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TLR8, TLR9 and Gfi-1 restrain TLR7-mediated lupus

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Résumé

Le lupus érythémateux disseminé (LED) est une maladie chronique auto-immune caractérisée par la production d'autoanticorps dirigés contre les antigènes nucléaires. L'étiologie du lupus n'est pas encore claire mais de nombreuses études indiquent un rôle des récepteurs Toll-like (TLR) et leurs voies de signalisation dans l'initiation et la mise en place du LED. Mon projet de thèse avait deux objectifs dont le premier était de comprendre comment le TLR8 et TLR9 contribuent au lupus dépendant de TLR7. Des études antérieures de notre laboratoire ont révélé que le TLR8 murin contrôle la fonction de TLR7 dans les cellules dendritiques et est aussi impliqué dans le lupus. D'autre part, TLR9 contrôle également le lupus dépendant de TLR7. Cependant, leur contribution respective dans le contrôle de TLR7 restait inconnue. En étudiant des souris doublement déficientes pour TLR8 et TLR9, nous avons constaté que les souris TLR8/9$^{-/-}$ présentent une maladie exacerbée avec une splénomégalie accrue, des niveaux d'autoanticorps circulants plus élevés et des dépôts de complexes immuns plus importants au niveau des reins par rapport aux souris déficientes pour TLR8 ou TLR9 uniquement. En outre, nous avons révélé que TLR8 contrôle l'expression de TLR7 dans les cellules dendritiques, tandis que le TLR9 contrôle la fonction de TLR7 dans les cellules B. Ainsi, TLR8 et TLR9 ont un effet additif dans le contrôle du lupus dépendant de TLR7 et leur action est spécifique d'un type cellulaire donné. Le deuxième objectif était d'étudier l'implication du répresseur transcriptionnel Gfi-1 dans la signalisation des TLR et le développement de lupus en utilisant des souris Genista. Ces souris portent une mutation ponctuelle dans le gène Gfi-1 qui code pour un répresseur transcriptionnel régulant de façon négative la voie TLR4 et potentiellement impliqué dans l'auto-immunité. Nous avons constaté que les souris Genista développent un lupus dépendant de TLR7, caractérisé par l'augmentation des auto-anticorps circulants et l'apparition d'une pathologie rénale. De plus, nous avons montré que Gfi-1 agit comme un répresseur de la transcription en aval de TLR7 et contrôle l'expression d'Interféron de type I dépendante des TLR. Ainsi, le déséquilibre des interactions entre TLR ainsi que les facteurs transcriptionnels en aval de ces TLR peuvent conduire à des mécanismes d'inflammation et d'auto-immunité qu'il est important de prendre en compte dans le développement d'approches thérapeutiques nouvelles ciblant les TLRs.

Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by production of autoantibodies against nuclear antigens. The etiology of SLE is still unclear, but many studies indicate a role for Toll-like receptors (TLRs) and their signaling pathways in the initiation and establishment of SLE. The project of my thesis had two aims. The first was to understand how the cooperation of TLR8 and TLR9 contributes to TLR7-mediated lupus. Previous studies in the laboratory revealed that murine TLR8 controls TLR7 function and that TLR8 deficiency in mice leads to TLR7-dependent lupus. Moreover, TLR9 also controls TLR7-mediated lupus. However, it was unknown if TLR8 and TLR9 act in parallel or in series in the same or different cell types and if they have an additive effect or not in controlling TLR7 function. By studying double TLR8/9$^{-/-}$ mice, we found that TLR8/9$^{-/-}$ mice had exacerbated disease as revealed by increased splenomegaly, higher levels of antibodies in sera and accumulation of immunocomplexes in kidneys compared to single TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. Moreover, we revealed that TLR8 controls TLR7 expression and function on DCs, while TLR9 restrains TLR7 responses in B cells. Thus, TLR8 and TLR9 have an additive effect on controlling TLR7-mediated lupus, and that their function is cell type specific. The second aim of my thesis was to study the implication of the transcriptional repressor Gfi-1 in TLR-signalling and lupus development using Genista mice. Genista mice carry a single-point mutation in the Gfi-1 gene. Gfi-1 encodes a transcriptional repressor that negatively regulates the TLR4 pathway, and data suggest that might be implicated in autoimmunity. We found that Genista mice develop lupus characterized by elevated circulating autoantibodies and renal pathology and that the disease is TLR7-dependent. In addition, we demonstrated that Gfi-1 act as a transcriptional repressor downstream of TLR7 and controls TLR7- dependent type-I IFN expression. Thus, unbalancing TLR-interactions and transcription factors downstream of TLRs can lead to inflammation and autoimmunity. These mechanisms have to be taken into account when novel therapeutic approaches are developed that target TLRs.

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List of Abbreviations

A

ANA	antinuclear antibody
AP1	activator protein 1
AP3	adaptor protein 3
APC	antigen presenting cells

B

BCR	B cell receptor
BMDC	bone-marrow derived dendritic cell

C

CARD	caspase recruitment domain
CD	cluster of differentiation
cDC	conventional DC
CLP	commom lymphoid precursor
CMP	commom myeloid precursos
CoRest	corepressor for element-1-silencing transcription factor
CpG	cytidine-phosphateguanosine
CTLA4	cytotoxic T lymphocyte-associated antigen 4

D

DC	dendritic cell
DNA	deoxyribonucleic acid
Ds-DNA	double-stranded DNA

E

ELISA	enzyme-linked immunosorbent assay
ENU	N-ethyl-N-nitrosourea-induced
ERK	extracellular regulated kinase

F

FcRy	Fc-gamma-receptor

G

GC	Germinal Centers
Gfi-1	Grown-factor independent 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte macrophage precursors

H

HDAC-1	histone deacetylase1
HMGB1	high mobility group protein 1
HIV	immunodeficiency virus
HNP	human neutrophil peptide
HSC	hematopoietic stem cells
HSV-1	Herpes simplex virus

I

IC	immunocomplexes
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of nuclear factor B
IL	interleukin
IRAK	IL-1 receptor-associated kinases
IRF	interferon regulator factor

J

JNK	JUN N-terminal kinase

L

LBP	lipopolysacharide-binding protein
LPS	lipopolyssaccaride
LRR	leucine-rich repeats
LSD-1	lysine specific demethylase-1

M

MAL	MyD88 adaptor protein
MAMP	microbe-associated molecular pattern
MAPKs	mitogen-activated protein kinases
MHC	major histocompatibility complex
MRL	Murphy Roths Large
MS	multiple sclerosis
MyD88	myeloid differentiation primary-response gene 88
MZ	marginal zone

N

NEMO	NF-kappaB essential modulator
NET	neutrophil extracellular traps

NF-κB	nuclear factor kappa B
NK	natural killer
NZB	New Zeland black
NZW	New Zeland white

O

ODN	olidodeoxynucleotide
OspA	outer-surface lipoprotein

P

PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononucleated cell
PD-1	programmed death-1
pDCs	plasmacytoid dendritic cell
PIK3	phosphatidylinositol-4, 5-bisphosphate 3-kinase
Poly I: C	polyinosinic-polycytidylic acid
PRAT4A	protein associated with TLR4 A

Q

qPCR	quantitative polymerase chain reaction

R

RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end-products
RIP1	receptor interacting protein 1
RNA	ribonucleic acid

S

SARM	armadillo-motif-containing protein
SLE	systemic lupus erythematosus
Sm	smith antigen
SmRNP	Small nuclear ribonucleoprotein
SNP	single nucleotide polymorphisms
SOCS	supressor of cytokine signaling
ssRNA	single-stranded RNA

T

TAK	transforming-growth-factor-b-activated kinase
TBK1	TANK binding kinase 1
TCR T	T cell receptor
Tg	transgenic
Th	T helper

TGF-b	transforming growth factor 1
TIR	Toll/IL-1 receptor
TIRAP	TIR associated protein
TLR	toll-like receptor
TNF	tumor necrosis factor
TRADD	tumor necrosis factor-receptor 1-associated death domain protein
TRAF 6	TNF receptor-associated factor 6
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRIFF	TIR domain-containing adaptor inducing IFN-β
TWEAK	TNF-like weak inducer of apoptosis
T reg	regulatory T cell

U

UNC93B1	unc-93 homolog B1

W

WT	wild-type

Y

Yaa	Y-linked autoimmune accelerator locus

Introduction

1. Innate and Adaptive Immunity

The immune system is a network of cells, tissues and organs that work together to protect the body from infection. Host defense mechanisms consist of innate immunity which mediates the initial protection against infections and adaptive immunity which develops more slowly and provides more specialized and effective defense against infection (Janeway C.A. Jr., 2001)

The basis of innate immunity was formed by the work of Metchnikov in starfish larvae. He observed that white blood cells are able to engulf and destroy bodies such as bacteria and this process was called phagocytosis (Metchnikov, 1884). Innate immunity is a fast, basic and conserved ancient defense system and the first line of defense against infection. The characteristics of the innate immune response include non-specific killing of microbes by phagocytosis, leukocyte recruitment to sites of infection and initiation of inflammation.

The innate immune system relies on a limited repertoire of receptors called Pattern Recognition Receptors (PRRs) for the detection of invading pathogens but compensates for this limited number of invariant receptors by targeting conserved microbial components that are shared by large groups of microorganisms called Pathogen-associated molecular patterns (PAMPs). Innate immunity plays a central role in activating the subsequent adaptive immune response which sets in place B and T cell responses for the elimination of the pathogen that have been encountered (Akira et al., 2006; Janeway and Medzhitov, 2002). The adaptive response is specific and is characterized by an immunological memory that will lead to a more efficient and rapid destruction at a second contact with the same pathogen (Pulendran and Ahmed, 2006).

The first discoveries about adaptive immunity were due to von Behring and Kitasato who produced passive immunity to diphtheria and tetanus in animals that received injections of blood serum from other animals affected by the disease (Behring and

Kitasato, 1890). Subsequently, Erhlich proposed a theory that predicted the mechanism of antibody production by B cells in response to antigens. Based on this proposal, the body produces abundant side-chains (antibodies) able to react with specific bacterial toxins (Ehrlich, 1900). Some years later, the adaptive immunity started to be categorized in humoral and cellular immunity. In adaptive or acquired immunity, pathogen-specific receptors are "acquired" during the lifetime of the organism (Janeway and Medzhitov, 2002). The diversity of antigen receptors is due to V(D)J recombination which is an irreversible genetic recombination of antigen receptor gene segments, and also somatic hypermutation, a process of accelerated mutations (Tonegawa, 1983). Because gene rearrangements lead to an irreversible change in the DNA of each cell, all the progeny of that cell will inherit genes encoding receptors with same specificity. This is particularly relevant for the memory B and T cells that are the keys to long-lived specific immunity

For long time, the innate and adaptive immune response had been considered independent from each other and much attention had been focused on the field of adaptive immunity. Charles Janeway proposed an integrative theory suggesting a close link between innate and adaptive immunity (Medzhitov and Janeway, 1999). Currently, innate immunity is no more seeing as “nonspecific immunity” but as a proud partner with the adaptive immune system in protecting mammalian hosts from infections. Furthermore, much evidence has demonstrated that the activation of innate immunity and its cells is a prerequisite for the proper induction of adaptive immunity. This paradigm shift changed the thinking on pathogenesis and treatment of many diseases (Medzhitov and Janeway, 1999).

2. Autoimmunity and how the immune system can cause disease

The immune system is made to recognize and to respond to several pathogens, but at the same time it should remain unresponsive to self-antigens since immune responses against self-antigens lead to autoimmunity. The normal consequence of an immune response against a foreign antigen is the clearance of the antigen from the body.

Virus-infected cells, for example, are destroyed by cytotoxic T cells, whereas soluble antigens are cleared by formation of immune complexes of antibody and antigen, which are taken up by cells of the mononuclear phagocytic system such as macrophages (Atassi and Casali, 2008). When an adaptive immune response develops against self-antigens, it is usually impossible for immune effector mechanisms to eliminate the antigen completely, thus provoking a sustained response. As a consequence the effector pathways of immunity cause chronic inflammatory injury to tissues, which may prove lethal. Some mechanisms of tissue damage in autoimmune diseases are similar to those that operate in protective immunity and in hypersensitivity diseases (Albani, 1994). Nevertheless, most people do not develop autoimmune disease because the pathogenicity of these self-reactive cells is counterbalanced by regulatory mechanisms that are constantly at work suppressing potentially damaging responses, thus maintaining tolerance to self (Mueller, 2010).
There are two major types of autoimmune diseases, those in which the expression of autoimmunity is restricted to specific organs of the body, known as "organ-specific" autoimmune diseases, and those in which many tissues of the body are affected, the "systemic" autoimmune diseases (Janeway C.A. Jr., 2001). Examples of autoimmune diseases and their prevalence in the world (**Figure 1**) include:

- **Rheumatoid arthritis (RA)** - affects mainly the joints but the eyes, lungs and heart may also be affected. Symptoms include swollen and deformed joints.
- **Psoriasis** - affects the skin. Features include the development of thick, reddened skin scales.
- **Crohn's disease** - is a type of inflammatory bowel disease that can affect any part of the gastrointestinal tract from mouth to anus, causing a wide variety of symptoms.
- **Diabetes (Type I)** - affects the pancreas. Symptoms include thirst, frequent urination, weight loss and an increased susceptibility to infection.
- **Graves' disease** - affects the thyroid gland. Symptoms include weight loss, elevated heart rate, anxiety and diarrhea.

- **Inflammatory bowel disease** - including ulcerative colitis and, possibly, Crohn's disease. Symptoms include diarrhea and abdominal pain.
- **Multiple sclerosis (MS)** - affects the nervous system. Depending on which part of the nervous system is affected, symptoms can include numbness, paralysis and vision impairment.
- **Systemic lupus erythematosus (SLE)** - affects connective tissue and can strike any organ system of the body. Symptoms can include joint inflammation, fever, weight loss and a characteristic facial rash.

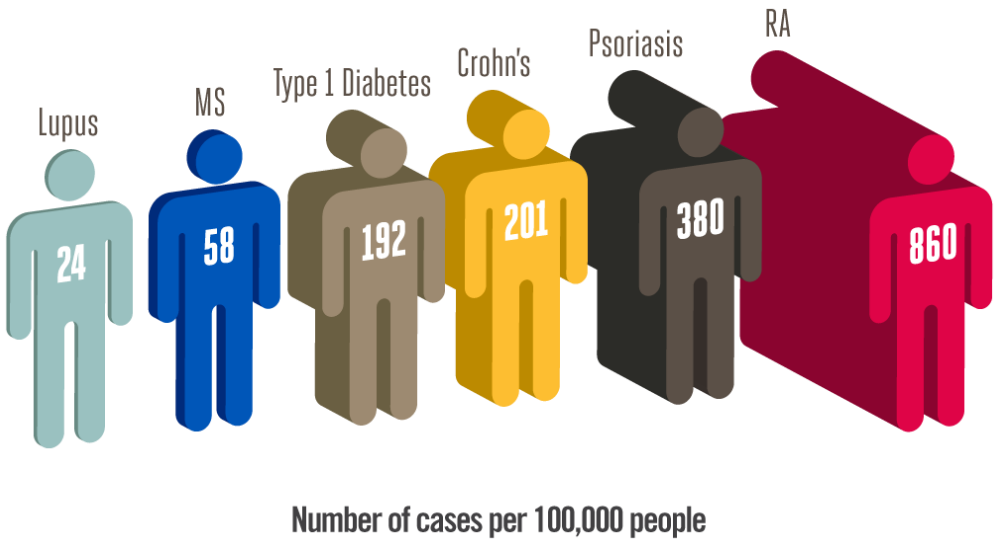

Figure 1: Prevalence of most common autoimmune diseases in the world (Reumathoidarthritis.net).

Usually autoimmunity is not set off by a single cause but can be triggered by a variety of immunological, environmental and genetic mechanisms. Gender, sex hormones and infections can also be risk factors to trigger autoimmunity. However, even if a given autoimmune disease were to be initiated primarily by a single trigger, other events and regulating mechanisms come into play, thereby adding complexity to the process (Atassi and Casali, 2008). Some of the mechanisms that can cause autoimmune diseases include escape from central and peripheral tolerance, production of autoantibodies, disruption in the level or activity of inhibitory

molecules and dysregulated apoptosis. More details on these mechanisms are presented below:

2.1 Escape from Central and Peripheral Tolerance

Although autoreactive T lymphocytes are constantly generated in the thymus, many of these cells are eliminated before they complete their maturation. However, this process is imperfect, because normal healthy individuals have circulating T cells that are capable of mounting pathogenic immune responses directed against self-antigens (Davis et al., 2011).

Immunological tolerance is broadly classified in central and peripheral. Central tolerance occurs via clonal selection in the thymus and the bone-marrow, the primary lymphoid organs where T and B cells develop, respectively. In the bone-marrow, autoreactive B cells that interact strongly with membrane-bound self-antigens are negatively selected or deleted (Cyster and Goodnow, 1995). To avoid cell loss, B cells have a tolerogenic process called "receptor editing" which involves a modification in the light chain of the antigen receptor that changes its specificity and avoids cell loss during clonal selection (Halverson et al., 2004). In the thymus, antigen presenting cells (APCs) interact with developing thymocytes by presenting self-antigens via major histocompatibility complex (MHC). T cells expressing a receptor with high affinity for self-antigens are negatively selected or deleted (Burnet, 1959; Hogquist et al., 2005). However, some autoreactive B/T cells can escape from the central tolerance and leave the primary lymphoid organs. Still, this escape does not automatically lead to autoimmunity because in the periphery there is a second tolerogenic mechanism. Peripheral tolerance is mediated by regulatory T cells (Treg) and tolerogenic dendritic cells (DCs) (Blanco et al., 2008; Wing and Sakaguchi, 2010).

2.2 Natural antibodies

Natural antibodies or autoantibodies arise independently of known and/or deliberate immunization. They bind a variety of exogenous antigens, such as bacteria, viruses, fungi as well as self-antigens, such as nucleic acids, erythrocytes, serum proteins, cellular components, insulin or thyroglobulin (Casali and Schettino, 1996). Most natural autoantibodies are IgM and polyreactive, they bind to several unrelated antigens, generally with moderate intrinsic affinity, although natural mono-reactive antibodies also exist (Casali and Schettino, 1996; Harindranath et al., 1993; Phillips-Quagliata et al., 2001). Despite the low-to-moderate intrinsic affinity of their antigen-binding sites, natural IgM antibodies possess a high overall binding avidity because of their decavalency, a feature that makes these antibodies particularly effective in binding antigens with a repetitive structure on the surface of cells, tissues, bacteria and viruses. Natural autoantibodies are produced mainly by B-1 cells (Casali et al., 1987), the predominant lymphocytes in the neonatal B-cell repertoire, and by marginal zone B cells (Casali and Notkins, 1989; Duan and Morel, 2006; Kasaian et al., 1991). B-1 cells are highly effective in presenting antigen (Mohan et al., 1998) and can have an important function in the production of pathogenic auto-antibodies in several autoimmune diseases (Kasaian and Casali, 1993, 1995).

2.3 Apoptosis

Apoptosis, a process of programmed cell death and removal of damaged cells, results in the release of cell components that are made accessible to immune recognition. This process exposes cytoplasm as well as nuclear components normally protected inside the cell, and can thus trigger immune responses to components of the cytoplasm, the membrane and/or the nucleus (Kerr et al., 1972). Apoptosis is essential for the prevention of autoimmunity because it controls the disposal of autoreactive T cells and enables the homeostasis and downregulation of immune responses against self-antigens (Elmore, 2007). Several autoimmune disorders have been linked to

apoptosis (Franz et al., 2006; Navratil et al., 2006; Viorritto et al., 2007). Defects in T cell apoptosis have been reported in autoimmunity associated with type 1 diabetes (Gronski and Weinem, 2006) and apoptotic loss of pancreatic β-cells has been reported to cause autoimmune responses that contribute to the onset and progression of type 1 diabetes (Lee and Pervaiz, 2007). In the presence of defective clearance of cellular debris or subcellular particles, apoptosis can be a significant trigger of autoimmune responses against nuclear components (Martelli et al., 2001). Defects in phagocytosis and/or other clearance of necrotic cells and subcellular particles in the germinal centers of secondary lymphoid organs can induce autoimmunity (Cline and Radic, 2004), and play a role in the pathogenesis of systemic autoimmune responses, such as those in SLE (Gaipl et al., 2006; Wu et al., 2001).

2.4 Inhibitory molecules

The immune system is also regulated by molecular interactions that organize, direct and control its functions. Molecular and/or cellular changes that compromise the correct performance of this network have been found to be associated with autoimmune diseases. The T and B cell activity is affected by inhibitor receptors, like cytotoxic T lymphocyte-associated antigen-4 (CTLA4) and programmed death-1 (PD-1) (Fife and Bluestone, 2008) for T cells and FcγRII, CD22 and PD-1 for B cells. CTLA4 is a member of the immunoglobulin superfamily, which is expressed on the surface of helper T cells and transmits an inhibitory signal to T cells. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86 on antigen-presenting cells (APCs). CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal (Magistrelli et al., 1999; Waterhouse et al., 1995). T cell activation through the T cell receptor and CD28 leads to increased expression of CTLA-4, an inhibitory receptor for B7 molecules. Certain alleles of the CTLA4 gene have been proposed to act as nonspecific costimulatory (exacerbating) elements in autoimmunity. CTLA4 gene polymorphism is associated with increased predisposition to certain autoimmune diseases (Vigano et al., 2005). In a similar way as CTLA4, PD-1 interactions with PD-L1 and PD-L2 downmodulate

immune responses (Fife and Bluestone, 2008). The role of the PD-1/PD-L1 pathway in autoimmunity has been investigated in vivo in several experimental animal models including insulin-dependent diabetes mellitus, systemic lupus erythematosus, myocarditis, encephalomyelitis, rheumatoid arthritis and inflammatory bowel diseases. With the advent of candidate gene and genome-wide association studies, single nucleotide polymorphisms (SNPs) in the PD-1 gene in humans have demonstrated relevant associations with a higher risk for developing autoimmune diseases in certain ethnic groups (Gianchecchi et al., 2013).

FcγRII is a receptor for the Fc portion of IgG5, a member of the Ig superfamily and the only Fc receptor expressed by B cells. FcγRII inhibits signaling through the B cell receptor (BCR) when B cells interact with immune complexes containing IgG. Upon interaction with IgG, FcγRII activates molecules that decrease the recruitment of BCR activators to the signalosome complex (Malbec et al., 1999). Evidence of a role for defective FcγRII inhibition in the pathogenesis of autoimmunity is found in studies of FcγRII deficient mice, in mouse models of autoimmune disease and in SLE patients (Boackle et al., 2001; Bolland and Ravetch, 2000).

3. SLE in humans

Systemic lupus erythematosus (SLE or lupus) is a systemic autoimmune disease that can affect any part of the body. SLE most often harms the heart, joints, skin, lungs, blood vessels, liver, kidneys and nervous system. The course of the disease is unpredictable, with periods of illness, also called flares, alternating with remissions (Rahman and Isenberg, 2008). One of the most severe manifestations in SLE is lupus nephritis, which remains a cause of substantial morbidity and mortality, either secondary to kidney disease, or as a result of intense immunosuppressive drug toxicity (Bernatsky et al., 2006; Borchers et al., 2004; Borchers et al., 2010; Hiraki et al., 2012).

The overall incidence of SLE generally ranges from 1 to 5 in 100.000 and the prevalence from 20 to 150 in 100.000 people, with women being affected 9 times

more frequently than men (Borchers et al., 2010). The frequency of SLE is 2- to 8-fold higher in non-European populations, particularly those of African ancestry. The onset of SLE in most populations occurs most commonly during the third and fourth decade of life and somewhat later in populations of European descent. Approximately 15–20% of patients experience the onset of SLE prior to 18 years of age. The frequency of renal involvement is particularly high in juvenile-onset SLE, ranging from 50% to 80% in most cohorts described today (Abdwani et al., 2008; Barron et al., 1993; Font et al., 1998; Hoffman et al., 2009) and does not differ with ethnicity (Barron et al., 1993). In contrast, lupus nephritis is less frequent in patients with late-onset SLE (≥50 years), with fewer than 30% of patients affected (Boddaert et al., 2004). Renal disease may be the first manifestation of SLE, but most commonly occurs within a year of diagnosis, and almost always within 5 years, but can occur any time throughout the course of the disease (Seligman et al., 2002). Renal nephritis prevalence differs significantly by ethnicity, being 3.5 in 100.000 for white, 13.3 in 100.000 for Indo-Asian, 64.6 in 100.000 for Afro-Caribbean and 66.7 in 100.000 for Chinese patients. This is in agreement with numerous cohort studies demonstrating that SLE patients of European descent have the lowest frequency of renal disease (20–45%) (Alonso et al., 2011; Brugos et al., 2006; Cervera and Font, 1993), whereas 50–70% of SLE patients develop nephritis in African American and certain Asian, Arab, Hispanic, indigenous and mestizo populations (Borchers et al., 2010). Non-European patients, in particular those with African ancestry, often develop nephritis earlier in the course of the disease (Alarcon et al., 2006; Bastian et al., 2002; Burgos et al., 2011; Hopkinson et al., 2000; Pons-Estel et al., 2004; Seligman et al., 2002).

The diagnosis of lupus is considered complex because the symptoms vary among the patients and can appear at different times. Current guideline revised by the American College of Rheumatology requires that patient have 4 out of the 11 criteria (**Table 1**). For diagnosis various laboratorial tests should be done and the gold standard is the antinuclear antibody (ANA) test (Sullivan, 2000).

1	Malar Rash
2	Discoid Rash
3	Serositis, pleuritis, pericarditis
4	Oral ulcers: usually painless
5	Arthritis
6	Photosensitivity
7	Blood disorders
8	Renal Disease
9	ANA positive
10	Immunologic: Anti-ds DNA, SmRNP, anticardiolipin
11	Neurologic: seizure or psychosis

Table 1: 1997 American College of Rheumatology Revised Criteria for the Classification of SLE (Hochberg, 1997). Abbreviations: ds,double-stranded; SmRNP, a small nuclear ribonucleoprotein.

3.1 Autoantibodies in SLE

The central immunological disturbance in patients with SLE is autoantibody production. These antibodies are directed against several self-molecules found in the nucleus, the cytoplasm, and on the cell surface, in addition to soluble molecules such as coagulation factors (Mok and Lau, 2003). Antinuclear antibodies are most characteristic and present in more than 95% of patients. Anti-double-stranded DNA (ds-DNA) and anti-SmRNP antibodies are unique to patients with SLE (Marks and Tullus, 2012; Mok and Lau, 2003; Tan et al., 1982). The SmRNP antigen is designated as a small nuclear ribonucleoprotein (SmRNP) and is composed of a unique set of uridine rich RNA molecules bound to a common group of core proteins and other proteins associated with RNA molecules. Anti-SmRNP antibodies react

with smRNP core proteins, whereas anti-DNA antibodies bind to a conserved nucleic acid determinant widely present on DNA. Anti-DNA antibody titers frequently vary over time and disease activity, while anti-Sm antibody titers are usually constant.
The most remarkable feature of anti-DNA antibodies is their association with glomerulonephritis (Ehrenstein et al., 1995). Anti-DNA antibodies can be isolated in an enriched form from glomerular eluates of patients with active lupus nephritis (Ehrenstein et al., 1995). These antibodies show preferential deposition in the kidneys, suggesting that DNA-anti-DNA antibody immune complexes are the main mediators of inflammation (Foster et al., 1993). They can also bind to DNA adherent to the glomerular basement membrane via the histones or interact with additional glomerular antigens, such as C1q, nucleosomes, heparan sulfate and laminin. The binding of anti-DNA antibodies to these antigens may initiate local inflammation and complement activation, and may also anchor immune complexes to the kidney sites, whether formed in the circulation or *in situ*. However, the correlation between anti-DNA antibodies and lupus nephritis is not universal since some patients with active nephritis are negative for anti-DNA antibodies, whereas some patients with persistent high titers of anti-DNA may not show renal involvement (van Bruggen et al., 1997).

