females and TLR9"/" females (n=12), TLR7"/" males (n=10), TLR7"/" females (n=11) and TLR9"/" males (n=8). (B) WT males and females and TLR9"/" males and females (n=6). Data are representative of three independent experiments each.

Figure 2. TLR7 and TLR9 mRNA expression in MCMV-infected WT male and female mouse spleens. WT male and female mice were infected i.p. with 1x10^5 PFU of MCMV or left uninfected and spleens were harvested after 36 hours or 4 days. Total splenic RNA was isolated and the expression of TLR7, TLR9 and β-actin mRNA was determined by Q-PCR. For uninfected male and females spleens, day 4 males and female spleens (n=9), 36 hours male and female spleens (n=10). Data are the pool of 2 independent experiments. *p<0.05.

Figure 3. Viral organ load in MCMV-infected WT and TLR9"/" male and female mice. WT and TLR9"/" male and female mice were infected i.p. with 1x10^5 PFU of MCMV and four days after infection the viral load in spleen and liver was determined by Q-PCR with specific primers for IE-1 and GAPDH. The graph summarizes the mean ratio IE-1/GAPDH. For WT males and females, TLR9"/" males and females (n=9).

Figure 4. Proinflammatory cytokine-production in sera of MCMV-infected WT and TLR9"/" male and female mice. WT and TLR9"/" male and female mice were infected i.p. with 1x10^5 PFU of MCMV and sera were collected after 36 hours of infection. IL-6, TNFα, IL-12p70
and IFNγ were measured by CBA flex, and IFNα and IL-12p40 by ELISA. Data are representative of three independent experiments. For WT males and females, TLR9−/− males (n=9) and TLR9−/− females (n=8). *p<0.05, **p<0.01 and ***p<0.001.

**Figure 5.** Plasmacytoid and conventional dendritic cells of uninfected and MCMV-infected WT and TLR9−/− male and female mice. WT and TLR9−/− male and female mice were left uninfected or infected i.p. with 1x10^5 PFU MCMV and spleens were harvested 36 hours after infection. Total splenocytes were stained for B220, CD11c, 120G8 and Ly6C. Data are presented as mean ± SEM of 3 mice per group of 3 independent experiments. *p<0.05.

**Figure 6.** Analysis of marginal zone B cells in WT and TLR9−/− mouse spleens. WT and TLR9−/− male and female mice were left uninfected or infected i.p. with 1x10^5 PFU of MCMV and spleens were harvested 36 hours later. Total splenocytes were collected, stained with antibodies against B220, CD19, CD23, CD21, IgM and IgD and analyzed by flow cytometry. (A) Plots show expression levels of CD21 and CD23 on B220+CD19+ gated lymphocyte population. (B) Histograms show expression of CD21 cells on IgM^{high}IgD^{low} cells. Data are representatives of 3 mice per group of 3 independent experiments.

**Figure 7.** Distribution of neutrophils in MCMV infected WT and TLR9−/− mouse spleens. WT or TLR9−/− male and female mice were left untreated of infected with 1 x 10^5 PFU of MCMV and 3 days later mouse spleens were collected for immunoflourescent analysis. (A) Murine spleen sections were incubated with antibodies specific to neutrophils (7/4, blue or red), B cells (B220, red), marginal metalophilic macrophages (MOMA-1, green) and reticular fibroblasts (ER-TR7, green). (B) Number of neutrophils per white pulp area were counted on slides stained in A. *** p<0.001. Data are representative of 9 mice per group.
Figure 8. Chemokine production in mouse sera of MCMV-infected WT and TLR9<sup>−/−</sup> mice. WT or TLR9<sup>−/−</sup> male and female mice were infected i.p. with 1x10<sup>5</sup> PFU MCMV and sera were collected 36 hours after infection. KC, MIP-2 and MCP-1 were measured by CBA flex or ELISA. Data are representative of four independent experiments. *p<0.05, **p<0.01 and ***p<0.001.
**Table I.** Percentage of B, T, NK and NKT cells in WT or TLR9<sup>−/−</sup> male and female mouse spleens upon MCMV infection. WT and TLR9<sup>−/−</sup> male and female mice were left uninfected or infected i.p. with 1x10<sup>5</sup> PFU of MCMV. After, 36h spleens were harvested, total splenocytes were isolated, stained for B220, CD19, CD3, CD4, CD8 or NK1.1 and analyzed by flow cytometry. Data are mean ± SD of three mice per group, and are representative of two or three independent experiments.
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Résumé

Les récepteurs Toll-like (TLRs) détectent des structures conservées exprimées par différentes classes de microorganismes, jouant ainsi un rôle majeur dans la réponse immunitaire. Les TLRs localisés dans les endosomes (TLR3, 7, 8 et 9) reconnaissent principalement des acides nucléiques dérivés de microbes. Cependant, ils peuvent également être responsables de la reconnaissance d’acides nucléiques endogènes et contribuer au développement d’autoimmunité. À la différence du TLR8 humain, le TLR8 murin n’induit pas de réponse à l’ARN simple brin et a ainsi été considéré comme non fonctionnel. Le but de cette étude est d’étudier le rôle du TLR8 murin dans l’immunité. Nous avons montré que les cellules dendritiques déficientes en TLR8 surexpriment le TLR7 et présentent une réponse accrue à une stimulation de TLR7. Chez la souris, la déficience en TLR8 entraîne une augmentation des taux d’anticorps circulant (IgM, IgG, IgG2a), des autoanticorps, et au niveau rénal la présence de dépôts de complexes immuns. À l’inverse des souris TLR8−/−, les souris TLR7/8−/− sont protégées de tout symptôme. Nos résultats indiquent donc que chez la souris le TLR8 joue un rôle primordial dans la modulation de l’expression de TLR7, et cette régulation est cruciale dans le contrôle du développement d’autoimmunité spontanée.

Mots clef : récepteur Toll-like, autoimmunité, cellules dendritiques, lupus érythémateux systémique

Summary

Toll-like receptors (TLRs) detect conserved molecular products of microorganisms and play an essential role in the induction of immune responses. Endosomal TLRs (TLR3, 7, 8 and 9) sense nucleic acids derived from microbes. However they can also recognize self nucleic acids and thus be involved in the development of autoimmunity. Unlike human TLR8, murin TLR8 does not respond to single-stranded RNA suggesting that it could be not functional. In the current study, we investigated the role of murine TLR8 signaling in immunity. We found that TLR8−/− dendritic cells overexpress TLR7 and are hyperresponsive to various TLR7 ligands. In mice, TLR8 deficiency leads to increased levels of IgM, IgG, IgG2a circulating antibodies, autoantibodies and in the kidney to higher deposition of immunocomplexes while double TLR7/8−/− mice are protected from autoimmune features. These data provide evidence for a pivotal role of murine TLR8 in the regulation of murine TLR7 expression and this control is critical for the prevention of spontaneous autoimmunity development.

Key words: Toll-like receptors, autoimmunity, dendritic cells, systemic lupus erythematosus

Discipline: Immunologie

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