3.2 Treatment for SLE

There is no curative treatment available for SLE. The current treatments are effective at reducing morbidity and mortality and they frequently have adverse effects (Yildirim-Toruner and Diamond, 2011). An algorithm for the treatment of SLE is proposed in **Figure 2** (Xiong and Lahita, 2014). Antimalarials and non-steroidal anti-inflammatory drugs are currently used in almost all SLE patients, due to the many beneficial effects associated with their use as reported over the years; reduction in mortality, damage, flares, cardiovascular morbidity, and infections may occur due to the multiple immunomodulatory and metabolic effects of these compounds (Bertsias et al., 2008; Ruiz-Irastorza et al., 2010). Glucocorticoids are still common in the treatment of SLE in case of organ involvement; however, they have been associated with several adverse events including cardiovascular events, diabetes mellitus,

osteoporosis, osteonecrosis, infections, glaucoma, cataracts, psychological disorders, among several other deleterious effects (Ruiz-Irastorza et al., 2012).

Several immunosuppressive drugs have been used in SLE as steroid-sparing or to augment their effect when steroids alone have not been able to abate the manifestations associated with severe organ system involvement. For example, cyclophosphamide has been shown to be efficacious in patients with lupus nephritis and neurological involvement. However, as cyclophosphamide has been associated with various adverse events, there has been several proposals for reducing the time and dose of this medicine (Yildirim-Toruner and Diamond, 2011).

Mycophenolatemofetil has emerged as another option for the treatment of lupus nephritis, being at least as effective as cyclophosphamide for the induction phase. Other therapeutic options for these patients are azathioprine, methotrexate, cyclosporine, tacrolimus, and leflunomide. However, all these immunosuppressive drugs are also associated with several adverse events like infections and malignancies (Ugarte-Gil and Alarcon, 2014). Some advances in understanding the immunopathogenesis of SLE have led to the development of targeted immunotherapies, such as the anti-BAFF antibody belimumab, which has been approved as an add-on therapy for patients who have active disease despite receiving standard therapy (Sthoeger et al., 2014).

Despite these treatments, SLE patients still have a threefold increase in all-cause mortality compared to the general population (Yurkovich et al., 2013). In addition, there is still a big gap between what lupus patients need or expect and what current treatment options offer. Therefore, better understanding of the causes and mechanisms of SLE pathogenesis is pivotal for the development of novel therapeutic means with improved efficacy and lower toxicity.

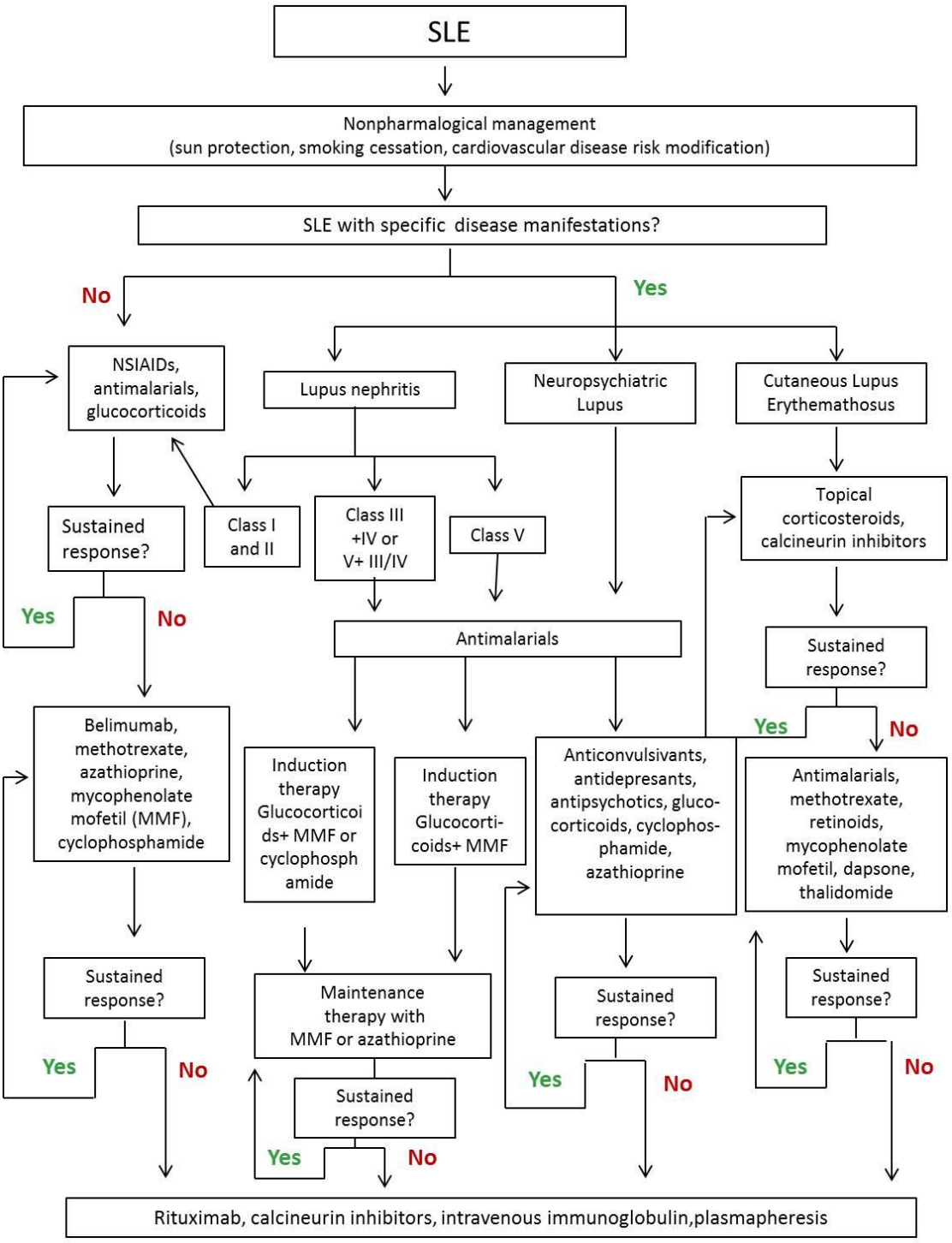

Figure 2: Algorithm for the treatment of SLE. Abbreviations: MMF: mycophenolate mofetil; NSAIDs: non-steroidal anti-inflammatory drugs. Adapted from (Xiong and Lahita, 2014).

4. Toll-like receptors (TLRs)

Innate immune responses are initiated by PRRs, which recognize microbial components that are essential for the survival of the microorganisms. Among the PRRs, Toll-like receptors (TLRs) are capable of sensing various microrganisms like bacteria, fungi, protozoa, and viruses, and they play a major role in innate and adaptive immunity (Hopkins and Sriskandan, 2005). The first receptor belonging to the family of TLRs was discovered in *Drosophila melanogaster*. Initially, its only identified function was its essential role for the formation of the dorsoventral axis of the fly embryo (Hashimoto et al., 1988). In the early 1990's, it was revealed that Toll had a cytosolic portion homologous to that of IL-1R (Bird et al., 1988; Dower et al., 1985; Gay and Keith, 1991), the active receptor for IL-1, a cytokine that plays a very significant role in several immune responses, mediating a variety of local and systemic effects. In 1996, Toll was found to be important for the antifungal defense of the adult fly by Hoffman's group (Lemaitre et al., 1996). In 1997, it was showed by Janeway's group that Toll has a homologue in humans (Medzhitov et al., 1997) and one year later, Beutler's group revealed a role for mammalian TLR4 in the specific detection of lipopolysaccharide (LPS), a constituent of the external membrane of Gram-negative bacteria (Poltorak et al., 1998a; Poltorak et al., 1998b).

TLRs are type I integral membrane glycoproteins composed of an extracellular, a transmembrane and an intracellular signaling domain (Gay and Gangloff, 2007). The extracellular N-terminal domain is specialized in the ligand recognition and consists of 16-28 leucine-rich repeats (LRRs), each of which consisting in 20-30 amino acids with the conserved motif "LxxLxLxxN" (Kajava, 1998; Kobe and Kajava, 2001). The intracellular C-terminal is known as the Toll/IL-1 receptor (TIR) domain and shows homology with that of the IL-1 receptor. This domain is required for the interaction and recruitment of various adaptor molecules that lead to the activation of downstream signaling pathways.

Until today, 13 mammalian TLRs have been identified (Hopkins and Sriskandan, 2005). They are expressed by APCs, lymphocytes and non-immune cells and their signaling leads to the secretion of cytokine and chemokines. So far, 10 and 12 functional TLRs have been identified in humans (TLR1-TLR10) and mice (TLR1-

TLR9, TLR11-TLR13), respectively, with TLR1–TLR9 being conserved in both species. Mouse TLR10 is not functional because of a retrovirus insertion, and TLR11, TLR12 and TLR13 are not present in the human genome (Kawai and Akira, 2010). Studies of mice deficient in each TLR as well as studies on humans have demonstrated that each TLR has a distinct function in terms of PAMP recognition and immune responses (Akira et al., 2006).

The recognition of PAMPs by TLRs occurs in various cellular compartments, including the plasma membrane, endosomes, lysosomes and endolysosomes (Akira et al., 2006). The proper cellular localization of TLRs is thought to be important for ligand accessibility and, maintenance of tolerance to self-molecules such as nucleic acids (Diebold et al., 2006). TLRs function as homodimers or heterodimers. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the cell surface and recognize mainly components from bacteria (**Figure 3**). TLR3, TLR7, TLR8, TLR9 and TLR11, TLR12 and TLR13 are expressed in intracellular vesicles such as endosomes, lysosomes, endolysosomes and endoplasmic reticulum (**Figure 3**). Intracellular TLRs span the endosomal membrane with the ligand-binding domains inside the lumen and the signaling domains in the cytoplasm. They mainly function as homodimers and recognize nucleic acids, from viral genomic nucleic acids and/or replication intermediates (Blasius et al., 2010; Diebold, 2010).

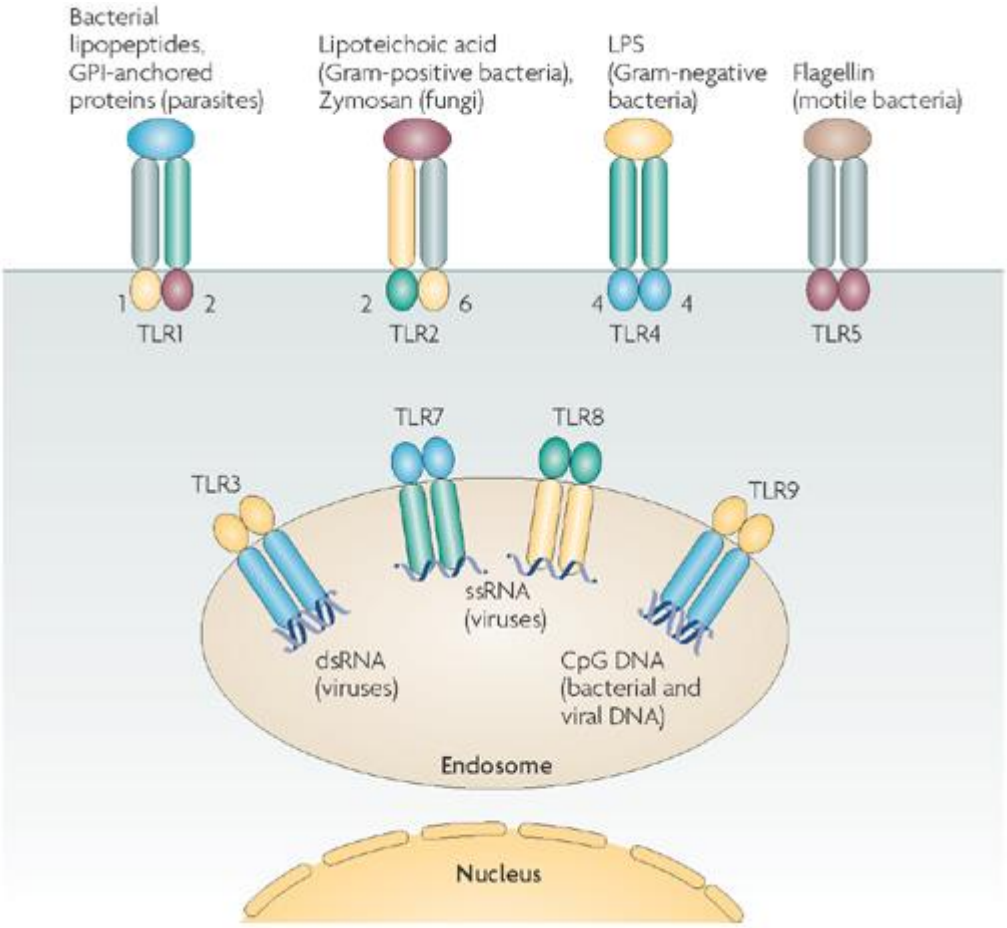

Figure 3: Mammalian TLRs and their ligands (Borden et al., 2007).

Despite the wide range of ligands recognized by TLRs, the receptors share a common structural framework in their extracellular, ligand-binding domains (Botos et al., 2011). These domains adopt horseshoe- or ring-shaped structures built from leucine-rich repeat motifs. Typically, upon ligand binding, two extracellular domains form an ‘‘m’’-shaped dimer sandwiching the ligand molecule (**Figure 4**) and bringing the transmembrane and cytoplasmic domains in close proximity to trigger a downstream signaling cascade. Although the ligand-induced dimerization of these receptors has many common features, the nature of the interactions of the TLR extracellular domains with their ligands varies markedly between TLRs. Clarification of the crystal structure of several TLR ectodomains has provided contributions to understanding the ligand recognition and signaling mechanisms of TLRs (Jin and Lee, 2008). PAMPs recognized by TLRs include lipids, lipoproteins, proteins and nucleic acids derived

from a wide range of microbes such as bacteria, viruses, parasites and fungi (Akira et al., 2006).

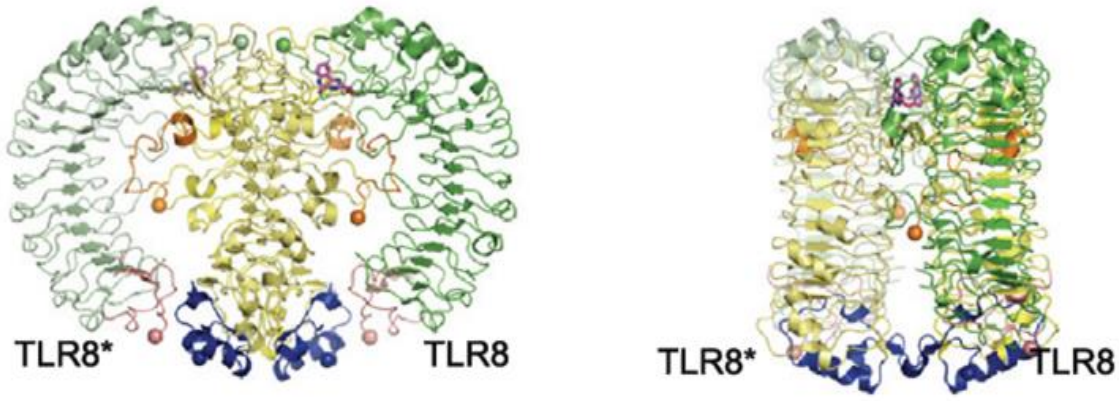

Figure 4: Structure of TLR8 activated dimer complex with the ligand CL097 in front (left panel) and side view (right panel). Upon binding of agonistic ligands, the TLR8 dimer undergoes a large structural rearrangement whereby the ligand molecule penetrates the dimerization interface of the unliganded form. In green, orange and blue color, TLR8 dimer; in magenta, CL097 (Tanji et al., 2013).

4.1 Extracellular TLRs

TLR2 is involved in the recognition of lipopeptides, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, and the hemagglutinin protein from measles virus (Aliprantis et al., 1999; Li et al., 2013). TLR2, **TLR1** and **TLR6** are closely related and form TLR2-TLR1 and TLR2-TLR6 heterodimers that are responsible for the subtle discrimination between lipoproteins (Kang et al., 2009). Moreover, TLR2 has the ability to act together with other cell surface coreceptors, such as CD36, which acts together with the TLR2-TLR6 heterodimer to mediate the sensing of some TLR2 agonists (Hoebe et al., 2005; Triantafilou et al., 2006). The TLR2-TLR1 heterodimer recognizes Gram-negative bacteria and mycoplasma, and also the outer-surface lipoprotein

(OspA) of the pathogen *Borrelia burgdoferi* both in mice and humans (Alexopoulou et al., 2002; Takeuchi et al., 2002). TLR2-TLR6 heterodimer recognizes Gram-positive bacteria and mycoplasma. Respiratory infection models of $TLR2^{-/-}$ mice with *Mycoplasma* and *Mycobacterium* showed increased bacterial burden, tissue damage, and death (Yim et al., 2006). The potentially protective role for TLR2 in mycobacterial infection was confirmed in humans, where TLR2 polymorphisms that decrease TLR2 expression were found to predispose people to tuberculosis (Yim et al., 2006) and non-tuberculosis mycobacterial lung disease (Yim et al., 2008). The importance of TLR2 in the host defense against Gram-positive bacteria has been demonstrated using $TLR2^{-/-}$ mice, which were found to be highly susceptible to challenge with *Staphylococcus aureus* or *Streptococcus pneumonia* (Echchannaoui et al., 2002; Takeuchi et al., 2002).

TLR4 detects bacterial lipopolysaccharide (LPS), a component of the Gram-negative bacteria cell wall that can cause septic shock (Poltorak et al., 1998a)**.** For LPS recognition, a complex formation of TLR4, MD2, CD14 and LBP is necessary (Shimazu et al., 1999). TLR4 is also involved in the recognition of respiratory syncytial virus fusion proteins, mouse mammary tumor virus envelope proteins, pneumolysin from *Streptococcus pneumoniae* and the plant-derived cytostatic drug paclitaxel (Kawai and Akira, 2010), although structural insights into the interaction between TLR4 and these ligands have not yet been provided. $TLR4^{-/-}$ mice are more susceptible to infection by protozoa *Leishmania major* and disseminated candidiasis (Kropf et al., 2004; Netea et al., 2002). Defect in TLR4 gene on mice leads to high susceptibility to Gram-negative bacteria such as *Salmonella typhimurium* (Hoshino et al., 1999; Poltorak et al., 1998b). In humans, mutations in the extracellular domain of TLR4 have been identified and studied for their association with infectious and inflammatory diseases, as with increased susceptibility to Gram-negative bacteremia and septic shock with Gram-negative organisms (Agnese et al., 2002; Lorenz et al., 2002).

Mammalian **TLR5** recognizes bacterial flagellin, a principal component from the flagella bacteria (Feuillet et al., 2006; Hayashi et al., 2001; Uematsu et al., 2006). Unlike other TLRs, TLR5 is expressed mainly on intestinal $CD11c^+$ lamina propria cells (LPCs). $CD11c^+$ LPCs detect pathogenic bacteria and secrete proinflammatory cytokines in a TLR5-dependent way (Uematsu et al., 2006). Moreover, TLR5 cooperates with TLR4 for the recognition and immune response against *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Feuillet et al., 2006).

A ligand for human **TLR10** has not been described yet. In mouse this receptor is disrupted by the insertion of an endogenous retrovirus. But in humans it is functional and similar to TLR1, TLR2 and TLR6 (Chuang and Ulevitch, 2001). TLR10 is not only able to homodimerize but also heterodimerized with TLR1 and TLR2. TLR10 is highly expressed in B cells from peripheral blood, plasmacytoid dendritic cells from tonsil and in a $CD1a^+$ DC subset that resembles the Langerhans cells of the epidermis (Hasan et al., 2005). Some studies identified TLR10 as a key mediator of the inflammatory response to *Listeria monocytogenes* in intestinal epithelial cells and macrophages and also in innate immune responses following viral infection (Lee et al., 2014; Regan et al., 2013).

4.2 Intracellular TLRs

TLR3 recognizes viral double-stranded (dsRNA) and the synthetic analog called polyinosinic-polycytidylic acid (poly I: C) (Alexopoulou et al., 2001; Schulz et al., 2005). In mouse studies, it was demonstrated that TLR3 can participate either as a protector against viral infection or as an infection aggravator. In infections with West Nile virus, a peripheral inflammatory response is initiated through TLR3, leading to disruption of the blood–brain barrier, which enables virus entry into the brain. As a consequence $TLR3^{-/-}$ mice are more resistant to lethal West Nile virus infection (Wang et al., 2004). In humans, TLR3 is vital for natural immunity to Herpes simplex virus (HSV-1). HSV-1 is a highly prevalent neurotropic virus that infects the central

nervous system and can generate herpes simplex encephalitis in children with inborn errors of TLR3 immunity (Zhang et al., 2007). To date, TLR3 is the only TLR that has been shown to play a non-redundant role for protection against viral infection in the central nervous system (Zhang et al., 2007).

Genes coding for **TLR7** and **TLR8** have a strong homology to each other and both in human and mice are located on the X chromosome 70 kb apart from each other. Single-stranded RNA (ssRNA) has been identified as the natural ligand of TLR7 and human TLR8 (Diebold, 2008; Diebold et al., 2004; Lund et al., 2004). They can also recognize small chemical compounds with antiviral and antitumor activity (**Figure 5**), such as imiquimod (R837), resiquimod (R848), CL097, loxoribine, bropirimine and CL075 (Heil et al., 2003; Hemmi et al., 2002; Lee et al., 2003).

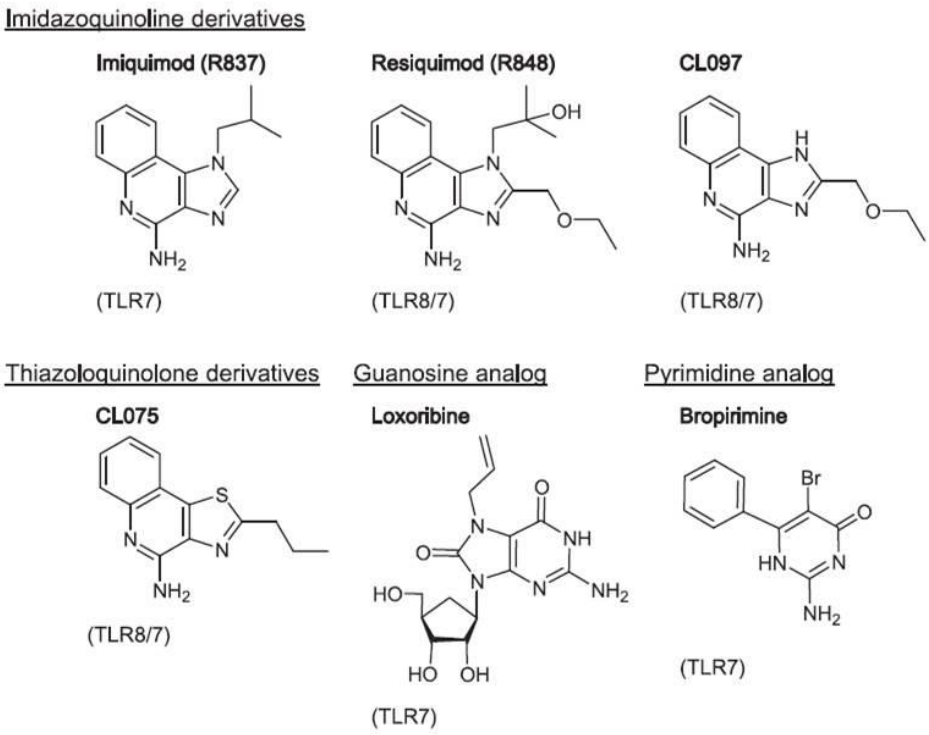

Figure 5: Chemical structures of synthetic TLR7 and TLR8 agonists.

TLR7 has been identified as a sensing receptor for several members of the retrovirus family, including HIV-1, foamy viruses, and murine gammaretroviruses (Beignon et al., 2005; Browne, 2011; Kane et al., 2011) . TLR7 also recognizes Influenza A and vesicular stomatitis viruses (Lund et al., 2004). It was show that $TLR7^{-/-}$ mice are more susceptible to *Pneumovirus* infection and predisposed to asthma pathology (Kaiko et al., 2013). *In vitro* studies have shown that retroviral particles stimulate secretion of some cytokines from myeloid and plasmacytoid dendritic cells in a TLR7-dependent manner (Beignon et al., 2005; Breckpot et al., 2010). HIV-1-derived ligands can contribute to the immune activation of NK cells and may play an important role in HIV-1 immunopathogenesis during acute and chronic viremic HIV infection (Alter et al., 2007). Nevertheless, the impact of TLR7 on HIV in vivo is unclear.

Human TLR8 mediates the recognition of viral ssRNA and various chemical compounds (Akira et al., 2006; Kawai and Akira, 2006), but not mouse TLR8. A study with HEK293 cells transfected with TLRs and a NF-κB luciferase reporter plasmid showed that human TLR8 could activate NF-κB after stimulation with R848 but not murine TLR8 (Jurk et al., 2002) . For this reason it was thought that murine TLR8 was nonfunctional in mouse. Some studies suggested that murine TLR8 could be activated by the imidazoquinoline 3M-002 (CL075) simultaneously with polyT oligonucleotides (Gorden et al., 2006), or can act as a supressor of neurite outgrowth (Ma et al., 2006) or recognize vaccinia virus (Martinez et al., 2010). However these studies remain controversial or have been not confirmed by other groups (Bauer et al., 2010).

Later, a study was performed to compare the activation of TLR8 from different species including mouse, rat, human, bovine, porcine, horse, sheep, and cat by ligand recognition. Only the TLR8 from rodent species (mouse and rat TLR8s) failed to respond to ligand stimulation in the absence of polyT-ODN (Liu et al., 2010). Multiple sequence alignment analysis suggested that these two rodents TLR8 lack a five-amino-acid motif that is conserved in the non-rodent species with varied sequence (Ohto et al., 2014). Deletion mutation analysis suggested that this motif is

essential for the species-specific ligand recognition of hTLR8, but not required for self-dimerization and intracellular localization of this receptor (Ohto et al., 2014).
Despite the general belief that mouse TLR8 is not functional, previous studies in our laboratory revealed an important role for mouse TLR8 as a regulator of TLR7 expression and signaling (Demaria et al., 2010). By generating and studying TLR8 $^{-/-}$ mice, our group showed that lack of TLR8 in mice leads lupus development due to increased expression and signaling of TLR7 by dendritic cells. (Demaria et al., 2010).

TLR9 recognizes unmethylated 2′-deoxyribo (cytidine-phosphateguanosine) (CpG) DNA motifs present in bacteria and viruses, but rare in mammalian cells (Hemmi et al., 2000; Lee et al., 2007). Recently, it was described that TLR9 senses RNA: DNA hybrids, a novel molecular pattern formed from intracellular replication intermediates generated during infection (Rigby et al., 2014). Hemozoin, a product formed from the digestion of blood by Plasmodium parasite, has been described as the first non-DNA ligand for TLR9 (Coban et al., 2005). DNA viruses such as murine cytomegalovirus (MCMV) and herpes-simplex viruses 1 and 2 (HSV-1/HSV-2) are recognized by TLR9 and induce production of inflammatory cytokines and type I IFN (Hochrein et al., 2004; Krug et al., 2004). The TLR9-mediated IFN-α response to HSV-1 and HSV-2 is limited to a subtype of dendritic cells, called plasmacytoid dendritic cell (pDCs) or natural interferon-α producing cells, which secrete high amounts of IFN-α in response to viral infection (Hochrein et al., 2004; Krug et al., 2004). Interestingly, one study showed that TLR9 activation is critical for the production of IFN-γ during infection with *Propionibacterium acnes* that is part of the human flora and associated with several human pathologies (Kalis et al., 2005). TLR9 dependent activation via *P. acnes* primes IFN-γ dependent enhanced resistance to murine typhoid fever, which is abolished in TLR9 deficient mice (Kalis et al., 2005). It was shown also that TLR9$^{-/-}$ mice are more susceptible to *Klebsiella pneumonie* infection (Bhan et al., 2007) .

TLR11 in the mice is highly expressed in the kidney and bladder (Lauw et al., 2005). Yarovinsky and colleagues demonstrated that a profilin-like molecule from *Toxoplasma gondii* activates TLR11, and this TLR has an important role during

infection in mice (Yarovinsky et al., 2005). Later it was demonstrated that TLR11 recognizes flagellin from both the uropathogenic *Escherichia coli* and *Salmonella spp* (Mathur et al., 2012). Interestingly, in addition to the nucleic acid–sensing TLRs, the protein-sensing receptor TLR11 interacts with UNC93B1, a chaperone-like protein indispensable for the proper localization and function of all RNA- and DNA-sensing TLRs and localizes to the endolysosomal compartment (Kim et al., 2008), which will be discusses later. Deficiency in UNC93B1 protein abolishes TLR11-dependent responses both *in vitro* and *in vivo* (Pifer et al., 2011).

Similar to TLR11, **TLR12** is an endolysosomal innate immune receptor that colocalizes and interacts with UNC93B1 (Lee et al., 2013). Biochemical experiments revealed that TLR11 and TLR12 directly bind to *Toxoplasma gondii* profilin and are capable of forming a heterodimer complex (Raetz et al., 2013). TLR12 is sufficient for recognition of *T. gondii* profilin by plasmacytoid dendritic cells (pDCs), whereas TLR11 and TLR12 are both required in macrophages and conventional DCs. In contrast to TLR11, TLR12-deficient mice succumb rapidly to T. gondii infection (Koblansky et al., 2013). TLR12-dependent induction of IL-12 and IFN-α in pDCs leads to production of IFN-γ by NK cells. Consistent with this observation, the partial resistance of $TLR11^{-/-}$ mice is lost upon pDC or NK cell depletion (Koblansky et al., 2013). Thus, TLR12 is critical for the innate immune response to *T. gondii*, and this TLR may promote host resistance by triggering pDC and NK cell function

TLR13 is a murine receptor that recognizes bacterial 23S ribosomal RNA, since small interfering RNA (siRNA) against TLR13 reduced cytokine induction by bacterial RNA in DCs (Hidmark et al., 2012). Moreover, Chinese hamster ovary cells transfected with TLR13 can activate NF-κB in response to bacterial RNA or *Streptococcus pyogenes* in an RNA-specific manner (Hidmark et al., 2012).

4.3 Adaptors in TLR signaling

The molecular adaptors downstream of TLRs are: MyD88 (Myeloid differentiation primary-response gene 88), TIRAP (TIR associated protein) also known as MAL (MyD88 adaptor like protein), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule), sterile and armadillo-motif-containing protein (SARM), which is a negative regulator of TRIF; and B cell adaptor for PI3K (BCAP), a newly identified inhibitory adaptor (Ni et al., 2012; O'Neill and Bowie, 2007; Troutman et al., 2012). Their recruitment leads to activation of various signaling pathways (**Figure 6**). MyD88 was the first identified member of the TIR family (Hultmark, 1994). It is universally used by all TLRs, except TLR3, and activates the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce the expression of inflammatory cytokines (Akira et al., 2006; Warner and Nunez, 2013). TRIF is used by TLR3 and TLR4 and induces alternative pathways that lead to activation of the transcription factors IRF3 and NF-κB, and consequently the induction of type I interferon and inflammatory cytokines. TRAM and TIRAP function as sorting adaptors that recruit TRIF to TLR4 and MyD88 to TLR2 and TLR4, respectively. Thus, TLR signaling pathways can be largely classified as either MyD88-dependent pathways, which drive the induction of inflammatory cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I interferons as well as inflammatory cytokines (Akira et al., 2006; Kawai and Akira, 2010).

TLR4 is the only TLR that uses all four adaptors and activates both the MyD88- and TRIF-dependent pathways (**Figure 6**). TLR4 initially recruits TIRAP at the plasma membrane and subsequently facilitates the recruitment of MyD88 to trigger the initial activation of NF-κB and MAPK (Jin and Lee, 2008; Kawai and Akira, 2006). TLR4 subsequently undergoes endocytosis and is trafficked to the endosome, where it forms a signaling complex with TRAM and TRIF to initiate the TRIF dependent pathway that leads to IRF3 activation as well as the late-phase activation of NF-κB and MAPK (Kawai and Akira, 2006; Rowe et al., 2006; Tanimura et al., 2008). Thus, TLR4 activates the MyD88-dependent pathway earlier than the TRIF-dependent pathway.

The activation of both the MyD88- and TRIF-dependent pathways is necessary for the induction of inflammatory cytokines via TLR4 signaling, which is in contrast to other TLRs, for which activation of either the MyD88- or the TRIF-dependent pathway is sufficient for the induction of inflammatory cytokines.

4.3.1 The MyD88-dependent pathway

After the activation of TLRs, MyD88 recruits the IL-1 receptor–associated kinases IRAK4, IRAK1, IRAK2 and IRAK-M (**Figure 6**). IRAK4 is activated initially and has an essential role in the activation of NF-κB and MAPK downstream of MyD88 (Akira et al., 2006; O'Neill et al., 2013). IRAK1 and IRAK2 are activated sequentially, and activation of both kinases is required for robust activation of NF-κB and MAPK. IRAK activation results in an interaction with TRAF6, which is an enzyme that activates TAK1 through polyubiquitination. TAK1 can signal through two different pathways. It can phosphorylate and activate the IKK complex, which is composed of the kinases IKKα, IKKβ and the scaffold protein NEMO. This complex phosphorylates the inhibitor of NF-kB, IκB, resulting in the polyubiquitination, and proteasome mediated degradation of the inhibitor, which frees nuclear translocation the NF-κB transcription factor dimers for nuclear translocation and target gene expression(Bhoj and Chen, 2009). Polyubiquitination of NEMO by the complex constituted by HOIL-1L and HOIP have been shown to be an important process for IKK activation (Tokunaga et al., 2009). TAK1 simultaneously activates the MAPKs by inducing the phosphorylation of MAPK kinases, which then activate various transcription factors, including AP-1. Activation of the MyD88-dependent pathway results in the induction of many genes, and some of these have critical roles in modulating NF-κB-dependent transcription (**Figure 6**) to facilitate IL-6 and IL-12p40 induction (Yamamoto et al., 2004).

4.3.2 The TRIF-dependent pathway

The TRIF-dependent pathway culminates in the activation of both NF-κB and IRF3, leading to the production of inflammatory cytokines and type-I interferon (**Figure 6**). TRIF forms a multiprotein signaling complex along with TRAF6, TRADD, Pellino-1 and RIP1 for the activation of TAK1, which in turn activates the NF-κB and MAPK pathways. TRIF also recruits a signaling complex involving the noncanonical IKKs TBK1 and IKKi (IKKε), which catalyze the phosphorylation of IRF3 and induce its nuclear translocation (Hacker and Karin, 2006). The activation of TBK1-IKKi by TRIF requires TRAF3. TRAF3 is also incorporated into the MyD88 complex during TLR4 signaling. However, this exposes TRAF3 to K48-linked ubiquitination and degradation via cIAP1 and cIAP2, which are both components of the MyD88 signaling complex but not of the TRIF signaling complex. TRAF3 degradation results in translocation of the membrane-proximal signaling complex to the cytoplasm, which leads to TAK1 activation (Tseng et al., 2010). These findings suggest that TRAF3 promotes IRF3 activation as well as inhibits the MyD88-dependent pathway.

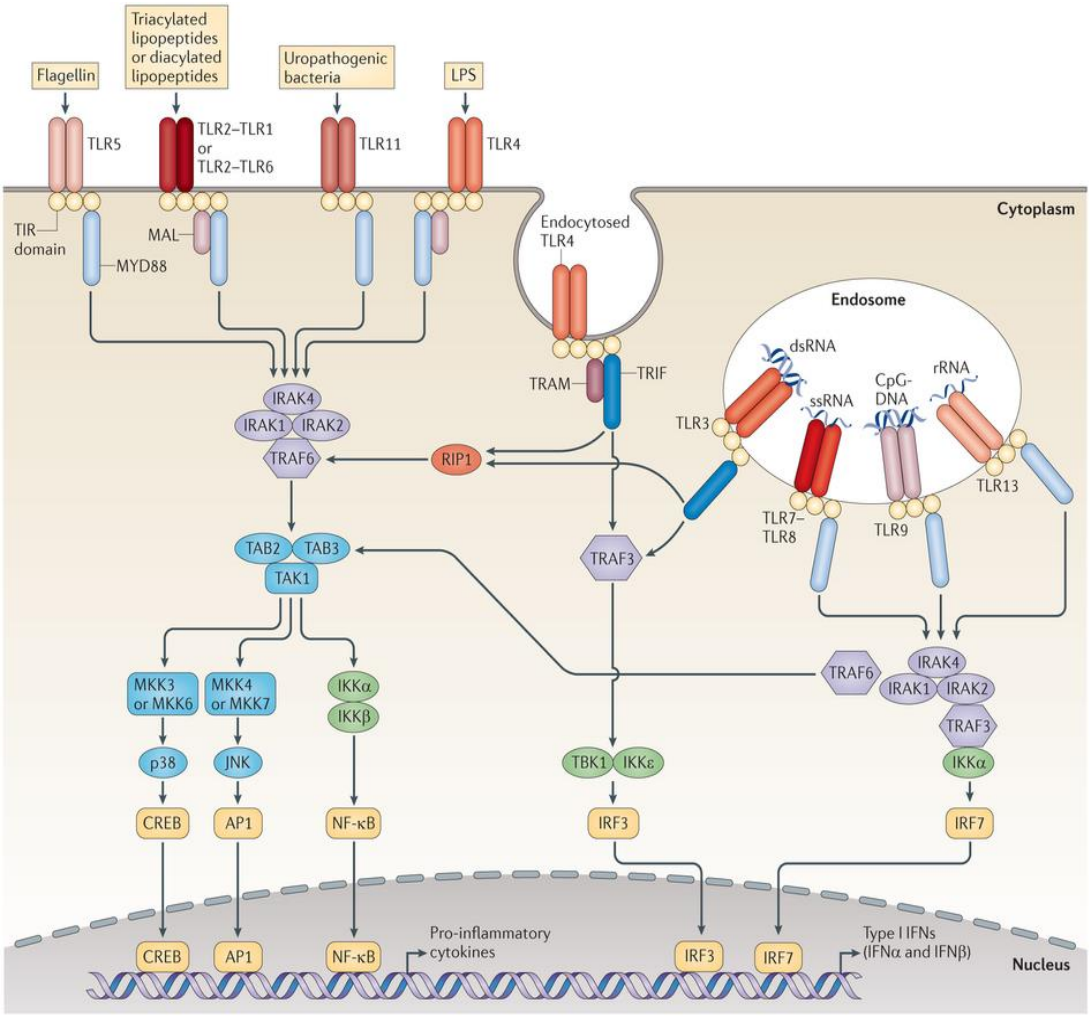

Figure 6: The TLR signaling pathways (O'Neill et al., 2013). TLR11 is represented as extracellular TLR, but later it was described as an endosomal TLR.

4.4 Recognition, location and trafficking of intracellular TLRs

The intracellular TLRs capable of nucleic acid recognition localize within endosomal compartments, which should be away from self-nucleic acids in the extracellular space (Barton and Kagan, 2009; Barton et al., 2006). While access of the cell surface TLRs to their extracellular ligands appears to be less complex, the ligands for the intracellular TLRs must be transported into the endolysosomal compartments in

which these TLRs reside. How the ligands reach the intracellular TLRs is not yet very well understood. The intracellular TLRs can be activated by the addition of their ligands to the extracellular medium, indicating that cells can transport nucleic acids from outside the cell into the TLR-containing compartments. Whether internalization is by phagocytosis or pinocytosis or even by receptor mediated endocytosis is not known. For TLR9, it was shown that responses to exogenously added CpG DNA were dependent on both endocytosis and on endosomal maturation (Hacker et al., 1998). CpG DNA is internalized and colocalized with early endosomal markers (Ahmad-Nejad et al., 2002; Hacker et al., 1998). Blocking endolysosomal compartment acidification by drugs, blocks intracellular TLR signaling suggesting that either the intra-compartment pH or the ability of the TLR containing compartments to fuse with other vesicles is necessary for TLR function (Ahmad-Nejad et al., 2002). CpG DNA has also been shown to be internalized from the extracellular medium via scavenger receptors on the cell surface. One such cell surface receptor that has been described to mediate the internalization of CpG DNA and subsequent activation of TLR9 in plasmacytoid dendritic cells (pDC) is termed the receptor for advanced glycation endproducts (RAGE) (Tian et al., 2007). RAGE associates to high mobility group protein 1 (HMGB1) that binds directly to CpG DNA. The internalization of HMGB1-CpG DNA complexes by RAGE results in the activation of the pDCs (Tian et al., 2007). How general the use of RAGE is in CpG DNA internalization by immune cells remains to be determined. It also remains to be determined if there are additional scavenger receptors for other intracellular TLR ligands.

At present, little is known about the mechanisms by which TLR agonists derived from viruses or bacteria that replicate within the cell reach the intracellular TLRs. For TLR7, recent evidence suggests that autophagy may play a role in providing the viral ssRNA and replication intermediates to TLR7-containing compartments (Into et al., 2012; Lee et al., 2007). Autophagy involves the sequestration of cytoplasmic material into membrane-enclosed compartments termed autophagosomes. The content of the autophagosomes is delivered to the lysosomes for degradation by direct fusion of autophagosomes with the lysosomes. It is proposed that during this sequestration

process TLR ligands, including viral nucleic acids and their replication intermediates, gain access to autophagosomes for subsequent delivery to TLRs in the endolysosomal compartments (Into et al., 2012; Lee et al., 2007). In addition, recent reports suggest that various TLR ligands stimulate autophagy in macrophages and DCs (Delgado et al., 2008; Sanjuan et al., 2007). However, it remains unclear if TLR ligand-induced autophagy is a general phenomenon to promote the trafficking of pathogenic nucleic acids to the TLRs.

The intracellular location of the TLRs within endosomes also suggests a possible regulatory mechanism to modulate ligand binding by controlling the microenvironment within the endosome. The subcellular localization of TLRs is important for its specificity and, in particular, for the discrimination of microbial from self DNA (Barton et al., 2006; Brencicova and Diebold, 2013). It was shown that intracellular localization of TLR9 is determined by its transmembrane α-helix. A chimeric receptor composed of the TLR9 ectodomain and the TLR4 transmembrane and cytoplasmic domain can traffic to cell surface. These chimeric TLRs responded to self, mammalian DNA and developed inflammation, whereas native TLR9 that was present in intracellular compartments responded only to microbial CpG-containing DNA. This study suggested that the ability of TLR9 to discriminate between microbial and self DNA is location dependent (Barton et al., 2006).

Endosomal TLRs require proteolytic processing in the endolysosome compartment to initiate signaling in response to ligands, and this processing was suggested as a mechanism of tight control of TLR signaling (Ewald et al., 2011; Manoury, 2013). Proteases from asparagine endopeptidase (AEP) or cathepsin family members cleave the ectodomains of TLR3, TLR7 and TLR9 in the endolysosome, leading to the activation of adaptors signaling (Sepulveda et al., 2009). Notably, although both the full-length and cleaved forms are capable of binding ligand, only the processed form recruits the signaling adaptors on activation (Ewald et al., 2011; Ewald et al., 2008; Garcia-Cattaneo et al., 2012; Park et al., 2008).

The finding that blockade of endolysosome acidification prevents TLR3-, TLR7- and TLR9-induced responses suggests that the delivery of internalized nucleic acids to the endolysosomes is pivotal to interaction with these TLRs. TLR9 and other intracellular

TLRs are exclusively sequestered in the ER in unstimulated cells and they need to be rapidly trafficked to endolysosomes after ligand stimulation. One protein responsible for this trafficking is called UNC93B1, which is a multi-pass transmembrane protein localized in the ER (Brinkmann et al., 2007; Kim et al., 2005). Mice homozygous for a nonfunctional UNC93B1 (H412R) allele (*Unc93b1*3d/3d) fail to respond to TLR3, TLR7, or TLR9 ligands, and mice and humans deficient in UNC93B1 are highly susceptible to viral infection (Casrouge et al., 2006; Lafaille et al., 2012; Tabeta et al., 2004). Later UNC93B1 was also implicated in the function of others intracellular TLRs such as TLR8, TLR11, TLR12 and TLR13 (Oldenburg et al., 2012; Pifer et al., 2011; Tabeta et al., 2006).

Reports suggest that endosomal TLR trafficking may be influenced at both ER and post-Golgi trafficking steps. Molecules like Gp96 functions as an ER folding chaperone for many TLRs while PRAT4A has been implicated in TLR trafficking from the ER (Lee et al., 2012; Takahashi et al., 2007; Yang et al., 2007). Additionally, the HRS/ESCRT pathway is involved in post-Golgi trafficking by sorting ubiquitinated TLR7 and TLR9 to endosomal compartments (Chiang et al., 2012). The adaptor protein-3 (AP-3) has been reported to target TLR9 and TLR7 to lysosome related organelles specialized for type I IFN induction (Blasius et al., 2010; Honda et al., 2005; Sasai et al., 2010).

UNC93B1 plays a direct role in facilitating exit of TLRs from the ER as well as a later role in recruitment of adaptor protein-2 (AP-2) to facilitate endocytosis of TLR9 from the plasma membrane. TLR7 does not have the same requirements as TLR9; it utilizes distinct trafficking machinery to reach endolysosomes (**Figure 7**). UNC93B1 enters the secretory pathway and directly controls the packaging of TLRs into COPII vesicles that bud from the ER (Lee et al., 2013). Unlike other COPII loading factors, UNC93B1 remains associated with the TLRs through post-Golgi sorting steps. Unexpectedly, these steps are different among endosomal TLRs. TLR9 requires UNC93B1-mediated recruitment of adaptor protein complex 2 (AP-2) for delivery to endolysosomes while TLR7, TLR11, TLR12, and TLR13 utilize alternative trafficking pathways (**Figure 7**) (Lee et al., 2013).

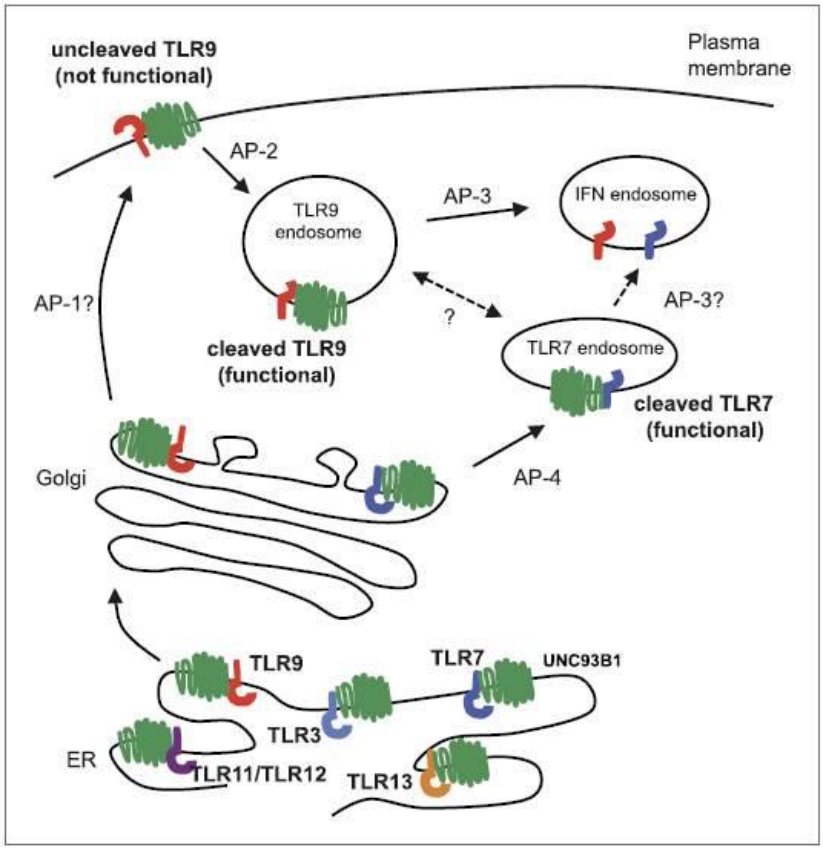

Figure 7: Trafficking pathways controlling localization of endosomal TLRs. UNC93B1 interacts with TLRs in the ER and facilitates loading into COPII vesicles. UNC93B1 remains associated with TLR9 and TLR7 after exit from the ER. Through its recruitment of AP-2, UNC93B1 is necessary for endocytosis of TLR9 from the plasma membrane into endosomes. TLR7 uses AP-4 to traffic directly to endosomes (Lee et al., 2013).

4.5 Negative regulators of TLRs

After TLR activation and establishment of the inflammatory response, there must be a checkpoint where TLR signaling is abolished and the system returns to a normal physiological state to avoid harmful response towards the host immune system. To avoid inappropriate inflammatory responses, negative regulatory mechanisms exist to attenuate TLR signaling and to maintain a balance between activation and inhibition

of this pathway. Some of TLR regulators of the MyD88-dependent pathway are represented in **Figure 8** can lead to persistent inflammation *in vivo*, if disrupted or mutated (Carty et al., 2006; Sheedy et al., 2010; Shi et al., 2008; Taganov et al., 2006; Tanaka et al., 2007) . So far, many negative regulators that suppress TLR signaling pathways at multiple levels have been identified and they can act through three major different mechanisms (Cadwell et al., 2008; Carrick et al., 2004; Croker et al., 2008; Hampe et al., 2007):

- Dissociation of adaptor complexes: the TIR domain adaptor proteins have several variants that act as antagonists to prevent association among intact forms of adaptors and block downstream signaling pathways. Among these regulators, A20 has been well studied in relation to autoimmune disordes such as inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus. A20 functions as a break on the immune system by regulating the termination of a number of proinflammatory signals upon TLR activation. $A20^{-/-}$ mice present multiorgan inflammation that is MyD88 dependent (Skaug et al., 2011).

- Degradation of signal proteins: mediated by ubiquitination and autophagy systems. Unlike in the case of dissociation of adaptor complexes, this mechanism is irreversible. Supressor of cytokine signaling (SOCS) proteins of the E3 ubiquitin ligase family are well characterized regulators that promote degradation of TIRAP: MyD88-adaptor-like (MAL) or TRAF proteins (Yoshimura et al., 2007).

- Transcriptional regulation: unlike other mechanisms for TLR inhibition, transcriptional regulation often enables control of a particular subset of TLR target genes without termination of TLR signaling. It can contribute to regulate the balance of immunity beyond suppression of TLR signaling. An example is TLR-inducible IκB protein (IκBNS or IκB delta) that negatively regulates induction of genes such as IL-6 and IL-12p40, but not TNF-α. It is selectively recruited to the IL-6 promoter and controls NF-kB activation. Mice

deficient for IκBNS are highly susceptible to LPS-induced endotoxin shock and intestinal inflammation (Kuwata et al., 2006).

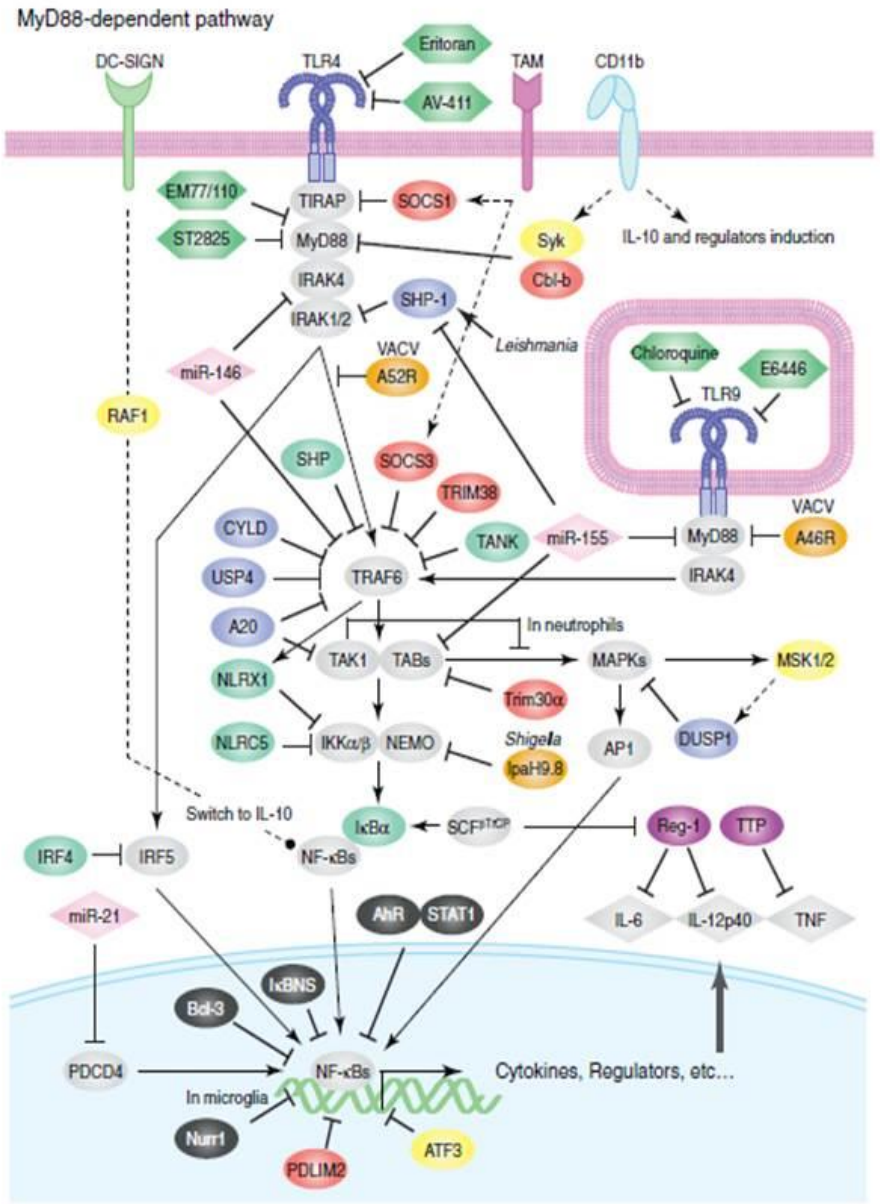

Figure 8: MyD88-dependent TLR pathway and its negative regulators. In Gray, signal molecule; lime green, competitor; red, ubiquitin ligase or autophagy protein; blue, deubiquitinase or phosphatase; black, transcriptional regulator;

purple, regulator of RNA stability; pink, miRNA; brown, pathogen derived; green, artificial antagonist; yellow, other categories (Kondo et al., 2012).

4.6 Endosomal TLRs and SLE

The first indications that TLRs might be involved in autoimmune phenomena came from studies of Leadbetter *et al*., which showed that chromatin-IgG complexes activate B cells by dual engagement of IgM and TLR9 (Leadbetter et al., 2002). Since then, various studies with animal models or humans suggested that improper engagement of TLRs by endogenous or exogenous ligands may lead to the initiation of autoimmunity responses and tissue injury (Papadimitraki et al., 2007).

4.6.1 Implication of endosomal TLRs in mouse models of lupus

SLE is a polygenic disease that results in a number of complex phenotypes. The understanding of disease development and TLR implication over the last decades has benefited from the study of murine models. There are numerous murine models that have long been employed in an effort to understand the cellular and genetic requirements for SLE induction. The classic models of spontaneous lupus include the F1 hybrid between the New Zealand Black (NZB) and New Zealand White (NZW) strains (NZB/W F1), the MRL/lpr, and BXSB/Yaa strains, whereas induced lupus models include the pristane-induced model and the chronic graft-versus-host-disease models (cGVHD) (Perry et al., 2011). All of these models have symptoms similar to those observed in human SLE patients, namely, autoantibody production, lymphoid activation and lupus nephritis.

Based on the findings that TLR9 is activated by SLE sera or immune complexed self-DNA, it was anticipated that the deletion of TLR9 gene in lupus-prone murine models would be beneficial in terms of anti-ANA antibody production and disease progression. However, the first study that depleted TLR9 in the MRL/lpr model

demonstrated that although anti-dsDNA and anti-chromatin autoantibodies were reduced, glomerulonephritis and immune complexes were still present and some disease parameters as lymphadenopathy and splenomegaly were even enhanced, suggesting that TLR9 has a protective role (Christensen et al., 2005; Wu and Peng, 2006). These results were further confirmed in other study with MRL/lpr mice and in another model of SLE, named Ali5, which is a model based on hyper-reactive B cell activation mediated by mutant phospholipase Cg2 (Nickerson et al., 2010; Yu et al., 2006).

The initial interest in TLR7 as a candidate gene for SLE began with the study of BXSB mice strain. These mice in male gender present a severe lupus phenotype with death at 5-6 months of age (Andrews et al., 1978). Later, it was found that this phenotype is due to the Y-linked autoimmune accelerating (Yaa) locus and this locus is a translocation of a cluster of 16 genes from the X on the Y chromosome, including TLR7 (Pisitkun et al., 2006; Subramanian et al., 2006). Once the translocation of TLR7 within the Yaa region had been established, other studies checked the necessity of TLR7 for the disease progression. Using knock-out strategies that diminished TLR7 expression, TLR7 was proven to be important for severe disease development in B6.Sle1Yaa and B6.Nba2/Yaa lupus models (Fairhurst et al., 2008; Santiago-Raber et al., 2008). Two studies using the MRL/lpr model also showed that depletion of TLR7 reduced the gravity of the disease and prevented the development of antibodies or nephritis, confirming the pivotal role of TLR7 in lupus pathogenesis (Christensen and Shlomchik, 2007; Nickerson et al., 2010).

Surprisingly, ablation of TLR3, a receptor for dsRNA, did not inhibit the formation of autoantibodies to either RNA- or DNA-containing antigens and did not alter the development of clinical autoimmune disease or nephritis in the MRL/lpr lupic mice (Christensen et al., 2005). **Table 2** summarizes the *in vivo* implication of TLRs in different mouse models of lupus.

Mouse model*	Genetic background	Effect of deficiency or increase in Toll-like-receptor expression on autoantibody titres‡	Renal disease	Survival
Myd88$^{-/-}$ *lpr/lpr*	Mixed	• HEp-2: no ANAs • Western blotting: no Sm-specific antibodies	ND	ND
Tlr9$^{-/-}$ *lpr/lpr*	Mixed or MRL	• HEp-2: ↓ dsDNA-specific antibodies • *Crithidia*: ↓ dsDNA-specific antibodies • Western blotting and ELISA: ↑ Sm-specific antibodies	Exacerbated	Decreased
	MRL	• ELISA: ↑ DNA-specific antibodies	Exacerbated	Decreased
	C57BL/6	• HEp-2: ↓ dsDNA-specific antibodies; ↑ nucleolus-specific antibodies • ELISA: ↑ DNA-specific antibodies; ↓ nucleosome-specific antibodies	Exacerbated	Decreased
Tlr9$^{-/-}$ *Ali5*	C57BL/6	• HEp-2: ↓ dsDNA-specific antibodies; ↑ nucleolus-specific antibodies • ELISA: ↔ DNA-specific antibodies; ↓ nucleosome-specific antibodies	Exacerbated	ND
Tlr3$^{-/-}$ *lpr/lpr*	Mixed	• No effect	Unchanged	ND
Tlr7$^{-/-}$ *lpr/lpr*	MRL (3–6 backcrosses)	• HEp-2: ↓ speckled nuclear staining by ANAs	Reduced	ND
Tlr7$^{-/-}$ 564Igi	C57BL/6	• ELISA: spontaneous secretion of 564 autoantibodies	ND	ND
Fcgr2b$^{-/-}$*Myd88*$^{-/-}$	C57BL/6	• ELISA: ↓ DNA-, GBM- and cardiolipin-specific IgG2a and IgG2b	Reduced	Prolonged
Fcgr2b$^{-/-}$*Myd88*$^{-/-}$ 56R	C57BL/6	• ELISA: ↓ DNA-, GBM- and cardiolipin-specific IgG2a and IgG2b	ND	ND
Fcgr2b$^{-/-}$*Tlr9*$^{-/-}$ 56R	C57BL/6	• ELISA: ↓ DNA-, GBM- and cardiolipin-specific IgG2a and IgG2b	ND	ND

Table 2: Implication of aberrant expression of TLRs in Mouse models of lupus (Marshak-Rothstein, 2006).

Since TLR9 and TLR7 can be engaged by nucleic acids released by dead cells and participate in the activation of cells and subsequent production of autoantibodies and cytokines, it was a mystery why TLR9 deficiency in mouse lupus models led to more severe disease, whereas TLR7 deficiency led to less severe disease (**Figure 9**).

Previous studies in our group revealed that in the C57BL/6 background (that is not prone to lupus) TLR8$^{-/-}$ mice develop a lupus phenotype, characterized by splenomegaly, increased serum levels of IgM and IgG2a, increased serum levels of autoantibodies against small nuclear ribonucleoproteins, ribonucleoprotein, dsDNA and RNA, as well as IgM, IgG and complement C3 immunodeposits in the kidneys (Demaria et al., 2010). Furthermore TLR8$^{-/-}$ mice presented defective development of marginal zone (MZ) and B1 B cells. The lupus phenotype in TLR8$^{-/-}$ mice was due to increased TLR7 expression and signaling in DCs, suggesting that TLR8 keeps under control TLR7 function and TLR7-mediated lupus (Demaria et al., 2010).

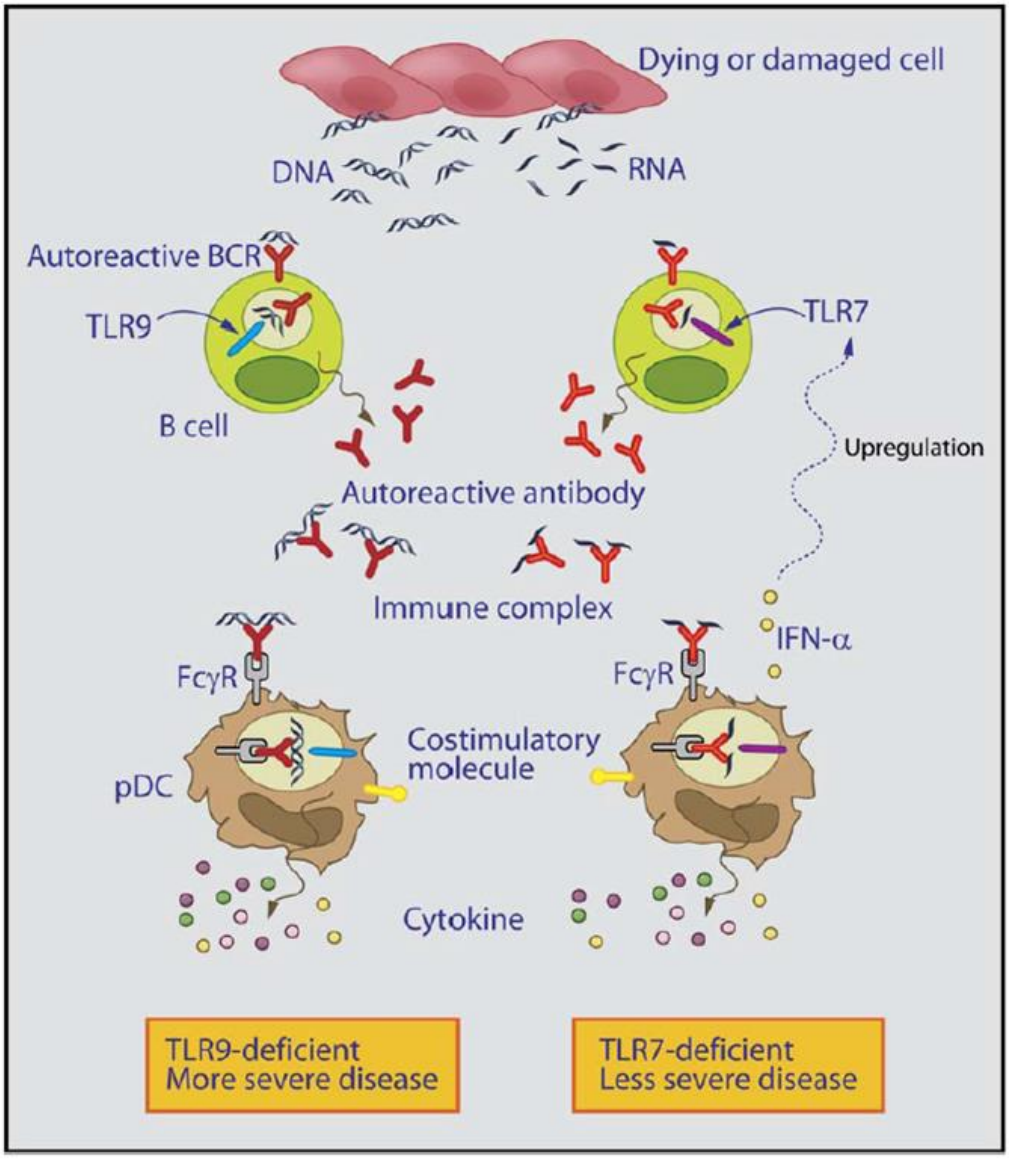

Figure 9: Controversial roles of TLR9- and TL7- deficient mice in the outcome of lupus disease (Marshak-Rothstein, 2006).

Parallel with our studies on TLR8$^{-/-}$ mice it was revealed that TLR9 also keeps under control TLR7. The disease exacerbation in MRL/lpr/TLR9-deficient mice was completely suppressed by the deletion of TLR7 (Nickerson et al., 2010). The enhanced disease in the B6.Nba2 mice deficient for TLR9 was correlated with upregulated expression of TLR7, as documented by an increase of TLR7-dependent

activation of B cells and plasmacytoid dendritic cells (Nickerson et al., 2010; Santiago-Raber et al., 2010).

Control of TLR7-function by TLR8 or TLR9 has been also observed with human TLRs. An *in vitro* study using HEK293 cells transfected with human TLR7, TLR8 or TLR9, revealed that in cells cotransfected with TLR7 and TLR8 or TLR7 and TLR9, TLR7 signaling is reduced compared to cells transfected only with TLR7, suggesting that both human TLR8 and TRL9 control TLR7 signaling (Wang et al., 2006).

Interestingly, the group of Miyake has shown that there is a competition of TLR7 and TLR9 for Unc93B1-dependent trafficking, where TLR9 predominates and has higher affinity than TLR7 for Unc93B1 (Fukui et al., 2009). Indeed, a D34A mutation of Unc93B1 does not affect TLR7 or TLR9 expression, but leads to increased TLR7 trafficking, and mice that carry the mutation Unc93B1-D34A develop TLR7-dependent systemic lethal inflammation due to increased response to TLR7 ligand (**Figure 10**). Thus, in mice both TLR8 and TLR9 keep under control TLR7-function and TLR7-mediated lupus.

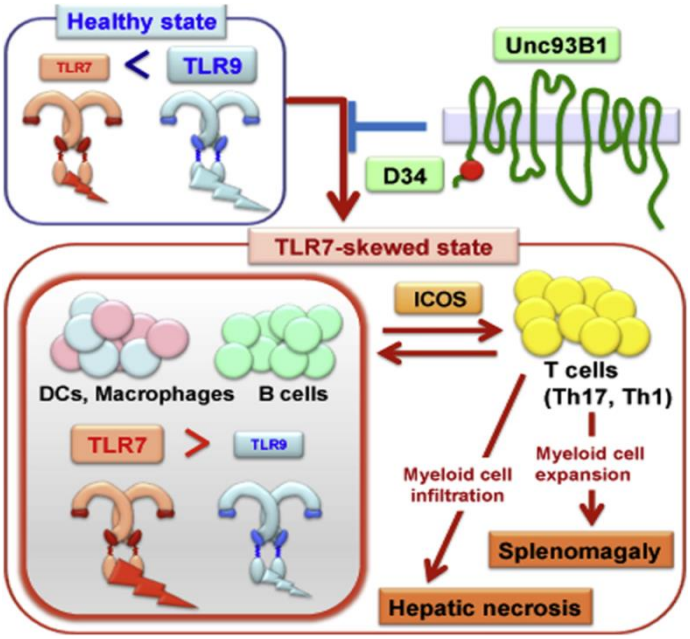

Figure 10: Mice harboring a D34A mutation in Unc93b1 suffer from systemic lethal inflammation due to aberrant trafficking of TLR7 and 9 (Fukui et al., 2011).

4.6.2 Involvement of TLR7 and TLR9 in human SLE

In humans, data incriminate TLR7 as an essential player in SLE pathology. Two independent studies reported that the SNP rs3853839 (C-G) at the 3′ untranslated region of TLR7 is associated with elevated transcript expression and increased risk for SLE in Eastern Asians (Kawasaki et al., 2011; Shen et al., 2010). Interestingly, further studies revealed that the risk G allele is also linked with increased TLR7 protein levels and is located within a predicted binding site of microRNA-3148 that is most likely responsible for the observed association with SLE in three populations of non-Asian ancestry (Deng et al., 2013). Furthermore, increased TLR7 copy number is a risk factor for childhood-onset of SLE in the Mexican population, and correlates significantly with increased TLR7 and IFN-α mRNA levels (Theofilopoulos et al., 2010).

Regarding TLR9, four polymorphisms have been associated with SLE in Japanese, Chinese, or Brazilian patients; however, none of these findings have been confirmed in additional studies (dos Santos et al., 2012; Huang et al., 2012; Ng et al., 2005; Tao et al., 2007). Interestingly, the GC haplotype that combines variants at positions -1174 and -1486 was shown to downregulate TLR9 expression by reporter gene assay, which would recapitulate mouse models where TLR9 deficiency exacerbates disease and suggests a protective role in SLE (Tao et al., 2007). However, SNPrs352140, which was associated with susceptibility in Chinese SLE patients (Xu et al., 2009), has been associated with higher TLR9 expression and increased IgM production in response to CpG in a study investigating primary biliary cirrhosis (Kikuchi et al., 2005). Recently, the C variant at position -1237 in the promoter region of TLR9 was associated with SLE in Brazilian patients with 3/4 European ancestry (dos Santos et al., 2012). However, the same polymorphism was not associated with SLE in UK and American Caucasian patients (De Jager et al., 2006; Demirci et al., 2007). More studies across multiple ethnicities together with clinical profiling of patients are necessary to clarify the role of TLR9 polymorphisms on expression, function and association or not with SLE.

4.6.3 Perpetuation of autoimmune responses in lupus

In SLE patients the balanced steady state is broken due to one or most probably several defects including increased cell death, decreased phagocytosis, and decreased or absent DNase activity (Celhar et al., 2012). This leads to increased nucleic acids that form immune complexes with auto-antibodies and additional components released from dead cells in the serum. Those immune complexes are able to stimulate B cells and pDCs to produce even more antibodies and IFN-α, which leads to a self-perpetuating stimulatory loop and severe autoimmunity(Banchereau and Pascual, 2006). Some of these impaired mechanisms that lead to TLR activation in lupus are presented below:

4.6.3.1 Augmented cell death, NETosis and impaired cell clearance

A high rate of cell death and a lack of clearance by myeloid cells result in increased cell debris and nucleic acids. Their rapid degradation by DNases and RNases, and inherent low uptake into cells, prevents an immune response. Accumulated apoptotic cells in either germinal centers of lymph nodes in the skin or bone marrow have been shown in subgroups of SLE patients (Gaipl et al., 2007; Hepburn et al., 2007). Freshly isolated SLE PBMCs and neutrophils are in a greater state of apoptosis than cells isolated from healthy donors and when left in culture the cells display accelerated apoptosis compared with healthy donor cells (Donnelly et al., 2006; Perniok et al., 1998; Ren et al., 2003). In addition, it has been reported that autoreactive T cells in lupus show an increased expression of the apoptotic ligands TRAIL, TWEAK, and Fas L that directly mediate the apoptosis of monocytes (Kaplan et al., 2002).

A specialized form of neutrophil cell death, termed NETosis, has been described a decade ago (Brinkmann et al., 2004). During NETosis, neutrophils extrude fibrillary networks called NETs (Neutrophil extracellular traps) that can be composed of DNA, citrullinated histones, and granule peptides such as neutrophil elastase,

myeloperoxidase, cathepsin G, LL37 and human neutrophil peptide (HNP). Data have suggested that the neutrophils participate, thought NETosis, in providing the TLR ligands that stimulate the immune system in SLE (Garcia-Romo et al., 2011; Lande et al., 2011). Enhanced NETosis has been reported in SLE patients, even in the absence of *in vitro* stimulation, while anti-LL37 and anti-HNP auto-antibodies can drive this process in healthy cells (Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011). Moreover, it has been reported that a subset of SLE patients cannot clear NETs efficiently, either because of the presence of DNase-I inhibitors or the production of anti-NETs antibodies, which prevent DNase-I from degrading NETs (Hakkim et al., 2010).

On the other hand, macrophages from SLE patients are characterized by impaired phagocytosis of apoptotic cells and other particles in vitro (Gaipl et al., 2007; Ren et al., 2003; Tas et al., 2006). Additionally, data obtained from SLE lymph node biopsies showed an in vivo defect in apoptotic cell clearance by tangible body macrophages in the germinal centers (Baumann et al., 2002). The complement system, and specifically, C1q, plays a crucial part in apoptotic cell clearance by the myeloid lineage (Korb and Ahearn, 1997; Navratil et al., 2001; Taylor et al., 2000). There is evidence for impaired C1q binding on apoptotic neutrophils isolated from SLE patients compared with healthy controls (Donnelly et al., 2006). C1q is also involved in the clearance of immune complexes (ICs) through its binding to the Fc regions on IgG and IgM. This is followed by rapid uptake by phagocytic cells that express C1q receptors and activation of the complement via the classical pathway (Carroll, 2004).

DNases and RNAses work rapidly to clear cell debris and nucleic acids under normal circumstances. DNase-I$^{-/-}$ mice develop lupus-like symptoms, including accumulation of immunocomplexes in the kidney and full-blown glomerulonephritis (Napirei et al., 2000). In agreement with mouse data, DNAse-I activity is lower in SLE patients compared with normal controls and in some patients this has been linked to mutations in the DNase-I gene (Martinez-Valle et al., 2009; Puccetti et al., 1995; Tinazzi et al., 2009; Yasutomo et al., 2001; Yeh et al., 2003). One study identified a rare monogenic form of lupus in pediatric patients linked to a loss-of-function mutation in the

DNASE1L3 gene (Al-Mayouf et al., 2011). Based on the information that RNA is a ligand for TLR7 and RNase treatment of apoptotic or necrotic extracts markedly reduces stimulation of type-1 IFN by pDC *in vitro*, a group created and studied RNase A transgenic (Tg) mice and crossed with TLR7 trangenic mice (that overexpress TLR7) to obtain TLR7RNase double Tg mice (Sun et al., 2013). Double transgenic TLR7/RNAse mice showed increased survival associated with reduced activation of T and B lymphocytes and reduced immunodeposition of IgG and C3. Moreover, the hepatic inflammation and necrosis were strikingly reduced in DTg mice indicating that high concentrations of serum RNase protect against immune activation and inflammation associated with TLR7 stimulation (Sun et al., 2013).

4.6.3.2 Increased formation of immunocomplexes

Patients with SLE have increased plasma levels of components, such as antibodies, LL-37, and HMGB1, that were shown to bind to self-nucleic acids and increase their uptake in vitro (Abdulahad et al., 2011; Barrat et al., 2005; Ganguly et al., 2009; Garcia-Romo et al., 2011; Lande et al., 2011; Ma et al., 2012; Means et al., 2005; Sun et al., 2011). The mechanisms of increased self-nucleic acid uptake that leads to TLR7 and/or TLR9 stimulation are represented in **Figure 11**.

LL37 functions primarily as an antimicrobial peptide and alarmin that recruits and activates inflammatory cells such as DCs and mononuclear phagocytes at the site of microbial infection. It is stored predominantly in the secondary granules of neutrophils and is released upon activation and degranulation as a component of NETs (Yang et al., 2009). LL-37 is detected in psoriatic skin lesions and as a component of NETs infiltrating the skin and kidneys of patients with SLE and small-vessel vasculitis (Kessenbrock et al., 2009; Lande et al., 2007; Villanueva et al., 2011). Additionally, LL37 can be found in DNA-containing immune complexes purified from SLE patients with high titers of anti-dsDNA antibodies (Lande et al., 2011). LL-37 forms complexes with self-nucleic acids in vitro, which results in aggregation and formation of condensed structures that are protected from degradation with nucleases. The aggregation into particles appears crucial for the delivery of RNA/DNA-LL-37 complexes into endosomal compartments of pDCs

where they can interact with TLR7, TLR8 and TLR9 (Ganguly et al., 2009; Lande et al., 2011; Lande et al., 2007). LL37-DNA complexes stimulate IFN-α production from pDCs in a TLR9-dependent manner (Lande et al., 2007), while self-RNA-LL37 complexes activate TLR7-dependent production of IFN-α (Ganguly et al., 2009). It was shown that when anti-DNA antibodies are present, the LL37-DNA/anti-DNA complexes are internalized via FcχRII (Lande et al., 2011). Surprisingly, in this study, DNA/anti-DNA complexes alone were unable to promote DNA uptake and pDC activation, unless LL37 was present, which is in contrast with previous observations (Barrat et al., 2005; Means et al., 2005). However, both studies used ICs purified from SLE serum, which does not exclude that other components were present in the complex besides DNA and anti-DNA antibodies, such as LL37, HMGB1, or unknown molecules. Based on their findings, Lande and co-workers propose that LL37-induced aggregation of DNA into insoluble particles protects DNA from degradation and it is required for the antibodies to promote internalization via receptor-mediated endocytosis (Lande et al., 2011).

HMGB1 is a chromatin-binding non-histone protein, located mainly in the cell nucleus (Stott et al., 2006) and is released from the cells during damage, cell death, or activation and thus serves as an alarmin or death (or damage)-associated molecular pattern (DAMP) (Krieg, 2007). *In vitro* studies revealed that IFNs promote the release of HMGB1 from macrophages (Jiang et al., 2007), which might lead to a perpetual loop in SLE patients with elevated IFN-α in the sera. Binding of HMGB1 to CpG-A enhances IFN-α and TNF-α production by pDCs, and similarly, binding of HMGB1 to CpG-B increases IL-6 secretion from bone marrow-derived macrophages and IL-6, IL-12, and TNF-α from cDCs in a strictly TLR9-dependent way (Ivanov et al., 2007). Furthermore, HMGB1 binds to DNA-containing immune complexes and induces IFN-α secretion from pDCs following engagement of (RAGE) on the surface of the cells (Brencicova and Diebold, 2013; Christen and von Herrath, 2005). SLE sera used for these studies contained anti-DNA antibodies, but undetectable levels of anti-RNP antibodies, so at present it is unknown whether HMGB1 can bind RNA-containing immune complexes that would activate TLR7. Nevertheless, mouse experiments supports this idea, since HMGB1 has been shown to function as an universal sentinel

for nucleic acids, binding B-form DNA, CpG-B, dsRNA (in the form of polyI:C), and ssRNA (poly(U)). Moreover, the absence of HMGB1 severely impaired the activation of TLR3, TLR7 and TLR9 by their respective nucleic acid ligands (Yanai et al., 2009).

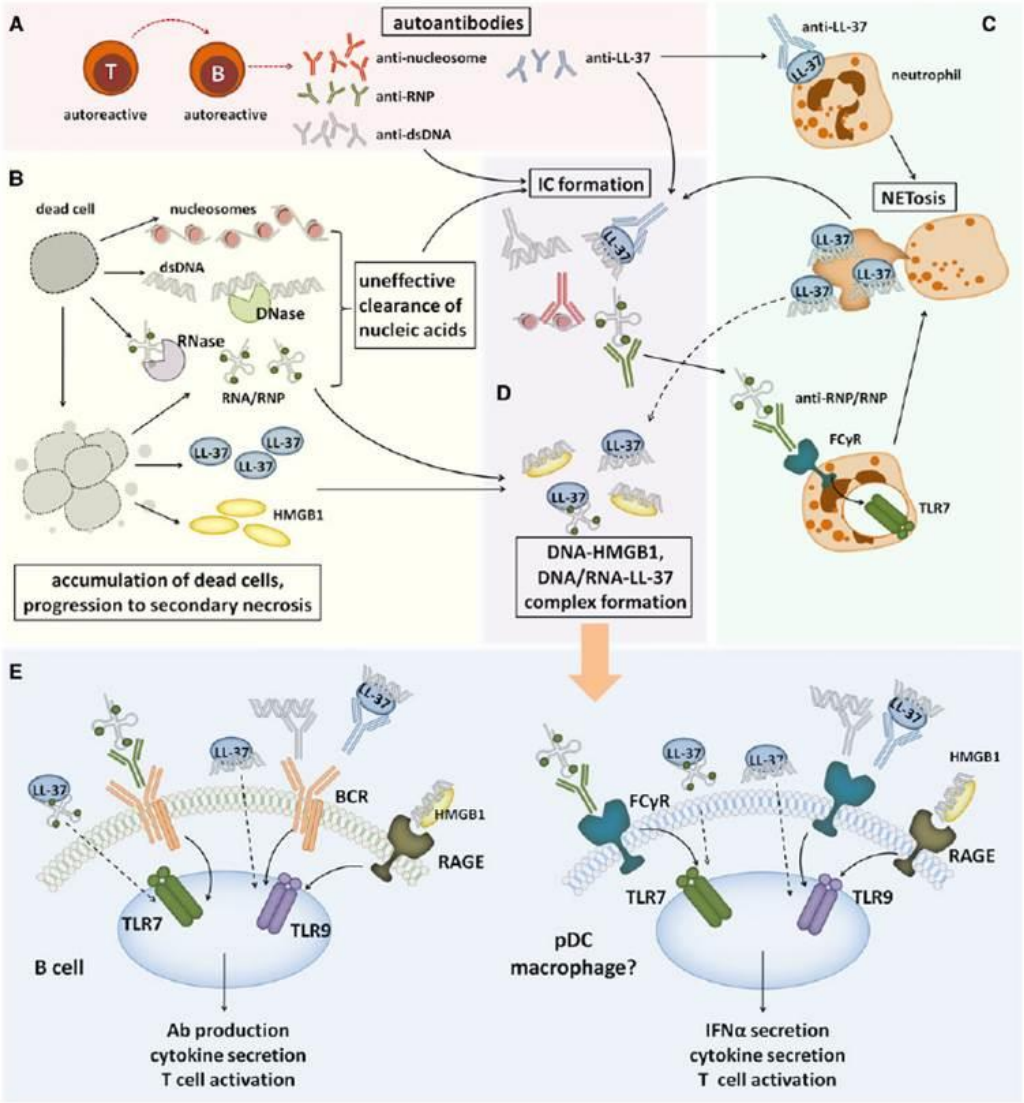

Figure 11: Break of self-tolerance in SLE patients results in autoantibody production (A). Anti-nuclear autoantibodies form immune complexes (IC) with nuclear material released from dead cells that have not been efficiently cleared (B). Dead cells release also proteins and peptides, such as HMGB1 and LL-37, which bind directly to self-nucleic acids (D). LL-37-DNA complexes are also being released in the process of NETosis that can be mediated by anti-LL-37 antibodies binding to LL-37 on the

surface of neutrophils or by TLR7 activation in neutrophils induced by RNA-containing ICs (D). The formed complexes, ICs, DNA-HMGB1, and DNA/RNA-LL-37 (D), mediate the uptake of self-nucleic acids into the endosomal compartments of cells by engaging different receptors, including BCR (B-cell receptor), FcχR and RAGE (E). RNA-containing complexes activate TLR7, while DNA-containing immune complexes activate TLR9 in B cells, pDCs, and murine macrophages (E) (Celhar et al., 2012).

5. Zinc finger transcription factor Growth factor independence 1 (Gfi-1)

Gfi-1 gene is localized on chromosome lp22 in humans and encodes a small nuclear protein of about 55 kDa that contains six c-terminal C2H2-type zinc-finger domains and an N terminal SNAG domain critical for its repressor activity (Zweidler-Mckay et al., 1996)(**Figure 12**). Zinc fingers 3–5 of Gfi-1 are necessary for binding to its cognate consensus DNA recognition sequence taAATCac(t/a)gca, zinc fingers 1, 2 and 6 very likely have a role in the interaction with other proteins (Bell et al., 1995; Duan and Horwitz, 2003; Gilks et al., 1993; Grimes et al., 1996; Hock et al., 2003; Person et al., 2003). The function or the structure of the part of the protein, which separates the SNAG domain from the zinc-finger is unknown and is also the least conserved part among the vertebrate Gfi-1 molecules. It is possible that this middle part of the Gfi-1 proteins serves as a specific platform for the interaction with co-factors.

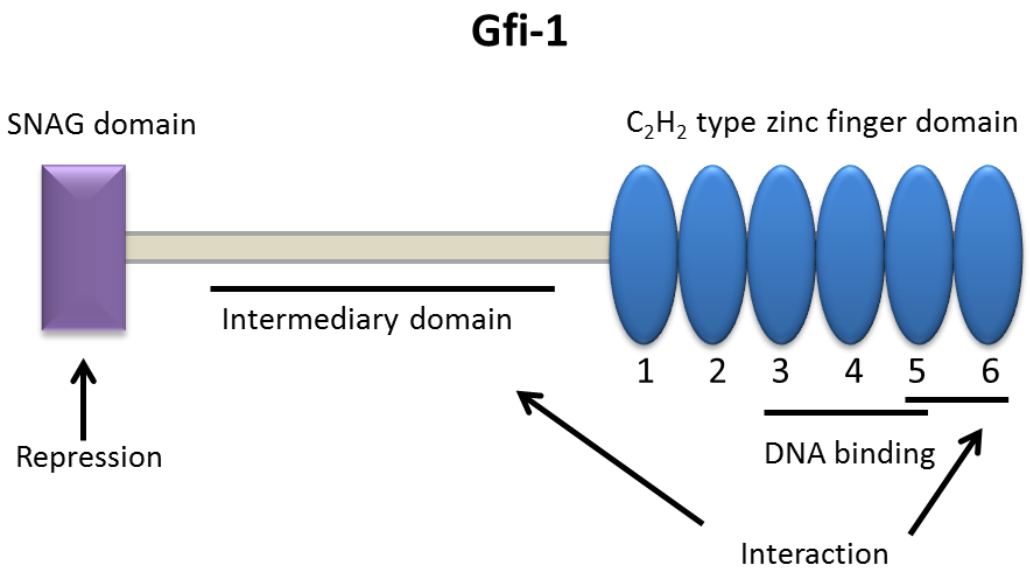

Figure 12: Schematic depiction of the domain structure of Gfi-1. Adapted from (Moroy and Khandanpour, 2011).

Gfi-1 exerts its role as transcriptional repressor by interacting with a number of histone modifying enzymes (Duan et al., 2005; Saleque et al., 2007). These enzymes and Gfi-1 are part of a large protein complex, which binds to a specific promoter area defined by the Gfi-1 binding site. The recruitment of this complex by Gfi-1 to specific target gene promoters leads to transcriptional silencing at this locus. The main components that Gfi-1 can recruit to target genes are the histone demethylase complex LSD-1/CoRest, which removes methyl groups from the histone H3 (H3K4) and the histone deacetylases HDAC-1 (**Figure 13**). Both demethylation and deacetylation of histones by these enzymes lead to a transcriptional repression, which may be reversible once the complex is dissolved or degraded (Duan et al., 2005; Saleque et al., 2007). Gfi-1 can also interact with the methyl transferase G9a that dimethylates Histone H3 at lysine 9 (H3K9), which in turn can recruit the chromatin protein HP-1 and may lead to a heterochromatinization of the locus (Duan et al., 2005; Saleque et al., 2007). A working model has been proposed in which these complexes form in a stepwise manner at Gfi-1 target gene promoters. It is not known if the Gfi-1 complex is directed to histones and in particular how it is reorganized

after DNA replication, but it can be speculated that other chromatin regulatory elements may be involved.

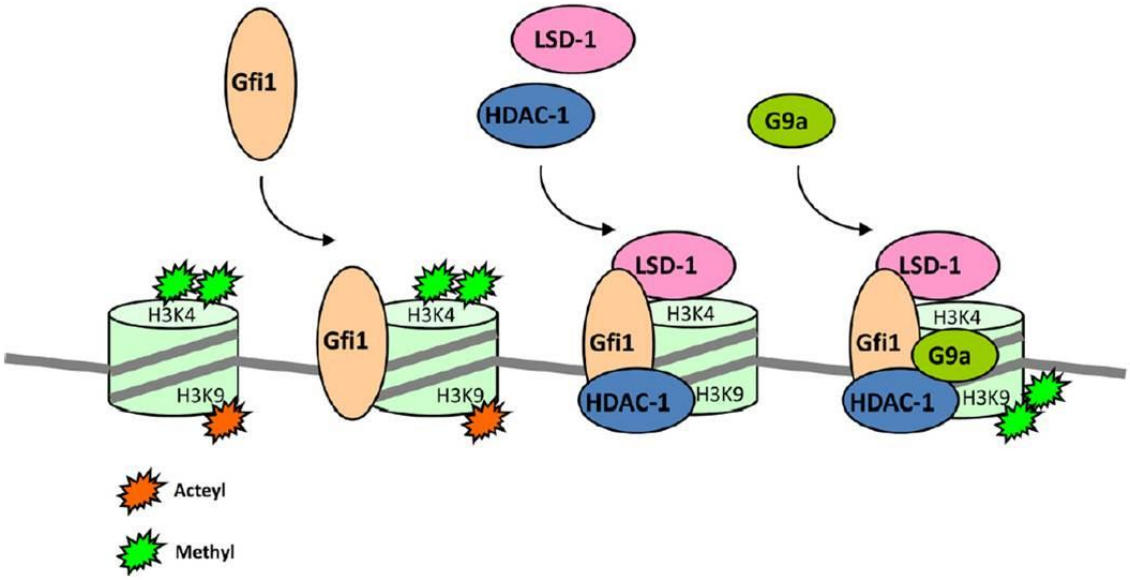

Figure 13: Gfi-1 recruits a multiprotein complex to DNA and enables histone modifications, in particular the de-methylation of histone H3 at K4, the deacetylation of histone H3 at various sites, and the methylation at histone H3K9 (Moroy, 2005).

Expression of Gfi-1 was first detected in cells of the immune system in particular thymic T-cells (Gilks et al., 1993). Protein and mRNA levels in the thymus are precisely regulated during the developmental stages of pre-T cells, and most surprisingly, do not show a direct linear correlation. This suggests that an additional level of post-transcriptional regulation of Gfi-1 gene expression takes place during pre T cell differentiation (Yucel et al., 2004). In contrast to the high, constitutive levels of Gfi-1 in most thymic T-cells, peripheral, naive T-cells have only a very low basic expression level of Gfi-1, which can be rapidly and transiently induced by T cell receptor-mediated activation (Karsunky et al., 2002a).

Less is known about naive B cells but it is likely that, as in T cells, Gfi-1 expression is induced upon antigenic stimulation. Gfi-1 is expressed not only in hematopoietic precursors and stem cells but also in more mature cells such as granulocytes, monocytes and DCs (Karsunky et al., 2002a; Rathinam et al., 2005). In mature

differentiated macrophages, Gfi-1 can be detected when stimulated with endotoxins, for instance with lipopolysaccharide, so Gfi-1 is an effector of TLR4 (Karsunky et al., 2002b).

In situ hybridization data revealed more recently that Gfi-1 is indeed even more widely expressed than previously thought and is by no means restricted to the immune system or hematopoietic cells. Clearly, Gfi-1 is expressed in the lung, sensory epithelia, neuronal precursors and in particular in the developing epithelia of the inner ear (Wallis et al., 2003).

5.1 Biological role of Gfi-1

The generation of Gfi-$1^{-/-}$ mouse strains helped to elucidate the biological role of Gfi-1. Studies have demonstrated that Gfi-1 plays an important role in hematopoietic stem cell (HSC), myeloid cell, B cell and T cells. Defects on Gfi-$1^{-/-}$ HSCs are characterized by their severely disturbed self-renewal and their inability to reconstitute hematopoietic lineages in a transplanted host (Hock et al., 2004; Moroy, 2005; Zeng et al., 2004). Two physiological functions of Gfi-1 may explain these observations. Gfi-1 restricts HSC proliferation by controlling the expression of the negative cell cycle regulator called $p21^{waf/cip1}$. The mechanisms underlying this regulation are unclear, but two studies demonstrated that Gfi-$1^{-/-}$ HSCs undergo more cell cycling and express reduced levels of $p21^{waf/cip1}$ compared to HSCs from WT mice. It is postulated that this increased proliferation impairs the function of Gfi-$1^{-/-}$ HSCs (Hock et al., 2004; Zeng et al., 2004).

Moreover Gfi-1 plays an important role in myeloid differentiation. Gfi-$1^{-/-}$ mice have increased numbers of common myeloid precursors (CMPs) and granulocyte macrophage precursors (GMPs) (Horman et al., 2009; Zeng et al., 2004). Gfi-1 seems to down regulate expression of genes that ensure a proper differentiation from CMPs to GMPs, and finally to neutrophil granulocytes. Loss of Gfi-1 leads to repression of genes favoring a development towards the monocytic lineage and inhibiting the development of granulocytes. Consequently, Gfi-$1^{-/-}$ mice are neutropenic, lack

granulocytes and display a strong expansion of atypical Mac-1^{+}, $Gr1^{lo}$ monocytes (Karsunky et al., 2002a). The requirement of Gfi-1 for the formation of neutrophil granulocytes is corroborated by a report that human patients with neutropenia carry germline mutations in the coding region of Gfi-1 affecting the zinc finger regions (Person et al., 2003).

Moreover, Gfi-1 plays a role in the early stages of B cell differentiation. Evidence for this comes mainly from the study of Gfi-$1^{-/-}$ mice that show reduced numbers of common lymphoid progenitors (CLPs) and a defective maturation of early B-lineage precursors, which leads to a reduced number of B cells in bone marrow and spleen (Rathinam and Klein, 2007; Yasukawa et al., 2000). In addition, Gfi-1 regulates the expression of PU.1, which is one transcription factor with an important role in both myeloid and lymphoid development. PU.1 enables precursors to differentiate into certain lineages and high levels favor myeloid over lymphoid development. In the absence of Gfi-1, PU.1 is thus hyperactive and drives precursors into the myeloid lineage while impeding the formation of lymphoid cells, in particular B-cells. By reducing PU.1 protein quantity in Gfi-$1^{-/-}$ mice, B cell differentiation defects can be overcome (Spooner et al., 2009). Based on these and other findings, interactive regulatory networks have been proposed, in which Gfi-1 favors B cell development whereas PU.1 and Egr1 inhibit B-cell development and favor differentiation. To fulfill all these regulatory tasks, Gfi-1 itself has to be induced upon initiation of B-cell lineage commitment. Ikaros, another transcription factor important for early B-cell differentiation, acts upstream of Gfi-1 and ensures its up-regulation after commitment of the progenitors to the lymphoid lineage (Spooner et al., 2009). Gfi-1 is also required for the maturation and activity of B-cells. Gfi-1 restricts an overshooting of antibody production after antigenic stimulation. When challenged with different antigens *in vivo*, Gfi-$1^{-/-}$ mice exhibited a higher number of germinal center B cells in the spleen and accentuated production of antigen specific IgG2a and IgG2b antibodies (Igwe et al., 2008). On the molecular level, increased level of TGF-β might explain this, as TGF-β promotes expression levels of different IgG subtypes. In accordance with disturbed regulation of the immune response, Gfi-$1^{-/-}$ mice are

characterized by an increased predisposition to develop autoimmunity (Park et al., 2005; Snapper et al., 1993).

Gfi-1$^{-/-}$ mice have a reduced number of thymocytes compared to littermate controls (Karsunky et al., 2002a). This is the result of a disturbed pre T-cell differentiation at different stages (Yucel et al., 2003; Yucel et al., 2004). As in the case of B cell development, one explanation for these deficiencies is a function of Gfi-1 in the regulation of IL-7R signaling. Gfi-1 is also implicated in the differentiation and activation of the mature peripheral T cell subpopulations. Generally, Gfi-1 is important for the proper function and development of CD4 T cells (Pargmann et al., 2007) and more specifically, within the CD4 T cell fraction, Gfi-1 plays a major role in Th2 and Th17 cells. Loss of Gfi-1 is associated with decreased number of Th2 cells, increased number of Treg cells and increased expression of Th17 gene in T cells (Ichiyama et al., 2009; Zhu et al., 2006) .

Gfi-1 is a critical transcription factor in DC differentiation. Gfi-1$^{-/-}$ mice showed a global reduction of DCs in all lymphoid organs whereas epidermal Langerhans cells were enhanced in number. *In vivo*, Gfi-1$^{-/-}$ DCs showed phenotypic and functional alterations such as decreased MHC class II expression, upregulation of costimulatory molecules (CD40, CD80 and CD86), constitutive secretion of IL-12 and impaired antigen presentation. *In vitro*, Gfi1$^{-/-}$ hematopoietic progenitor cells were unable to develop into DCs in the presence of GM-CSF or Flt3L (Rathinam et al., 2005).

5.1.1 Functions of Gfi-1 outside the hematopoietic system

Outside the hematopoietic system, Gfi-1 is required for the integrity and function of inner ear hair cells and in the central nervous system for Purkinje cells (Tsuda et al., 2005; Wallis et al., 2003). In addition, Gfi-1 plays a role in the lineage decision process during intestinal cell differentiation (Bjerknes and Cheng, 2010; Shroyer et al., 2005).

5.1.2 Role of Gfi-1 in endotoxin-mediated inflammation

It was noticed that Gfi-$1^{-/-}$ mice showed signs of inflammatory response, characterized by hunched posture, weight loss and eye infections at two months of age (Karsunky et al., 2002b). Histopathological examination of sick Gfi-$1^{-/-}$ animals revealed deposits of hyaline material around vessels in the lung that can be considered a consequence of toxic chock. Serum levels of inflammatory cytokines such as TNF-α and IL-1β in young, apparently healthy Gfi-$1^{-/-}$ mice varied but they were not significantly higher in Gfi-$1^{-/-}$ than in the wild type and that Gram-negative bacteria were absent in both Gfi-$1^{-/-}$ and WT mice. However, Gfi-$1^{-/-}$ macrophages produce enhanced levels of inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-1β, when stimulated with bacterial lipopolysaccharide (LPS) and that Gfi-$1^{-/-}$ mice succumb to low doses of this endotoxin that are tolerated by WT mice (Karsunky et al., 2002a).

Later it was showed that upon LPS stimulation, Gfi-1 interacts with p65 and inhibits p65-mediated transcriptional transactivation by interfering with p65 binding to target gene promoter DNA. Gfi-$1^{-/-}$ macrophages show abnormally high mRNA levels of the TNF gene and many other p65 target genes and a higher rate of TNF promoter occupancy by p65 than wild-type cells after LPS stimulation, suggesting that Gfi-1 functions as an antagonist of NF-κB activity at the level of promoter binding (Sharif-Askari et al., 2010).

5.2 Genista mice: a neutropenic model with a hypomorphic mutation in the Gfi-1 gene

Genista mice were generated by N-ethyl-N-nitrosourea-induced (ENU) mutagenesis resulting in a point mutation in the Gfi-1 gene (Ordonez-Rueda et al., 2012). This mutation is characterized by cysteine residue at position 318 of the third zinc finger domain of Gfi-1 into a tyrosine residue, thereby disrupting the Cys2His2 structural motif that constitutes the core component of zinc finger domains (**Figure 14**). The

levels of mutated protein expressed in Gfi-1 hypomorphic mutation in the Genista mice were comparable to the levels of Gfi-1 proteins found in WT mice. Genista mice are neutropenic but have normal viability and no weight loss (Ordonez-Rueda et al., 2012), in contrast to Gfi-$1^{-/-}$ mice (Karsunky et al., 2002b).

Figure 14: Representation of the Gfi-1 protein with the amino-terminal SNAG domain (gray) and the six C2H2-type zinc finger domains (pink) (Ordonez-Rueda et al., 2012).

It was demonstrated the Genista mutation likely corresponds to a partial loss of function mutation of the Gfi-1 gene. When compared to Gfi-$1^{-/-}$ mice, the Genista mice have less effect on hematopoietic precursors and the development of lymphoid cells (Ordonez-Rueda et al., 2012). Despite the two-fold decreased cellularity of the thymus, Genista mice have all stages of thymic T-cells development. The secondary lymphoid organs of Genista and WT mice contained identical T cell numbers and distribution of CD4 and CD8 cells. B cell development was not affected by the Genista mutation, thereby resulting in normal numbers of peripheral B cells. Regarding the myeloid compartment, the Genista mutation results in increase of monopoiesis, with 4-times more monocytes compared to WT mice in the bone-marrow (BM). Genista mice are devoid of $CD11b^{+}Ly\text{-}6G^{high}$ (mature neutrophils) but contains an atypical population of $CD11b^{+}Ly\text{-}6G^{int}$ cells the nuclear morphology of which resembled mature WT neutrophils but are less numerous than the $CD11b^{+}Ly\text{-}6G^{high}$ neutrophils found in WT. The Gfi$1^{-/-}$ mice also lack mature $Ly\text{-}6G^{high}$ neutrophils but differ from that of Genista mice in that they do not contain atypical $CD11b^{+}Ly\text{-}6G^{int}$ cells (**Figure 15**). The atypical neutrophils present in Genista mice are able to migrate from BM, express myeloperoxidase and are functional (Ordonez-

Rueda et al., 2012). Indeed, injection of K/BxN serum in WT mice induces arthritis by day 2. However, Gfi-$1^{-/-}$ mice do not develop arthritis, while Genista mice develop signs of arthritis by day 2, but the arthritis does not worsen over time, like in the case of WT mice (Ordonez-Rueda et al., 2012).

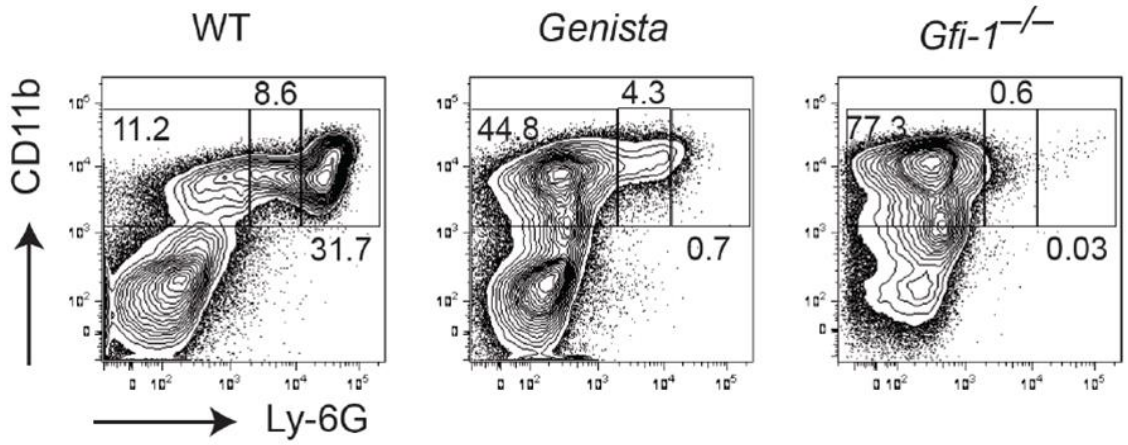

Figure 15: Genista mice lack mature neutrophils. BM cells from WT, Genista and Gfi-$1^{-/-}$ mice analyzed for CD11b and Ly-6G expression by flow cytometry. Gates correspond to mature ($CD11b^{+}Ly\text{-}6G^{high}$) and immature ($CD11b^{+}Ly\text{-}6G^{int}$) neutrophils, and to monocytes ($CD11b^{+}$ $Ly\text{-}6G^{-}Ly\text{-}6C^{+}$) (Ordonez-Rueda et al., 2012).

Objectives

The objective of my PhD was to study the implication of endosomal TLR interplay and signaling in the development of systemic lupus erythematosus (SLE). SLE is an autoimmune disease characterized by the production of autoantibodies against nuclear antigens (Rahman and Isenberg, 2008). The development of SLE is still not totally understood but recent studies implicate TLRs in the initiation and establishment of SLE (Kim et al., 2009). TLRs detect conserved microbial components and trigger immune responses leading to the engagement of signaling pathways and triggering of nuclear factor-κB (NF-κB), interferon-regulatory factors (IRFs) and MAP-kinases (O'Neill et al., 2013). A major consequence of TLR signaling is the induction of pro-inflammatory cytokines and type I-IFNs (Kawai and Akira, 2010). Many studies revealed that TLRs respond to endogenous molecules, most of which are released from dead cells (Brencicova and Diebold, 2013; Rifkin et al., 2005; Yu et al., 2010). Excessive TLR activation upon recognition of exogenous self-ligands by TLRs can disrupt immune homeostasis, and may be responsible for the development of autoimmune diseases (Kim et al., 2009; Pradhan et al., 2012). To avoid harmful and inappropriate inflammatory responses, TLR signaling is controlled by negative regulators, which are often induced by TLR ligands to terminate activation of signaling pathways (Kondo et al., 2012).

My PhD project had two aims:

Aim 1: Study the cooperation of TLR8 and TLR9 in controlling TLR7-mediated lupus.

Among the TLRs, the endosomal TLR7, TLR8, and TLR9 play a role in the initial activation and in the subsequent disease progression of lupus (Christensen and Shlomchik, 2007; Demaria et al., 2010). In mice TLR7-deficiency ameliorates SLE, but TLR8- or TLR9-deficiency exacerbates the disease due to increased TLR7 response (Deane et al., 2007; Demaria et al., 2010; Nickerson et al., 2010). Thus, both TLR8 and TLR9 control TLR7 function but is was unknown if these TLRs act in

parallel or in series in the same or different cell types and if they have an additive or not effect in controlling TLR7. In order to address these issues we generated double TLR8/TLR9-deficient (TLR8/9$^{-/-}$) mice and analyzed and compared the lupus phenotype between TLR8$^{-/-}$, TLR9$^{-/-}$ and TLR8/9$^{-/-}$ mice, using various criteria such as spleen size, autoantibody production, frequencies of marginal zone and B1 B cells, and renal pathology. Moreover, we studied the immune responses of TLR8$^{-/-}$, TLR9$^{-/-}$ and TLR8/9$^{-/-}$ or WT cell types, including dendritic cells, macrophages, splenocytes and B cells to TLR stimulation.

Aim 2: Investigate the implication of Gfi-1 in lupus and TLR signaling by studying Genista mice.

Growth Factor Independence 1 (Gfi-1) is a transcriptional repressor that controls NF-κB upon LPS stimulation (Sharif-Askari et al., 2010). Studies with Gfi$^{-/-}$ mice demonstrated that Gfi-1 plays an important role in hematopoietic stem cells (HSC), myeloid cells, B-cells, T-cells and neutrophils development (Hock et al., 2004; Moroy, 2005; Zeng et al., 2004). Moreover, Gfi$^{-/-}$ mice present symptoms of septic shock and general inflammation following LPS injection and have a life-spam of 2-3 months (Karsunky et al., 2002a). Recently, Genista mice carrying a single-point mutation in the Gfi-1 gene were generated and characterized as a novel neutropenic mouse model (Ordonez-Rueda et al., 2012). As Genista mice present normal lifespan, they represent an attractive alternative to study Gfi-1 function. Based on the fact that Gfi-1 deficient mice have an increased predisposition to develop autoimmunity (Igwe et al., 2008), we hypothesized that Genista mice will also develop lupus. Indeed we found that Genista mice present elevated immunoglobulins and autoantibodies in their sera and develop renal pathology compared to WT mice. So, the aim of my second aim was to characterize the lupus phenotype in Genista mice, study if the disease was TLR7-dependent and dissect the molecular pathways that are involved in Genista lupus pathology.

Results

Aim 1: Study the cooperation of TLR8 and TLR9 in controlling TLR7-mediated lupus

TLR8 on dendritic cells and TLR9 on B cells restrain TLR7-mediated spontaneous autoimmunity in C57BL/6 mice

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Systemic lupus erythematosus (SLE) is a complex autoimmune disease with diverse clinical presentations characterized by the presence of autoantibodies to nuclear components. Toll-like receptor (TLR)7, TLR8, and TLR9 sense microbial or endogenous nucleic acids and are implicated in the development of SLE. In mice TLR7-deficiency ameliorates SLE, but TLR8- or TLR9-deficiency exacerbates the disease because of increased TLR7 response. Thus, both TLR8 and TLR9 control TLR7 function, but whether TLR8 and TLR9 act in parallel or in series in the same or different cell types in controlling TLR7-mediated lupus remains unknown. Here, we reveal that double TLR8/9-deficient (TLR8/9$^{-/-}$) mice on the C57BL/6 background showed increased abnormalities characteristic of SLE, including splenomegaly, autoantibody production, frequencies of marginal zone and B1 B cells, and renal pathology compared with single TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. On the cellular level, TLR8$^{-/-}$ and TLR8/9$^{-/-}$ dendritic cells were hyperresponsive to TLR7 ligand R848, but TLR9$^{-/-}$ cells responded normally. Moreover, B cells from TLR9$^{-/-}$ and TLR8/9$^{-/-}$ mice were hyperresponsive to R848, but TLR8$^{-/-}$ B cells were not. These results reveal that TLR8 and TLR9 have an additive effect on controlling TLR7 function and TLR7-mediated lupus; however, they act on different cell types. TLR8 controls TLR7 function on dendritic cells, and TLR9 restrains TLR7 response on B cells.

knockout mice | innate immunity | endosomal TLRs

Systemic lupus erythematosus (SLE) is a complex chronic autoimmune disease that arises spontaneously and is characterized by production of autoantibodies against self-nucleic acids and associated proteins (1). These autoantibodies bind self-nucleic acids released by dying cells and form immune complexes that accumulate in different parts of the body, leading to inflammation and tissue damage. The kidneys, skin, joints, lungs, serous membranes, as well as, the cardiovascular, nervous and musculoskeletal system become targets of inflammation at onset or during the course of the disease (2). The etiology of SLE is unknown, yet genetics, sex, infectious agents, environmental factors, and certain medications may play a role in the initiation of the disease by causing alterations in lymphoid signaling, antigen presentation, apoptosis, and clearance of immune complexes (3, 4).

Toll-like receptors (TLRs) detect specific microbial components widely expressed by bacteria, fungi, protozoa, and viruses, and initiate signaling pathways critical for induction of immune responses to infection (5). In contrast to the cell surface TLRs that detect bacterial cell wall components and viral particles, nucleic acid-sensing TLRs are localized mainly within endosomal compartments (6). Human endosomal TLRs consist of TLR3, which senses viral double-stranded RNA (dsRNA) (7), TLR7 and TLR8, which recognize viral single-stranded RNA (8–10), and TLR9, which detects bacterial and viral unmethylated CpG-containing DNA motifs (11). Interestingly, these endosomal TLRs are also able to detect self-nucleic acids (12–14). Although the endosomal localization isolate TLR3, TLR7, TLR8, and TLR9 away from self-nucleic acids in the extracellular space, still self-RNA or -DNA can become a potent trigger of cell activation when transported into TLR-containing endosomes, and such recognition can result in sterile inflammation and autoimmunity, including SLE (4, 15, 16). The connection of the endosomal TLRs with SLE originates mainly from mouse models, where TLR7 signaling seems to play a central role. TLR7 gene duplication is the cause for the development of lupus in mice bearing the Y chromosome-linked autoimmune accelerating (Yaa) locus that harbors 17 genes, including TLR7 (17, 18). In TLR7 transgenic mouse lines, a modest increase in TLR7 expression promotes autoreactive lymphocytes with RNA specificities and myeloid cell proliferation, but a substantial increase in TLR7 expression causes fatal acute inflammatory pathology and profound dendritic cell (DC) dysregulation (17). In addition, studies in several lupus-prone mouse strains have revealed that TLR7-deficiency ameliorates disease, but TLR9-deficiency exacerbates it. Interestingly, this controversy can be explained by the enhanced TLR7 activity in the TLR9-deficient lupus mice (19, 20). Although murine TLR8 does not seem so far to be able to sense a ligand (21, 22), we have shown previously that it plays an important biological role in controlling TLR7-mediated lupus. Indeed, TLR8-deficiency in mice (on the C57BL/6 background that is not prone to lupus) leads to lupus

Significance

Toll-like receptors (TLRs) that sense microbial or endogenous DNA and RNA have been well implicated in systemic lupus erythematosus (SLE), a multisystem disease characterized by an autoimmune response to nuclear antigens. In mice, both TLR8 and TLR9 control TLR7-mediated lupus, but it is unknown if they have an additive effect in controlling TLR7. We demonstrate that double TLR8/9-deficient mice have increased abnormalities characteristic of SLE and that both TLR8 and TLR9 keep under control TLR7-mediated lupus, but they act on different cell types. TLR8 controls TLR7 function on dendritic cells, and TLR9 restrains TLR7 response on B cells. These TLR interactions have to be taken into account when novel therapeutic approaches are developed that target the blocking of TLRs.

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The authors declare no conflict of interest.

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development because of increased TLR7 expression and signaling in DCs (23). Thus, tight control and regulation of TLR7 is pivotal for avoiding SLE and inflammatory pathology in mice. Recent studies in humans have also revealed that increased expression of TLR7 is associated with increased risk for SLE (24–26).

Nucleic acid TLRs are expressed in many cell types, including DCs, plasmacytoid DCs (pDCs) and B cells, all of which play a central role in SLE development. TLR7, TLR8, and TLR9 signal through the adaptor molecule myeloid differentiation primary response gene 88 (MyD88), whereas TLR3 signals via the adaptor TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN-β) (5). MyD88-deficiency abrogates most attributes of lupus in several lupus-prone mouse strains (19, 27–29). Moreover, deficiency for Unc93B1, a multipass transmembrane protein that controls trafficking of TLRs from the endoplasmic reticulum to endolysosomes and is required for nucleic acid-sensing TLR function (30), also abrogates many clinical parameters of disease in mouse lupus strains, suggesting that endosomal TLRs are critical in this disease (31). Interestingly, TLR9 competes with TLR7 for Unc93B1-dependent trafficking and predominates over TLR7 (32). TLR9 predominance is reversed to TLR7 by a D34A mutation in Unc93B1 and mice that carry this mutation show TLR7-dependent, systemic lethal inflammation (32).

Thus, in mice both TLR8 and TLR9 control TLR7-mediated lupus, but it is unknown if these TLRs act in parallel or in series in the same or different cell types and if they have an additive effect or not in controlling TLR7. To address these issues, we generated double TLR8/TLR9-deficient (TLR8/9$^{-/-}$) mice and analyzed and compared the lupus phenotype in TLR8$^{-/-}$, TLR9$^{-/-}$, and TLR8/9$^{-/-}$ mice. Our data revealed that TLR8/9$^{-/-}$ mice have increased abnormalities characteristic of SLE and that both TLR8 and TLR9 keep TLR7-mediated lupus under control, but they act in different cell types. On DCs TLR7 function is ruled by TLR8, whereas on B cells TLR7 is mastered by TLR9.

Results

Splenomegaly and Reduced Innate B-Cell Populations in TLR8$^{-/-}$ and TLR8/9$^{-/-}$ Mice. To determine whether TLR8 and TLR9 have an additive effect in the development of lupus autoimmunity on the C57BL/6 background, we generated double TLR8/9$^{-/-}$ mice by intercrossing TLR8$^{-/-}$ to TLR9$^{-/-}$ mice. TLR8/9$^{-/-}$ mice had normal appearance, growth, and fertility, and showed no obvious behavioral abnormalities. Moreover, similar to TLR8$^{-/-}$ and TLR9$^{-/-}$ mice, TLR8/9$^{-/-}$ mice had normal survival rates up to 12 mo of age. We have shown previously that TLR8-deficiency in mice leads to lupus that is accompanied by splenomegaly and a reduction of the marginal zone (MZ) and B1 B cells (23), so first we evaluated these populations. TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice had splenomegaly compared with age- and sex-matched WT controls, whereas TLR8/9$^{-/-}$ mice showed the most exacerbated phenotype (Fig. 1*A* and Table S1). Moreover, both TLR8$^{-/-}$ and TLR8/9$^{-/-}$ mice had a defect on the MZ B frequencies (Fig. 1*B* and Table S2), and an expansion of the CD11c^{+} and CD19^{+} populations, whereas TLR9$^{-/-}$ mice looked normal (Table S1). Next, we evaluated the B1 B populations and noticed that B1a (B220^{lo}CD5^{int}) and B1b (B220^{lo}CD5^{lo}) B cells were reduced in TLR8$^{-/-}$ and TLR8/9$^{-/-}$ mice compared with WT or TLR9$^{-/-}$ mice, and the reduction of B1a B cells was more profound in TLR8/9$^{-/-}$ than in TLR8$^{-/-}$ mice (Fig. 1*C* and Table S2). Thus, TLR8/9$^{-/-}$ mice have a more severe defect on MZ and B1 B cells than TLR8$^{-/-}$ mice, although these populations appear to be unaffected in TLR9$^{-/-}$ mice.

Double TLR8/9$^{-/-}$ Mice Develop Stronger Lupus Phenotype than TLR8$^{-/-}$ or TLR9$^{-/-}$ Mice. To determine whether the augmented immunological phenotype we observed in TLR8/9$^{-/-}$ mice correlates with increased autoimmunity, serum levels of IgM and IgG2a isotypes were assessed by ELISA. All three TLR-deficient genotypes showed significantly increased IgM and IgG2a levels compared with WT mice, whereas both TLR8/9$^{-/-}$ and TLR9$^{-/-}$ mice also had significantly elevated IgM levels compared with

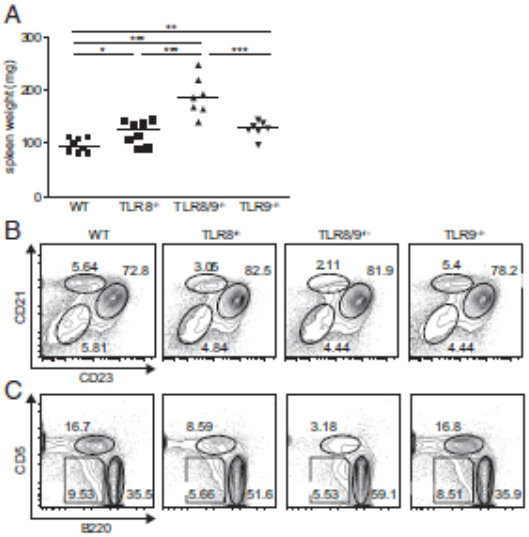

Fig. 1. TLR8/9$^{-/-}$ mice show increased splenomegaly and more severe defect on MZ and B1 B cells compared with TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. (*A*) Spleen weight of 4- to 6-mo-old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice. Each point represents one mouse (n = 7–8 mice per genotype) and horizontal bars denote the median. (*B*) Splenocytes from 12-wk-old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice were analyzed by flow cytometry for the expression of CD19, CD21, and CD23. Numbers denote the percentage of MZ (CD21^{hi}CD23^{lo}), follicular (CD21^{int}CD23^{hi}), and immature (CD21^{-}CD23^{-}) B cells in the indicated circles. (*C*) FACS analysis of B220 and CD5 expression on CD19^{+} gated cells of the peritoneal cavity shows the percentage of B1a (B220^{lo}CD5^{hi}), B1b (B220^{lo}CD5^{lo}), and B2 (B220^{hi}CD5^{-}) B cells. Data in *B* and *C* are representative of two to three independent experiments (n = 3–4 per group). *P < 0.05, **P < 0.01, ***P < 0.001.

TLR8$^{-/-}$ mice (Fig. 2*A*). Next, we evaluated the levels of IgG autoantibodies against dsDNA, RNA, Smith ribonucleoprotein (SmRNP) and RNP and found that TLR8$^{-/-}$ and TLR8/9$^{-/-}$ mice had significantly increased titers compared with WT sera (Fig. 2*B*). Interestingly, TLR8/9$^{-/-}$ mice had also significantly higher levels of anti-dsDNA, -RNA, and -RNP production versus TLR8$^{-/-}$ mice, whereas TLR9$^{-/-}$ mice resembled TLR8$^{-/-}$ mice regarding anti-dsDNA, -RNA, and -SmRNP levels, and showed normal levels of anti-RNP (Fig. 2*B*). Then, we evaluated the percentages of antibody-producing B cells and found that the B220^{lo}CD138^{+} population was increased in all three TLR-deficient genotypes compared with WT controls, where TLR8/9$^{-/-}$ mice showed the highest increase, followed by TLR8$^{-/-}$ and TLR9$^{-/-}$ mice (Fig. 2*C*). Moreover, we noticed that there was a twofold increase in the percentage of spontaneous splenic germinal centers in TLR8$^{-/-}$ and TLR8/9$^{-/-}$ mice compared with WT controls (6.9 ± 0.5% and 7.8 ± 0.6%, respectively, vs. 3.0 ± 0.9%), and TLR9$^{-/-}$ mice showed slight increase compared with WT mice (4.5 ± 0.5% vs. 3.0 ± 0.9%) (Fig. 2*D*). Further analysis of autoantibody production showed that although antinuclear antibody (ANA) patterns varied somehow, TLR8$^{-/-}$ sera appeared mainly nucleolar, and TLR8/9$^{-/-}$ and TLR9$^{-/-}$ sera were both cytoplasmic and nucleolar (Fig. 3*A*).

To evaluate renal pathology, we used semiquantitative pathological scoring of glomerular and interstitial nephritis. TLR8/9$^{-/-}$ mice had increased glomerular cellularity, glomerular deposits, and interstitial infiltrations compared with TLR8$^{-/-}$ or TLR9$^{-/-}$ mice (Fig. 3*B*, Fig. S1, and Table S3). In addition, we observed increased IgG and IgM glomerular depositions in TLR8/9$^{-/-}$

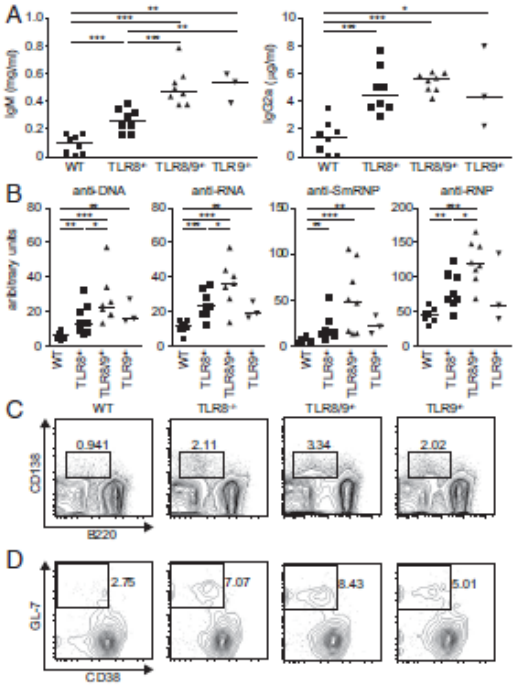

Fig. 2. Increased autoantibodies, plasmablasts, and germinal centers in TLR8/9$^{-/-}$ vs. TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. Sera from 10- to 13-wk-old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice were used for the evaluation of (*A*) serum levels of IgM and IgG2a and (*B*) dsDNA-, RNA-, smRNP-, and RNP-specific autoantibody production by ELISA. Each point represents value from one mouse and horizontal bars denote the median. Representative flow cytometry plots of (*C*) B220^{lo}CD138^{+} plasmablasts and (*D*) B220^{+}GL7^{+}CD38^{-} germinal center B cells in 5 mo old male WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice. Data in *C* and *D* are representative of two independent experiments (n= 3–4 per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

kidneys followed by TLR8$^{-/-}$ samples, whereas TLR9$^{-/-}$ mice showed reduced deposition compared with TLR8$^{-/-}$ mice, but stronger than in WT controls (Fig. 3*C* and Fig. S1*B*). Collectively, these data indicate that TLR8/9$^{-/-}$ mice show increased autoimmune phenotype compared with TLR8$^{-/-}$ or TLR9$^{-/-}$ mice, suggesting that both TLR8 and TLR9 contribute in lupus development in an additive manner.

Increased Response to R848 and Spontaneous Activation of TLR8$^{-/-}$ and TLR8/9$^{-/-}$ DCs and T Cells. We have shown previously that TLR8-deficiency in mice leads to lupus development because of increased TLR7 expression and signaling in DCs, but not in macrophages (23). Therefore, we evaluated the responses of bone marrow-derived dendritic cells (BM-DCs) to R848 (TLR7 ligand), LPS (TLR4 ligand), and poly I:C (TLR3 ligand). In response to R848, both TLR8$^{-/-}$ and TLR8/9$^{-/-}$ BM-DCs produced similar and significantly higher amounts of IL-6 (Fig. 4*A*) and TNF (Fig. S2*A*), compared with WT or TLR9$^{-/-}$ cells, whereas the response to LPS or poly I:C was similar in all four genotypes (Fig. 4*A* and Fig. S2*A*). Moreover, R848 stimulation induced IFN-β mRNA expression that was significantly higher in TLR8$^{-/-}$ and TLR8/9$^{-/-}$ BM-DCs vs. WT or TLR9$^{-/-}$ cells at 4 h, and by 8 h the expression was reduced and became similar in all four genotypes (Fig. 4*B*). The higher cytokine response of TLR8$^{-/-}$ and TLR8/9$^{-/-}$ BM-DCs compared with WT or TLR9$^{-/-}$ cells was accompanied by significantly higher TLR7 mRNA expression in untreated cells that was sustained upon R848 stimulation at 2 or 4 h (Fig. 4*C*). Bone marrow-derived macrophages from TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice produced normal levels of IL-6 in response to R848 or poly I:C (Fig. S2*B*).

We next tested the responses of BM-pDCs to R848, Pam_3CSK_4 (TLR2 ligand), and CpG (TLR9 ligand). TLR8$^{-/-}$ and TLR8/9$^{-/-}$ BM-pDCs showed similar but significantly higher IL-6 protein levels to R848 stimulation than WT or TLR9$^{-/-}$ cells, whereas all genotypes responded similarly to the TLR2 agonist (Fig. 4*D*). As expected, WT and TLR8$^{-/-}$ BM-pDCs showed similar response to CpG, but this response was absent in TLR8/9$^{-/-}$ or TLR9$^{-/-}$ cells (Fig. 4*D*). Regarding activation, untreated BM-pDCs had similar levels of CD86 or MHC II; however, upon R848 stimulation, TLR8$^{-/-}$ and TLR8/9$^{-/-}$ BM-pDCs showed higher activation than WT or TLR9$^{-/-}$ cells (Fig. 4*E*). Thus, ex vivo BM-DCs and BM-pDCs from TLR8$^{-/-}$ and TLR8/9$^{-/-}$ mice show higher response to R848 stimulation than their WT or TLR9$^{-/-}$ counterparts.

Next, we evaluated the status of splenic DCs and pDCs. No significant differences were observed regarding the percentages of splenic DCs (CD11c^{hi}MHC-II^{+}), pDCs (CD11c^{low}Siglec-H^{+} or B220^{+}CD11c^{low}), CD11b^{+}-like DCs (CD11c^{high}CD11b^{+} B220^{-}SIRPα^{+}), or CD8α^{+}-like DCs (CD11c^{hi}CD11b^{low} B220^{-}SIRPα^{-}CD24^{+}) (33) between the four genotypes (Fig. S2*D* and Table S1). Nevertheless, we found that TLR8$^{-/-}$ and TLR8/9$^{-/-}$ splenic pDCs showed increased CD86 expression upon R848 stimulation compared with WT or TLR9$^{-/-}$ cells, and CD11b^{+}-like DCs or CD8α^{+}-like DCs showed similar response in all four genotypes (Fig. S2*C*). Furthermore, we evaluated TNF and IL-12 cytokine production upon R848 stimulation by FACS analysis and found that TLR8$^{-/-}$ and TLR8/9$^{-/-}$ pDCs, as well as TLR8/9$^{-/-}$ CD11b-like DCs, produced significantly higher TNF amounts compared with WT or TLR9$^{-/-}$ cells (Fig. S3 *A* and *B*, and Table S4). TLR8$^{-/-}$ and TLR8/9$^{-/-}$ CD11b-like DCs produced also higher amounts of IL-12 compared with WT or TLR9$^{-/-}$ cells (Fig. S3*C* and Table S4). Nevertheless, the three splenic DC subsets showed similar TNF and IL-12 production in response to poly I:C stimulation in all four mouse genotypes (Fig. S3 and Table S4). Hence, certain splenic TLR8$^{-/-}$ and TLR8/9$^{-/-}$ DCs subsets show higher CD86 expression and ability

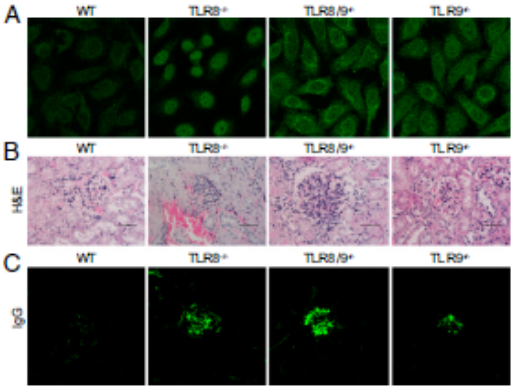

Fig. 3. Increased renal pathology in TLR8/9$^{-/-}$ mice compared with TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. (*A*) ANA staining patterns on Hep2 human epithelial cells for sera derived from 9-wk-old mice (Original Magnification 400×). Kidney sections from 5- to 6-mo-old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice were stained with (*B*) H&E (scale bars, 50 uM) or (*C*) immunofluorescence anti-IgG (Original Magnification 100×). Data in *A* and *C* are representative of two independent experiments (n = 3–4 per group).

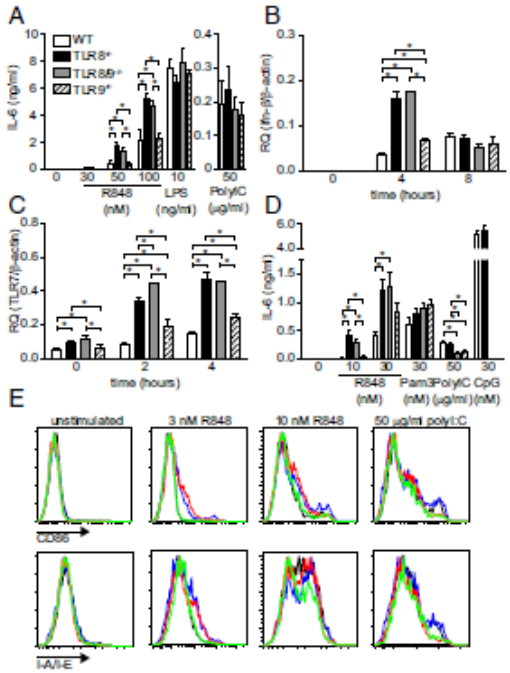

Fig. 4. Enhanced responses of TLR8$^{-/-}$ and TLR8/9$^{-/-}$ DCs and pDCs to TLR7 ligand. (*A*–*C*) BM-DCs from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice were stimulated with R848, LPS, or poly I:C. (*A*) After 16 h, the concentration of IL-6 in the culture supernatants was assessed by ELISA. (*B* and *C*) BM-DCs were left untreated or stimulated with 50 nM R848 for the indicated time points. Total RNA was extracted from the cells and the expression of (*B*) IFN-β or (*C*) TLR7 was assessed by quantitative PCR. (*D* and *E*) BM-pDCs from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice were stimulated with R848, Pam_3CKS_4, poly I:C, or CpG for 16 h. (*D*) The concentration of IL-6 in culture supernatants was assessed by ELISA. (*E*) The surface expression of CD86 and MHC class II was analyzed by flow cytometry on B220$^+$CD11c^{int}CD11b$^-$ cells from WT (black line), TLR8$^{-/-}$ (blue line), TLR8/9$^{-/-}$ (red line), and TLR9$^{-/-}$ (green line) mice. Data in all panels are representative of two to four independent experiments ($n = 3$–4 per group). *$P < 0.05$.

to produce TNF and IL-12 in response to R848 than WT or TLR9$^{-/-}$ cells.

Because DCs play a central role in T-cell activation, we also tested the activation status of splenic T cells and noticed a significantly increased percentage of CD44^{hi} CD4 T cells in TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice compared with WT controls (Fig. 5*A*), and of CD44^{hi} CD8 T cells in TLR8$^{-/-}$ and TLR8/9$^{-/-}$ compared with WT or TLR9$^{-/-}$ mice (Fig. 5*B*). Thus, the percentages of activated/memory CD4 and CD8 T cells are significantly increased in TLR8/9$^{-/-}$ mice compared with WT and TLR9$^{-/-}$ mice, and TLR8$^{-/-}$ mice show significant increased values compared with WT mice.

Increased Response to R848 Stimulation of TLR9$^{-/-}$ and TLR8/9$^{-/-}$ B Cells. The nucleic acid components of SLE autoantigens have previously been shown to activate B cells through TLR7 and contribute to lupus disease (34). To investigate the contribution of B cells in the lupus phenotype that we observed in TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice, total splenocytes were stimulated with R848 and the activation status of B cells were assessed by FACS analysis. Upon stimulation with R848, both TLR8/9$^{-/-}$ and TLR9$^{-/-}$ B cells showed a similar and significant up-regulation of CD69 and CD86, compared with TLR8$^{-/-}$ or WT B cells; all four genotypes showed similar response to LPS (Fig. 6*A* and Fig. S4*A*). Furthermore, TLR8/9$^{-/-}$ and TLR9$^{-/-}$ splenocytes showed increased production of IL-6 upon R848 stimulation compared with TLR8$^{-/-}$ or WT splenocytes, and all four genotypes responded similarly to LPS stimulation (Fig. 6*C*). Thus, upon TLR7-stimulation, TLR8/9$^{-/-}$ and TLR9$^{-/-}$ B cells in splenocytes are more responsive than WT or TLR8$^{-/-}$ cells. We wondered if this phenotype was accompanied by increased TLR7 expression, so we tested TLR7 expression in isolated B cells. To our surprise we found that TLR7 mRNA levels were significant higher in TLR8$^{-/-}$ and TLR8/9$^{-/-}$ B cells compared with WT or TLR9$^{-/-}$ cells (Fig. S4*B*). Moreover, we tested the responses of splenic TLR8$^{-/-}$, TLR8/9$^{-/-}$, TLR9$^{-/-}$, and WT isolated B cells and found that all four genotypes showed similar CD69 up-regulation and IL-6 production upon R848 or LPS stimulation (Fig. 6 *B* and *D*). Thus, in splenocyte cultures TLR8/9$^{-/-}$ and TLR9$^{-/-}$ B cells have the capacity to respond higher to R848 stimulation than TLR8$^{-/-}$ or WT B cells, but this capacity is lost in isolated B-cell cultures, suggesting that other factors that are provided from the surrounding splenocytes are necessary. Indeed, it has been shown previously that B-cell responses to the TLR7 ligand depend on type I IFNs (35, 36). Therefore, to test if the lost capacity of isolated TLR9$^{-/-}$ B cells to respond higher to TLR7 ligand than WT cells (as we observed with total splenocytes) could be restored by providing type I IFN, isolated B cells from WT and TLR9$^{-/-}$ mice were stimulated with R848 in the presence of IFN-α. Indeed, IFN-α increased the capacity of B cells to respond to the TLR7 ligand, as assessed by up-regulation of CD69; however, both TLR9$^{-/-}$ and WT isolated B cells showed similar activation (Fig. S4*C*). Overall, the data demonstrate an increased response of TLR8/9$^{-/-}$ and TLR9$^{-/-}$ B cells to TLR7 ligand, that is lost in isolated B cells and cannot be restored by IFN-α priming.

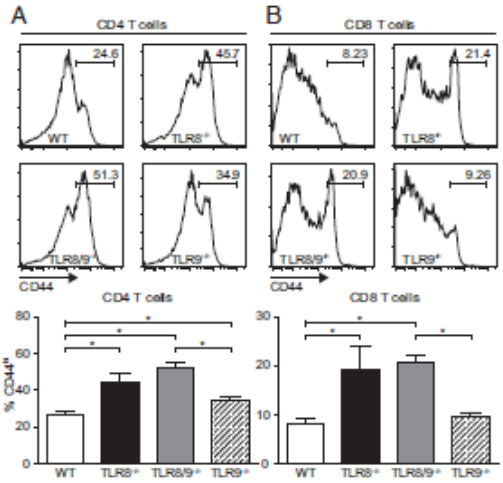

Fig. 5. Increased activated memory T cells in TLR8$^{-/-}$ and TLR8/9$^{-/-}$ mice. Flow cytometric histograms (*Upper*) and graphical analysis (*Lower*) on splenocytes from 7-mo-old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice, analyzed for the expression of CD3, CD4, CD8, and CD44. CD44 staining profiles of gated (*A*) CD4$^+$ and (*B*) CD8$^+$ T cells to identify activated memory (CD44^{hi}) subpopulations. Data are representative of three independent experiments ($n = 4$ per group). *$P < 0.05$.

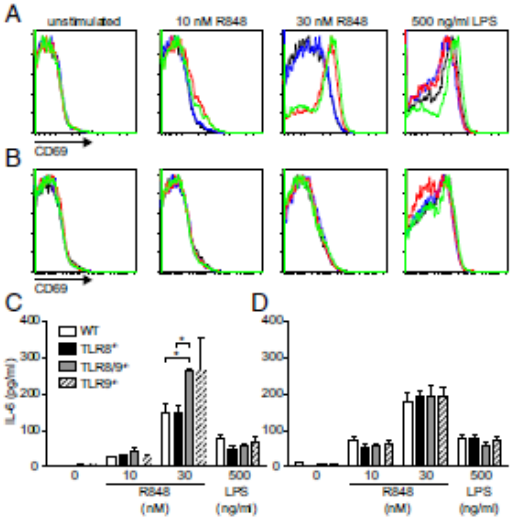

Fig. 6. Enhanced response of TLR9$^{-/-}$ and TLR8/9$^{-/-}$ B cells to TLR7 stimulation. Total splenocytes (*A* and *C*) or isolated B cells (*B* and *D*) from WT (black line), TLR8$^{-/-}$ (blue line), TLR8/9$^{-/-}$ (red line), and TLR9$^{-/-}$ (green line) mice were left untreated or stimulated with R848 or LPS for 16 h. Representative flow cytometry plots of CD69 on (*A*) CD11b^{+}B220^{+} splenocytes or (*B*) isolated B cells. IL-6 production on culture supernatants of (*C*) splenocytes or (*D*) isolated B cells. Data are representative of two to four independent experiments ($n = 3$ per group). *$P < 0.05$.

Discussion

Nucleic acid-sensing TLRs play an important role in SLE not only through their direct signaling upon ligand binding, but also by regulating the expression and function of each other. In the present study we show that in the C57BL/6 genetic background double deficiency for TLR8 and TLR9 leads to spontaneous lupus autoimmunity that is greater than the one that develop single TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. Interestingly, the lupus disease in the TLR8/9$^{-/-}$ mice is caused by increased TLR7 function, where both TLR8 and TLR9 keep under control TLR7 activity, but they act on different cell types. TLR8 controls TLR7 function on DCs, and TLR9 restrains TLR7 response on B cells. Moreover, we are unique in showing that TLR9$^{-/-}$ mice on the C57BL/6 background develop signs of autoimmunity that include splenomegaly, increased serum levels of IgM, and autoantibodies against DNA, RNA, and SmRNP increased frequencies of plasmablasts and renal immunodeposits compared with WT mice.

In vitro studies in HEK293 cells transfected with human TLRs in a pairwise combination have shown that both TLR8 and TLR9 inhibit TLR7, but not vice versa (37). Furthermore, our recent previous studies have revealed that the inhibitory action of TLR8 on TLR7 has important consequences in vivo, because TLR8-deficiency in mice leads to lupus development as a result of increased TLR7 function (23). Studies on C57BL/6 mice that carry the Yaa locus (B6.Yaa mice) revealed that TLR9-deficiency leads to increased TLR7 expression on B cells and BM-pDCs (20). In the same study the authors showed that TLR9-deficiency in C57BL/6 mice congenic for the Nba2 locus (B6.Nba2) in the presence of the Yaa mutation (TLR9$^{-/-}$ B6.Nba2.Yaa mice) is accompanied by increased TLR7-dependent activation of B cells and pDCs, suggesting that TLR9 controls TLR7 function both in B cells and pDCs. However, here we showed that in the C57BL/6 background TLR9-deficiency leads to increased TLR7-dependent activation of B cells, but not of DCs. These discrepancies might be because of the comparison of different autoimmune accelerating loci or might be the outcome of differences in ex vivo culturing conditions. We found that both TLR8$^{-/-}$ and TLR8/9$^{-/-}$ DCs and pDCs show similar but increased response to TLR7 ligand compared with TLR9$^{-/-}$ or WT cells. On the other hand, TLR9$^{-/-}$ and TLR8/9$^{-/-}$ B cells show similar but increased activation and response upon TLR7 stimulation compared with TLR8$^{-/-}$ or WT cells (Fig. 6). Hence, the increased lupus phenotype in TLR8/9$^{-/-}$ mice is the outcome of the additive effect of TLR8 and TLR9 on controlling TLR7 on DCs and B cells, respectively.

Our data suggest that in vivo both TLR8 and TLR9 control TLR7 function, but what is the mechanism by which the absence of TLR8 or TLR9 affect TLR7-mediated lupus-like disease? Unc93B1 associates with and delivers nucleic-acid–sensing TLRs, including TLR7, TLR8, and TLR9, from the endoplasmic reticulum to endolysosomes for ligand recognition; however, the mode of regulation of TLR localization differs for each TLR (30, 38). Elegant studies by the Miyake group have shown that there is a competition of TLR7 and TLR9 for Unc93B1-dependent trafficking, where TLR9 predominates and has higher affinity than TLR7 for Unc93B1 (39). Interestingly, a D34A mutation of Unc93B1 does not affect TLR7 or TLR9 expression, but leads to increased TLR7 trafficking, and mice that carry this D34A mutation develop TLR7-dependent systemic lethal inflammation because of increased response to TLR7 ligand (32). Thus, Unc93B1 controls homeostatic TLR7 activation by balancing TLR9 to TLR7 trafficking. We can hypothesize that TLR8 or TLR9-deficiency decreases the competition of endosomal TLRs for association with Unc93B1 and allows higher availability for TLR7, which results to increased TLR7 trafficking and response that ultimately leads to autoimmunity. In addition, we have shown previously that in the C57BL/6 background TLR8-deficiency leads to TLR7 overexpression in DCs, B cells, macrophages, and MZ B cells (23). However, from all of these cell types only TLR8$^{-/-}$ DCs show increased response to TLR7 stimulation, whereas macrophages, B cells or MZ B cells have a normal response (23). Thus, depending on the cell-type deletion of TLR8 or TLR9 may alter not only TLR7 trafficking, but also TLR7 expression, which dictate TLR7's ultimate function. Keeping in mind the complexity in expression pattern of the TLRs in a particular cell type and cell compartments, the variation in distribution and response to a given stimuli in different cell types, it is not surprising that TLR8 and TLR9 may control TLR7 function by mechanisms that can vary depending on the cell type.

Cumulative data suggest that from the nucleic-acid–sensing TLRs, TLR7 seems to be the most pathogenic regarding lupus (40); possible explanations could be the increased availability of exogenous or endogenous RNA-containing particles that can be sensed by TLR7 or stronger downstream signaling by TLR7 compared with TLR8 or TLR9. Most of the evidence that endosomal TLRs are implicated in SLE and that tight control and regulation of TLR7 is pivotal for avoiding SLE and inflammatory pathology is coming from studies in various lupus-prone mouse models. However, in our current studies we have used mice on the C57BL/6 background, which is not prone to lupus; we were still able to demonstrate that TLR7 dysregulation because of TLR8- and TLR9-deficiency can lead to autoreactivity and inflammatory pathology. In humans, recent data also incriminate TLR7 as an essential player in SLE pathology. Indeed, two independent studies reported that the SNP rs3853839 (C-G) at the 3′ untranslated region of TLR7 is associated with elevated transcript expression and increased risk for SLE in Eastern Asians (25, 26). Interestingly, further studies revealed that the risk G allele is also linked with increased TLR7 protein levels, and is located within a predicted binding site of microRNA-3148 that is most likely responsible for the observed association with SLE in three populations of non-Asian ancestry (24). Furthermore, increased TLR7 copy number is a risk factor for childhood-onset of SLE in the Mexican population, and correlates significantly with increased TLR7 and IFN-α mRNA levels (41). However, no correlation with

SLE has been found regarding the TLR7 SNP rs179008 in Spanish population or variations in the copy number of the TLR7 (42, 43).

In conclusion, TLR8 and TLR9, on the top of their importance in direct signaling upon ligand recognition, also have an essential role in keeping under control TLR7 function to prevent spontaneous triggering of harmful autoreactive and inflammatory responses. Thus, the mechanisms by which TLR8 and TLR9 control TLR7 seem to depend on the given TLR and cell type, and these important TLR interactions have to be taken into account when novel therapeutic approaches are developed that target the blocking of one versus the other endosomal TLR to avoid unwanted consequences.

Materials and Methods

Detailed materials and methods are provided in *SI Materials and Methods*.

Mice and Serological Analysis. Double TLR8/9$^{-/-}$ mice were generated by intercrossing TLR8$^{-/-}$ with TLR9$^{-/-}$ mice (11, 23). All mice used in the studies were in the C57BL/6 background. Evaluation of IgM and IgG2a, and IgG autoantibodies on serum samples were performed as described previously (23).

Preparation of Cells and Quantification of Cytokines. Bone marrow cells from mice were cultured with GM-CSF or M-CSF for the production of BM-DCs or BM-macrophages, respectively, as previously described (23). IL-6 and TNF in culture supernatants were measured by ELISA kits.

RNA Isolation and Quantitative PCR. Total RNA was isolated with TRIzol reagent or RNA easy kit, reverse-transcribed with Superscript II reverse transcriptase and quantitiative PCR for TLR7, IFN-β, and β-actin was performed as described previously (23).

Flow Cytometric Analysis. Cell suspensions were incubated with 24G2 hybridoma supernatant and then stained using immunofluorescence BM-DC antibodies. For intracellular cytokine staining, cells were fixed with Cytofix/Cytoperm and stained using antibodies for TNF and IL12p40/p70. Flow cytometry was conducted using an LSR2 and data were analyzed with FlowJo.

Histology, Immunofluorescence, and ANA Staining. Kidneys were fixed in formalin, embedded in paraffin, and tissue sections were stained with H&E or periodic acid-Schiff. Renal biopsies were analyzed in a blinded fashion by a pathologist. Immunofluorescence IgG and IgM staining on kidney sections was performed as described previously (23). ANA in mouse sera were tested with Hep2 cells fixed on slides.

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Supporting Information

Desnues et al. 10.1073/pnas.1314121111

SI Materials and Methods

Mice. Toll-like receptor (TLR)8$^{-/-}$ and TLR9$^{-/-}$ mice were generated as described previously (1, 2). TLR9$^{-/-}$ mice were obtained from S. Akira (Osaka University, Osaka, Japan). Double TLR8/9$^{-/-}$ mice were generated by intercrossing TLR8$^{-/-}$ with TLR9$^{-/-}$ mice. All three TLR-deficient mouse genotypes were backcrossed on the C57BL/6 background for more than 10 generations. C57BL/6 mice were purchased from the Charles River Laboratories. Unless otherwise specified, age- and sex-matched mice were used for each experiment. Mice were housed under specific pathogen-free conditions at the Centre d'Immunologie de Marseille-Luminy and experiments were conducted in accordance with institutional guidelines for animal care and use and European directives.

Reagents. R848, LPS from *Escherichia coli* 0111-B4, Pam_3CSK_4, CpG ODN 1826, and poly I:C were purchased from Invivogen. Recombinant mouse IFN-α was from Calbiochem.

Preparation of Cells and Quantification of Cytokines. Bone marrow cells from mice were extracted from femur and tibias and cultured with GM-CSF or M-CSF for the production of bone marrow dendritic cells (BM-DCs) or BM-macrophages, respectively as previously described (1). For the production of bone marrow-plasmacytoid DCs (pDC), bone marrow cells depleted of erythrocytes were cultured in RPMI, 10% (vol/vol) FBS, 2 mM GlutaMAX (Gibco), 1 mM sodium pyruvate, 1% (vol/vol) MEM Non-Essential Amino Acids (Gibco), 50 μM β-mercaptoethanol, and 2.5% (vol/vol) B16-Flt3L cell line supernatant (source of Flt3 ligand) at 2×10^6 cells/mL in six-well plates for 9 d (3). B cells were purified from spleen by negative selection using the B-cell isolation kit (Miltenyi). Purity was assessed by FACS and above 96%. Cytokines levels in culture supernatants were measured by IL-6 and TNF (eBioscience) ELISA kits. For intracellular cytokine staining splenocytes were treated with the indicated stimuli in the presence of Brefeldin A (e-Biosciences) for 4 h.

RNA Isolation and Quantitative PCR. Total RNA was isolated with TRIzol reagent (Gibco, Invitrogen) or RNA easy kit (Qiagen). RNA was reversed-transcribed with Superscript II reverse transcriptase (Invivogen) and quantitative PCR for TLR7, IFN-β, and β-actin was performed as described previously (1).

Serological Analysis. Evaluation of IgM and IgG2a, and IgG autoantibodies against DNA, RNA, ribonucleoprotein (RNP) and Smith ribonucleoprotein (smRNP) on serum samples were performed as described previously (1).

Flow Cytometric Analysis. Cell suspensions were incubated with 24G2 hybridoma supernatant and then stained using immunofluorescence-labeled antibodies against the following antigens: B220, CD3, CD4, CD8, NK1.1, CD19, CD11b, CD11c, Ly6G, Ly6C, CD21, CD23, CD5, CD44, CD86, CD38, CD138, CD24, and CD172a (SIRPα) from BD Biosciences, IA/IE (MHC class II), GL7 from eBioscience, and SiglecH from Biolegend. For intracellular cytokine staining cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 20 min on ice and stained using immunofluorescence-labeled antibodies for TNF and IL12p40/p70 (BD Biosciences). Flow cytometry was conducted using an LSR2 (BD Biosciences) and data were analyzed with FlowJo (Tree Star).

Histology, Immunofluorescence, and Antinuclear Antibody Staining. For histopathology studies, kidneys were fixed in formalin and embedded in paraffin. For light microscopy, 3- to 4-μm-thick tissue sections were stained with H&E and periodic acid-Schiff. To determine the extent of renal damage, all renal biopsies were analyzed in a blinded fashion by a pathologist. Typical glomerular active lesions of lupus nephritis were evaluated: glomerular cellularity (mesangial cell proliferation, endocapillary proliferation, and inflammatory cells), glomerular deposits, extracapillary proliferation, as well as, tubulointerstitial chronic lesions: tubular atrophy, interstitial fibrosis, and interstitial infiltrates. Lesions were graded semiquantitatively using a scoring system from 0 to 4 (0, no changes; 1, mild; 2, moderate; 3, high; 4, severe). The total histological score for each specimen was derived from the sum of all of the described parameters. Immunofluorescence IgG and IgM staining on kidney sections was performed as described previously (1). Antinuclear antibodies (ANA) in mouse sera were tested with Hep-2 cells fixed on slides (Biomedical Diagnostics). Sera were diluted in PBS 1:60 and an Alexa Fluor 488 anti-mouse IgG antibody (Invitrogen) was used to detect mouse antibodies.

Statistical Analysis. Statistics were calculated using Prism 5 (GraphPad Software) by Mann–Whitney U test with P values indicated throughout as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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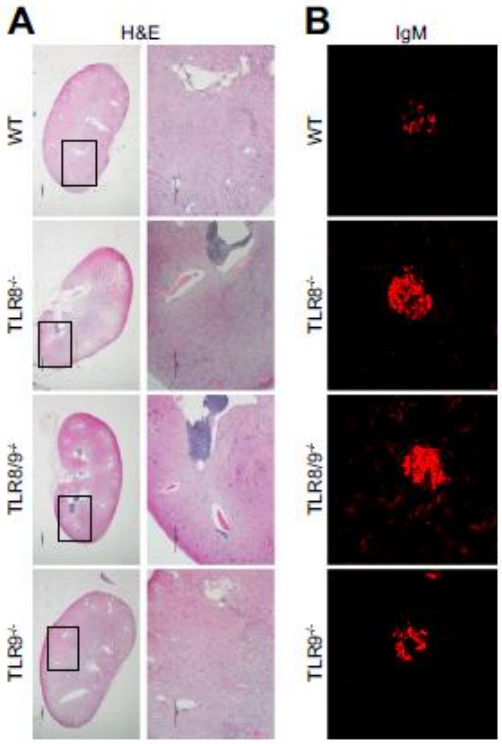

Fig. S1. Increased renal pathology in TLR8/9$^{-/-}$ mice compared with TLR8$^{-/-}$ or TLR9$^{-/-}$ mice. (*A*) H&E-stained kidney sections from 6 mo old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice ($n = 3$ per group). (*Left*) (Scale bars, 1 mm.) (*Right*) Magnified details of black bordered areas. (Scale bars, 0.5 mm.) (*B*) Kidney sections from 5-mo-old female WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, or TLR9$^{-/-}$ mice ($n = 3$ per group) were stained with immunofluorescence anti-IgM (original magnification × 100).

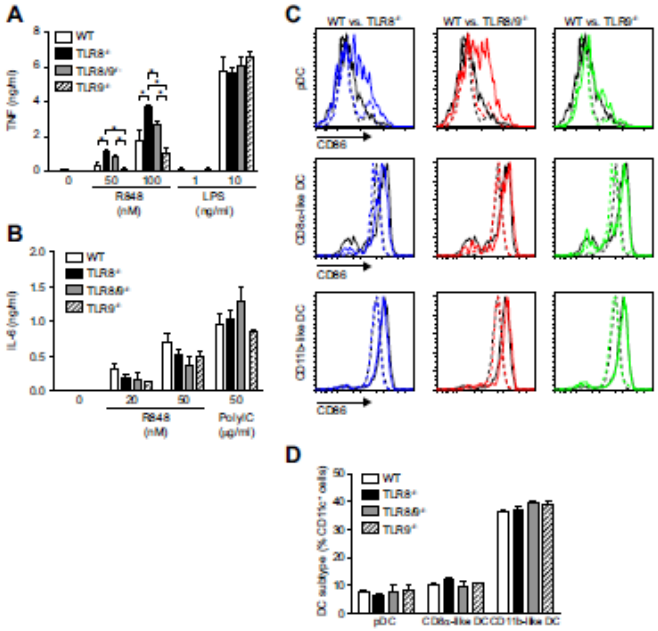

Fig. S2. Cytokine production and CD86 expression by WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ DCs or macrophages. (*A*) BM-DCs or (*B*) BM-macrophages from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice left untreated or stimulated with the indicated amounts R848, LPS, or poly I:C. After 16 h, the concentration of TNF (*A*) or IL-6 (*B*) in culture supernatants was assessed by ELISA. Data are representative of two to three independent experiments ($n = 3$ per group). (*C*) Total splenocytes were left untreated or stimulated with 30 nM R848. After 16 h, the surface expression of CD86 was analyzed by FACS analysis on splenic pDCs, CD8α-like DCs, or CD11b-like DCs from WT (black line), TLR8$^{-/-}$ (blue line), TLR8/9$^{-/-}$ (red line), or TLR9$^{-/-}$ (green line) mice ($n = 3$ mice per group). Untreated cells (dotted lines), R848 treated cells (full lines). $n = 3$ mice per group. (*D*) Frequencies of splenic pDCs, CD8α-like DCs, or CD11b-like DCs in the four genotypes. Data are average percentages ± SD ($n = 3$ mice per group). *$P < 0.05$.

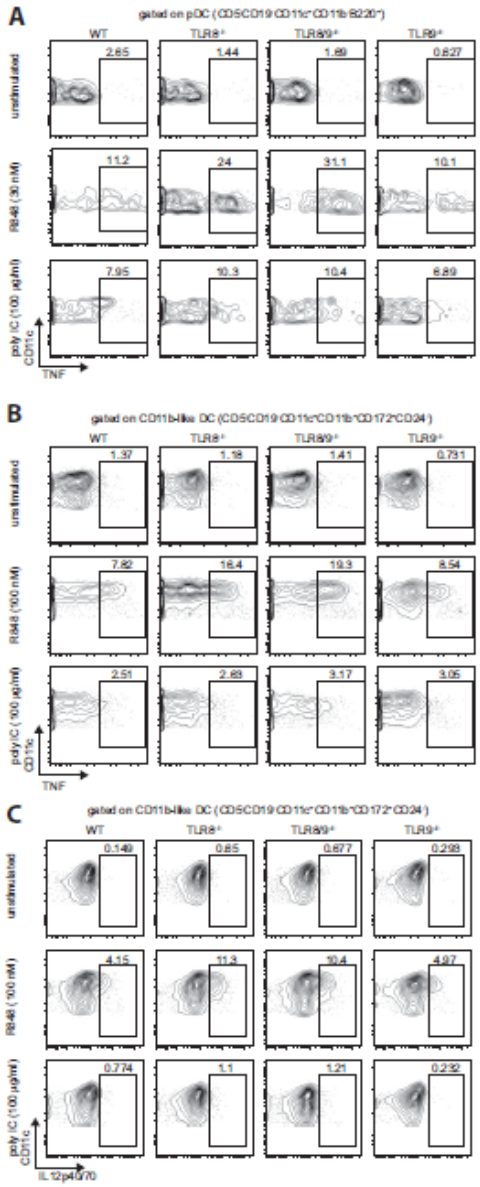

Fig. S3. Analysis of TNF and IL-12p40/70 cytokine production by splenic pDCs and CD11b-like DCs. Total splenocytes were left untreated or stimulated with R848 or poly I:C. After 4 h, intracellular staining for TNF and IL-12p40/p70 was performed. Expression of TNF was evaluated after gating on (*A*) pDCs and (*B*) CD11b-like DCs, and expression of IL-12P40/70 was evaluated after gating on (*C*) CD11b-like DCs from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, or TLR9$^{-/-}$ mice ($n = 3$ per genotype). Numbers in dot plots represent the percentage of cells that produce TNF or IL-12. Plots are representative of the data shown in Table S4.

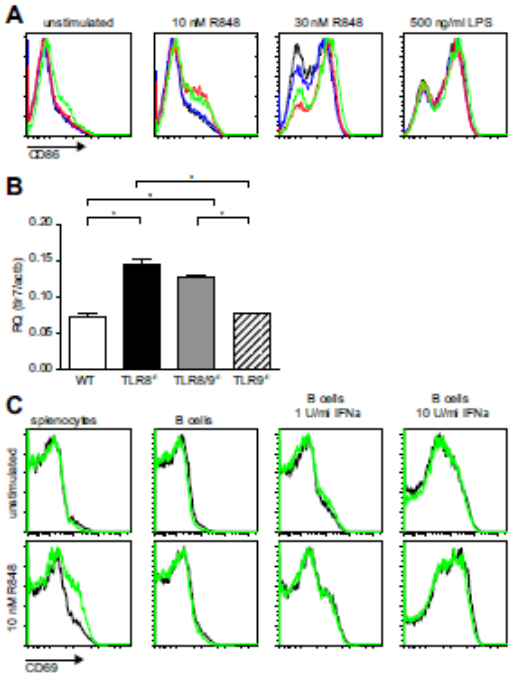

Fig. S4. CD86 expression by splenocytes and TLR7 expression and response of isolated B cells from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice. (*A*) Total splenocytes were left untreated or stimulated with R848 or LPS for 16 h. Representative flow cytometry plots of CD86 on WT (black line), TLR8$^{-/-}$ (blue line), TLR8/9$^{-/-}$ (red line), and TLR9$^{-/-}$ (green line) CD119^{+}B220^{+} splenocytes. (*B*) Total RNA was extracted from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, or TLR9$^{-/-}$ splenic isolated B cells and the expression of TLR7 and β-actin were evaluated by quantitative PCR. *$P < 0.05$. (*C*) Splenocytes or isolated B cells from WT (black line) or TLR9$^{-/-}$ (green line) mice were left untreated or stimulated with the indicated amount of R848 in the presence or absence of IFN-α. The surface expression of CD69 was analyzed by flow cytometry on CD19^{+} cells. Data are representative of two independent experiments ($n = 3$ per group).

Table S1. Spleen weight, total cell count, and major cell populations of WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice

		WT	TLR8$^{-/-}$	TLR8/9$^{-/-}$	TLR9$^{-/-}$
Spleen weight (mg)		77.0 ± 19.5	116.9 ± 29.1*,†	145.7 ± 50.8*,†,§	103.7 ± 17.8*,†
Total cell count (×10^{6})		67.7 ± 26.4	108.6 ± 32.8*,†	143.0 ± 46.8*,†	113.4 ± 34.1*
Cell type	Cell surface markers	Frequency (% of total)			
T cells	CD3^{+}	25.7 ± 3.7	20.7 ± 5.4§	21.3 ± 3.4§	26.1 ± 1.8†,‡
CD4 T cells	CD3^{+}/CD4^{+}	14.5 ± 2.7	12.3 ± 3.0§	12.8 ± 1.7§	15.9 ± 1.2†,‡
CD8 T cells	CD3^{+}/CD8^{+}	9.7 ± 1.3	6.7 ± 2.3*	6.5 ± 1.6*	8.4 ± 1.1
NKT cells	CD3^{+}/NK1.1^{+}	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	ND
NK cells	CD3^{-}/NK1.1^{+}	2.2 ± 0.1	1.8 ± 0.4	1.5 ± 0.2*	ND
B cells	CD19^{+}	58.8 ± 3.7	65.9 ± 5.1*	66.8 ± 5.5*	63.4 ± 6.0
CD11c^{+} cells	CD11c^{+}	3.9 ± 0.4	5.0 ± 0.5*	5.3 ± 0.6*	5.4 ± 0.9
DC	CD11c^{hi}/IA-IE^{+}	1.6 ± 0.1	1.8 ± 0.1	2.1 ± 0.5	2.0 ± 0.1
pDC	CD11c^{int}/SiglecH^{+}	0.7 ± 0.1	0.9 ± 0.3	0.8 ± 0.3	1.0 ± 0.2
Monocytes	CD11b^{+}/Ly6G^{-}/Ly6C^{+}	0.4 ± 0.3	1.1 ± 0.4*	0.8 ± 0.2§	1.1 ± 0.2*,†
Neutrophils	CD11b^{+}/Ly6G^{+}/Ly6C^{-}	0.4 ± 0.3	0.9 ± 0.2*,†	0.5 ± 0.3§	0.9 ± 0.2*

Data are from 4- to 7-mo-old male or female mice. No differences were observed between males and females. Values shown are average of at least four mice ± SD. ND, not determined.
*$P < 0.05$ vs. WT.
†$P < 0.05$ vs. TLR8/9$^{-/-}$.
‡$P < 0.05$ vs. TLR8$^{-/-}$.
§$P < 0.05$ vs. TLR9$^{-/-}$.

Table S2. Frequency of splenic marginal zone (MZ) B cells and peritoneal B1a and B1b B cells of WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice

Genotype	MZ B cells	B1a B cells	B1b B cells
WT	4.7 ± 0.2 (n = 8)	18.8 ± 1.2 (n = 9)	10.9 ± 0.4 (n = 5)
TLR8$^{-/-}$	2.1 ± 0.2*,† (n = 8)	9.1 ± 1.2*,†,§ (n = 8)	5.1 ± 1.0† (n = 4)
TLR8/9$^{-/-}$	1.5 ± 0.3*,† (n = 8)	4.7 ± 0.6*,†,§ (n = 7)	7.2 ± 1.7*,† (n = 3)
TLR9$^{-/-}$	4.8 ± 0.5‡,§ (n = 3)	14.9 ± 1.5‡,§ (n = 7)	11.7 ± 0.8‡,§ (n = 3)

Data are from 4- to 7-mo-old female mice. Values shown are average ± SD. In parenthesis is the number of mice per group.
*P < 0.05 vs. WT.
†P < 0.05 vs. TLR9$^{-/-}$.
‡P < 0.05 vs. TLR8/9$^{-/-}$.
§P < 0.05 vs. TLR8$^{-/-}$.

Table S3. Renal scores for WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ female 6-mo-old mice

	Glomerular lesions			Tubulo-interstitial lesions			
Genotype	Glomerular cellularity	Extracapillary proliferation	Glomerular depositis	Tubular atrophy	Fibrosis	Interstitial infiltration	Total score
WT	0	0	0	0	0	0	0
TLR8$^{-/-}$	1.33 ± 0.57	0	1.00 ± 0.00	0	0	0.66 ± 0.57	2.9
TLR8/9$^{-/-}$	1.66 ± 0.57	0.33 ± 0.57	1.66 ± 0.57	0	0	1.00 ± 0.00	4.5
TLR9$^{-/-}$	1.00 ± 0.00	0	1.00 ± 0.00	0	0	0	2

Pathologist scored H&E and periodic acid-Schiff stained paraffin embedded kidney sections displayed as mean ± SD, n = 3 mice per group shown. Pathologist scoring method and definitions can be found in *SI Materials and Methods*.

Table S4. Percentages of TNF- and IL-12p40/p75-expressing splenic pDCs, CD8α^{+}-like DCs, and CD11b^{+}-like DCs from WT, TLR8$^{-/-}$, TLR8/9$^{-/-}$, and TLR9$^{-/-}$ mice upon R848 or poly I:C stimulation

Genotype	Untreated	R848	Poly I:C	Untreated	R848	Poly I:C
	TNF by pDC			IL-12 by pDC		
WT	2.5 ± 0.7	10.9 ± 2.3	7.4 ± 1.3	0.3 ± 0.2	5.0 ± 2.8	0.4 ± 0.2
TLR8$^{-/-}$	1.4 ± 0.1*	44.1 ± 21.5*,†	10.2 ± 1.3*	0.5 ± 0.2	8.2 ± 1.6	0.9 ± 0.1*
TLR8/9$^{-/-}$	2.9 ± 1.4*	30.1 ± 4.0*,†	12.9 ± 4.5	0.2 ± 0.1	12.4 ± 2.1*	1.2 ± 0.4
TLR9$^{-/-}$	0.8 ± 0.1†,‡,§	11.5 ± 0.7‡,§	5.8 ± 0.6‡	0.2 ± 0.1	4.9 ± 1.0§	0.6 ± 0.2‡
	TNF by CD8α^{+}-like DC			IL-12 by CD8α^{+}-like DC		
WT	3.5 ± 2.3	2.7 ± 1.5	6.7 ± 0.8	2.1 ± 1.0	3.4 ± 1.4	10.9 ± 2.4
TLR8$^{-/-}$	1.0 ± 0.3	1.5 ± 0.3	9.7 ± 1.5	4.2 ± 0.8	5.4 ± 0.6	13.8 ± 0.4*
TLR8/9$^{-/-}$	1.3 ± 0.1*	2.1 ± 0.3*	7.2 ± 0.4	4.4 ± 0.1*,†	5.4 ± 0.8	13.2 ± 1.4
TLR9$^{-/-}$	0.9 ± 0.1†,§	1.2 ± 0.2§	8.2 ± 1.9	3.8 ± 0.2†,§	4.5 ± 0.8	9.9 ± 1.5‡
	TNF by CD11b^{+}-like DC			IL-12 by CD11b^{+}-like DC		
WT	2.1 ± 1.1	7.1 ± 1.9	2.1 ± 0.2	0.1 ± 0.0	3.9 ± 1.5	0.7 ± 0.1
TLR8$^{-/-}$	1.2 ± 0.2*	14.6 ± 3.7	2.2 ± 0.1§	0.6 ± 0.1*,†	10.7 ± 0.6*,†	1.0 ± 0.2
TLR8/9$^{-/-}$	1.6 ± 0.4*	16.5 ± 1.4*,†	3.0 ± 0.2†,§	0.6 ± 0.1*,†	12.9 ± 1.9*,†	0.8 ± 0.3
TLR9$^{-/-}$	0.7 ± 0.1†,‡,§	8.1 ± 1.2§	2.4 ± 0.4	0.2 ± 0.1‡,§	5.3 ± 1.0‡,§	0.1 ± 0.1

Data represent the average percentage ± SD of TNF or IL-12p40/p70- expressing pDCs, CD8α^{+}-like DCs, or CD11b^{+}-like DCs after stimulation of splenocytes from three mice per group with 30 nM R848 for pDCs and CD8α^{+}-like DCs, 100 nM R848 for CD11b^{+}-like DCs, or 100 μg/mL poly I:C.
*P < 0.05 vs. TLR9$^{-/-}$.
†P < 0.05 vs. WT.
‡P < 0.05 vs. TLR8/9$^{-/-}$.
§P < 0.05 vs. TLR8$^{-/-}$.

Aim 2: Investigate the implication of Gfi-1 in lupus and TLR signaling by studying Genista mice

Introduction

Toll-like receptors (TLRs) sense microbial components as well as endogenous molecules, mostly released by dead cells, and play a critical role in activating the innate and adaptive immune responses (Kawai and Akira, 2010). The main consequence of this sensing is the production of inflammatory cytokines and type-I interferons. TLRs play a role not only in antimicrobial defense, but also to the induction of autoimmunity, including systemic lupus erythematosus (SLE) (Kim et al., 2009). Activation of TLRs is regulated to avoid disruption of immune homeostasis and negative regulators act at different levels in the TLR signaling pathways (Kondo et al., 2012). Several lines of experimental data support a role for the nuclear transcriptional factor Gfi-1 as a regulator of the TLR4 pathway, and as an essential modulator preventing an exaggerated inflammatory response to LPS stimulation (Karsunky et al., 2002a).

Gfi-1 is a transcriptional repressor that acts by binding to DNA recognition sequences in target genes promoters (Grimes et al., 1996). Gene knock out studies revealed that Gfi-1 is important in many cellular processes such as the development of granulocytes and in macrophage-dependent cytokine production (Karsunky et al., 2002a). Gfi-$1^{-/-}$ mice are susceptible to LPS-induced septic shock and stimulation of Gfi-$1^{-/-}$ macrophages with LPS leads to an increased production of proinflamatory cytokines, including TNF-α and IL-1β (Karsunky et al., 2002b). The explanation for these observations is that Gfi-1 binds to the p65subunit of NF-κB and controls its DNA binding activity, thereby regulating the expression of a large numbers of NF-κB target genes (Sharif-Askari et al., 2010). Interestingly, it has been reported that Gfi-$1^{-/-}$ mice have higher levels of IgG1, IgG2a and IG2b than WT mice, suggesting that Gfi-1 might be also implicated in autoimmune responses (Igwe et al., 2008).

Based on the data regarding the importance of TLR7 in the immunopathology of SLE and the fact that Gfi-1 seems to be implicated in autoimmune pathology, the goal was

to study the role of the transcriptional repressor Gfi-1 in TLR signaling and lupus-development. To do so, we studied Genista mice that carry a hypomorphic mutation of the Gfi-1 gene (Ordonez-Rueda et al., 2012). Our studies revealed that Genista develop lupus that is TLR7-dependent, while *in vitro* studies demonstrated that DCs from Genista mice produced higher levels of IFN-β upon R848 or LPS stimulation. Therefore, our results suggest that Gfi-1 acts as a transcriptional repressor downstream of TLRs, and controls not only NF-κB genes like TNF-α, but also type-I IFN expression.

Results

Genista mutation leads to development of TLR7-dependent lupus

To determine whether Genista mice that carry a point mutation in Gfi-1 gene develop lupus, we used Genista mice in the C57Bl/6 background (Ordonez-Rueda et al., 2012). These mice are viable, fertile and do not show any obvious phenotypical alterations. First, we evaluated by ELISA the levels of IgM and anti-RNA autoantibodies in sera of 9 weeks old female WT and Genista mice. Genista mice presented significantly increased levels of IgM (**Figure 16A**) and anti-RNA autoantibodies (**Figure 16B**) compared to WT mice. Kidney sections from WT and Genista mice stained with immunofluorescence anti-IgG antibody revealed that Genista mice had an increased deposition of IgG in their kidneys compared to WT mice (**Figure 16C**). Thus, Genista mice develop lupus.

Based on the importance of TLR7 in the immunopathology of lupus in mice (Demaria et al., 2010), we were interested to evaluate if the lupus phenotype in Genista mice is TLR7 dependent. To do so, we crossed Genista mice with TLR7$^{-/-}$ mice on the C57BL/6 background and we generated Genista/TLR7$^{-/-}$. These mice did not present any phenotypical abnormalities up to 6 months of age, so we were able to use them for our studies. First, serum levels of IgM and IgG2a isotypes were assessed by ELISA. The sera from Genista mice showed significantly increased IgM and IgG2a levels compared to sera from WT mice, whereas Genista/TLR7$^{-/-}$ sera had normal levels (**Figure 17A**). Next, we evaluated the levels of IgG autoantibodies against

dsDNA, RNA and Smith ribonucleoprotein (SmRNP) and found that Genista mice presented increased titers compared to WT mice (**Figure 17B**), whereas Genista/TLR7$^{-/-}$ mice resembled WT mice (**Figure 17B**). These data suggest that the increased immunoglobulins and autoantibody production observed in Genista mice is TLR7-dependent. Moreover, we investigated the frequencies of plasmablasts and germinal centers (GCs) in 4 months old WT, Genista and Genista/TLR7$^{-/-}$ by flow cytometry. We found that frequencies of both plasmablasts and GCs were significantly increased in Genista compared to WT or Genista/TLR7$^{-/-}$ mice (**Figure 17 C and D and Table3**). Next, we evaluated the deposition of IgG in kidneys and noticed that Genista mice had increased deposition compared to WT mice, while Genista/TLR7$^{-/-}$ mice presented decreased deposition versus WT mice (**Figure 17E**). Thus, our data suggest that Genista mice develop lupus that is TLR7-dependent.

It has been shown MZ and B1 B (B1a and B1b) cells are altered in lupic mice and that TLR7 plays an important role (Demaria et al., 2008; Amano et al, 2003; Deane et al 2007). So we evaluated theses populations and found that both Genista and Genista/TLR7$^{-/-}$ mice had a significant increase of the splenic MZ B cells frequencies compared to WT mice (**Figure 18A and Table 4**). In the case of peritoneal cavity B1 B cells, we found a dramatic reduction of both B1a (B220^{lo}CD5^{int}) and B1b (B220^{lo}CD5^{lo}) B cells, of these cell populations in Genista and Genista/TLR7$^{-/-}$ mice compared to WT mice. However the frequency of B1 B cells in Genista/TLR7$^{-/-}$ mice were statistical significant increased compared to Genista mice (**Figure 18B and Table 4**), suggesting that TLR7 signaling contributes partially to the reduction of B1 B cells in Genista mice.

Frequencies and activation status of immune cells in Genista, Genista/TLR7$^{-/-}$ and WT mice

DCs, pDCs, B cells and T cells play an important role in the pathophysiology of SLE, so we evaluated the frequencies and activation status of these cells by flow cytometry in WT, Genista and Genista/TLR7$^{-/-}$ spleens. We found that both cDCs and pDCs frequencies were significantly increased in Genista mice compared to WT mice,

while in the Genista/TLR7$^{-/-}$ mice the frequencies of these cells were normal (**Figure 19A**). Next, we accessed the activation status of cDCs (CD11-b like and CD8α-like) and pDCs by evaluating CD86 expression. We found that the CD11-b like and pDCs expressed higher CD86 levels in Genista spleens than WT mice, while Genista/TLR7$^{-/-}$ behaved similar to WT (**Figure 19B**). In the case of CD8α–like DCs, both WT and Genista cells presented similar CD86 activation, while Genista/TLR7$^{-/-}$ cells presented reduced activation compared to Genista cells (**Figure 19B**). Next, we tested the frequencies of naïve (CD62L^{+}CD44^{-}) and memory-like (CD62L^{-}CD44^{hi}) splenic CD4 and CD8 T cells populations. Genista presented significant reduction of naïve CD4 T cells compared to WT mice. Interestingly, Genista/TLR7$^{-/-}$ mice had also reduced CD4 naïve T cells compared to WT mice, but showed a significant increase compared to Genista mice (**Figure 19C**). The frequency of naïve CD8 T cells was similar in Genista and Genista/TLR7$^{-/-}$ mice and significant reduced compared to WT mice. The memory-like CD4 T cells were increased in Genista mice versus WT and this phenotype was partially TLR7-dependent for CD4 T cells, since there is a reduction of the frequency in Genista/TLR7$^{-/-}$compared to Genista mice but the frequency is still significant higher than in WT mice (**Figure 19D**). The CD8 T memory-like cells, Genista and Genista/TLR7 were similar between them and higher than WT mice (**Figure 19D**). Moreover, Genista mice showed higher CD86 expression on B cells compared to WT or Genista/TLR7$^{-/-}$ cells (**Figure 19E**).
In addition, since TLR7 is important for the lupus development in Genista mice, we tested the expression of TLR7 in spleens derived from WT, Genista and Gfi-1$^{-/-}$ mice. All three mouse groups presented similar levels of splenic TLR7 mRNA expression (**Figure 20**).

Role of Gfi-1 as a transcriptional repressor in TLR signaling

Previous studies have shown that upon LPS (TLR4 ligand) stimulation, expression of Gfi-1 is induced in macrophages and that Gfi-1 is a negative regulator of endotoxin-initiated innate immune response via NF-κB (Sharif-Askari et al., 2010). However, it is still unknown if Gfi-1 expression is induced by all TLRs and if Gfi-1 acts as a

negative regulator downstream of all TLRs. To address these issues, we first evaluated Gfi-1 mRNA expression in WT BMMs and BMDCs upon stimulation with R848 (TLR7 ligand), LPS (TLR4 ligand), Pam_3CSK_4 (TLR1/2 ligand), poly I:C (TLR3 ligand) and CpG (TLR9 ligand) for 0, 2, 4, 6 and 16 hours using semi-quantitative RT-PCR. In BMMs, Gfi-1 was expressed upon stimulation with R848 (at 4 and 6 hours), LPS (at 6 and 16 hours) and Pam_3CSK_4 (at 6 hours), but not upon poly I: C or CpG stimulation (**Figure 21A**). In BMDCs, Gfi-1 was expressed upon stimulation with R848 (at 4 and 6 hours), LPS (at 4, 6 and 16 hours), Pam_3CSK_4 (at 4 hours) and poly I: C (at 6 and 16 hours) (**Figure 21B**).

Next, we performed an electrophoretic mobility shift assay (EMSA) to check NF-κB binding in nuclear extracts from WT, Genista and Gfi-$1^{-/-}$ BMMs stimulated with LPS or R848 for 30 and 60 minutes. Similar to previous published data, we observed that upon LPS stimulation, Gfi-$1^{-/-}$ BMMs presented increased NFκ-B binding compared to WT cells (**Figure 22A**). Moreover we found that R848 also induced increased NFκ-B binding in Gfi-$1^{-/-}$ cells to WT BMMs (**Figure 22A**). However, upon stimulation with R848 or LPS Genista BMMs showed similar NFκ-B binding like WT cells (**Figure 22A**).

Injection of LPS in Gfi-$1^{-/-}$ mice leads to increased sera levels of TNF-α (Karsunky et al., 2002). As we found that Gfi-1 is downstream of TLR7, we injected WT and Genista mice with R848 and collected sera after 2 and 4 hours and accessed the levels of TNF-α. We observed that in both time-points, Genista and WT mice produced similar levels of TNF-α in their sera (**Figure 22B**), suggesting that Genista mutation does not lead to increased TNF production upon TLR7 stimulation.

Increased activation and IFN-β expression by Genista BMDCs

It was showed previously that Gfi-1$^{-/-}$ progenitor cells fail to generate BMDCs upon culture with GM-CSF (Rathinham et al., 2005). So, we tested if Genista progenitor cells are able to generate BMDCs. We plated equal number of WT and Genista bone-marrow cells in the presence of GM-CSF and at day 6 we counted the total number of cells and evaluated cell viability by flow cytometry. We observed that Genista cells presented significant reduced cell counts and viability compared to WT cells (**Figure 23A**). Next, we accessed the maturation of DCs on CD11c^{+} cells based on MHCII expression: immature (CD11c^{+} MHCII^{lo}), intermediate (CD11c^{+} MHCII^{int}) and mature (CD11c^{+} MHCII^{high}) DCs. Genista BMDCs had reduced frequency of immature DCs and increased frequencies of intermediate and mature DCs compared to WT cells (**Figure 23B**). In addition, Genista CD11c^{+} DCs presented higher CD86 expression compared to WT cells (**Figure 23C**). In order to check if the increased activation observed in Genista DCs is correlated with increased TLR7 expression, we tested the TLR7 mRNA expression in WT and Genista BMDCs stimulated with R848 or LPS for 0, 2, 4, 6 or 16 hours. The Genista BMDCs have reduced levels of TLR7 mRNA expression in all time-points and with both stimuli compared to WT cells (**Figure 23D**).

In order to evaluate the response of Genista DCs upon TLR stimulation, we evaluated by Q-PCR the TNF-α, IL-6, IFN-β and ISG15 mRNA expression in WT and Genista BMDCs stimulated with 50nM of R848 or 1ng/ml of LPS for 0, 2, 4, 6 or 16 hours. Both ligands induced the expression of all the tested genes in both genotypes. Regarding TNF-α, R848 induced similar expression in WT and Genista cells, while LPS stimulation induced higher expression of this cytokine in WT than in Genista at 2 hours, but the expression became similar in both genotypes for the later time-points (**Figure 24**). The IL-6 expression was increased upon R848 stimulation in Genista cells compared to WT cells at 4, 6 and 16 hours, while LPS stimulation induced increased expression in WT versus Genista cells at 2 hours, but the expression became similar between the two genotypes at later time points. IFN-β expression was increased upon R848 stimulation in Genista cells compared to WT cells at 2, 4 and 6 hours, while LPS stimulation induced higher IFN-β expression in Genista compared to WT at 4 and 6 hours. We also measured the expression of ISG15, an IFN-

stimulated gene (Korant et al., 1984). Interestingly after R848 stimulation, there was an increased expression of ISG15 by Genista cells compared to control cells at 2, 4 and 6 hours, while upon LPS stimulation, cells of both genotypes behaved similarly (**Figure 24**). Overall the data demonstrated a significant increased IFN-β and IL-6 expression in Genista BMDCs upon stimulation with R848 (TLR7 ligand) or LPS (TLR4 ligand).

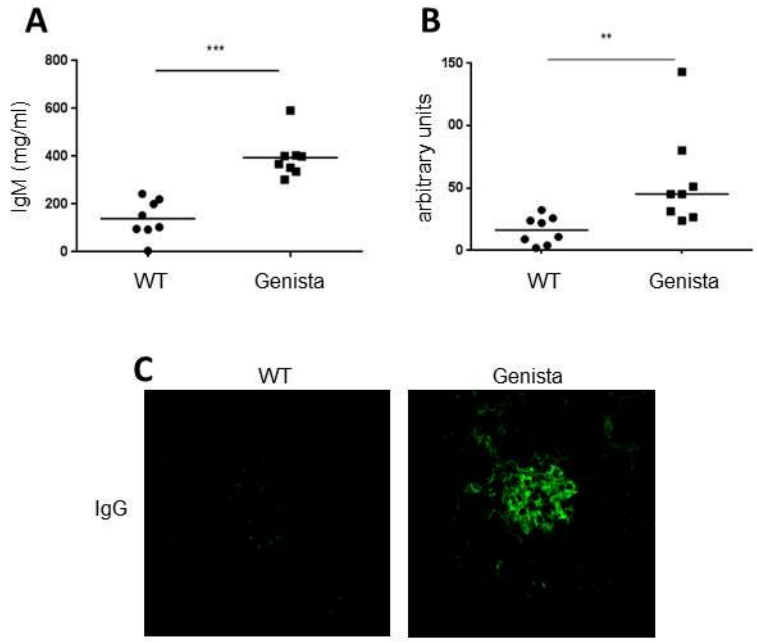

Figure 16: Genista mice develop lupus. Sera from 9 weeks-old female WT and Genista mice were used for evaluation of (A) serum levels of IgM and (B) RNA-specific autoantibody production by ELISA. Each point represents one mouse and horizontal bars denote the median. (C) Kidney sections from 4 months old female mice WT and Genista stained with immunofluorescence anti-IgG antibody. Data in C are representative of two independent experiments (n=3 mice per group).

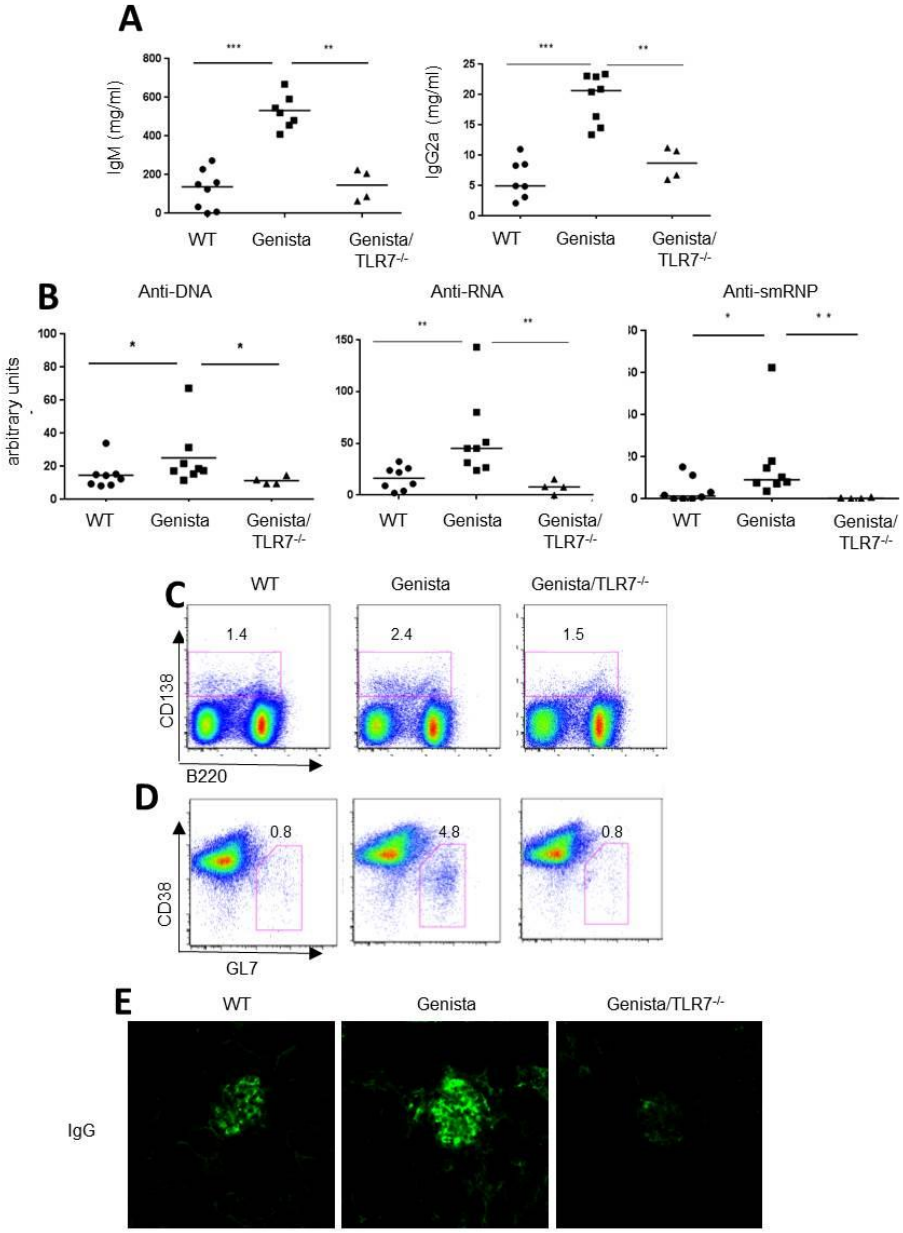

Figure 17: Genista mice develop lupus phenotype that is TLR7-dependent. Sera from 9 weeks-old female WT, Genista and Genista/TLR7$^{-/-}$ mice were used for evaluation of (A) serum levels of IgM and IgG2a and (B) ds-DNA-, RNA- and smRNP- specific autoantibody production by ELISA. Each point represents one mouse and horizontal bars denote the median. Representative flow cytometry plots from spleens of (C) B220^{lo}CD138^{+} plasmablasts and (D) B220^{+}GL7^{+}CD38^{-} germinal center B cells in 4 months old female WT, Genista and Genista/TLR7$^{-/-}$ mice. (E) Kidney sections stained with immunofluorescence anti-IgG antibody. Data in (C, D and E) represent 2 independant experiments (n=3 per group).

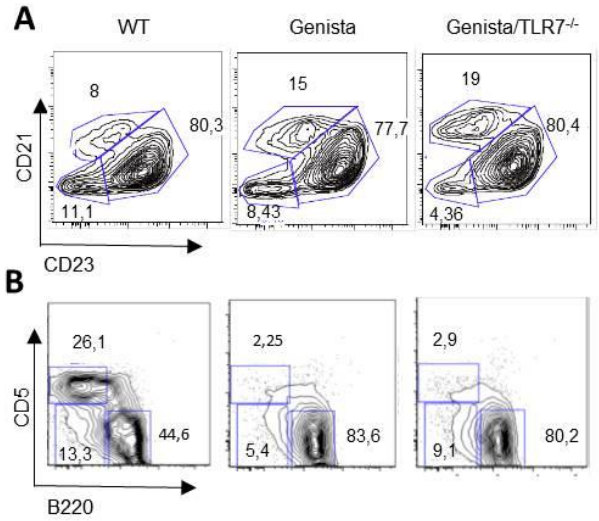

Figure 18: Genista and Genista/TLR7$^{-/-}$ mice show abnormal MZ and B1 B cell populations compared to WT mice. (A) Splenocytes from 16 weeks old female WT, Genista and Genista/TLR7$^{-/-}$ mice were analyzed by flow cytometry for the expression of CD19, CD21 and CD23. Numbers denote the percentage of MZ (CD21^{hi} CD23$^{lo/-}$), follicular (CD21^{int}CD23^{hi}) and immature (CD21^{-}CD23^{-}) B cells in the indicated gates. (B) FACS analysis of B220 and CD5 expression on CD19^{+} gated cells of the peritoneal cavity shows the percentage of B1a (B220^{lo}CD5^{int}), B1b (B220^{lo} CD5^{lo}) and B2 (B220^{hi} CD5^{-}) B cells. Data on (A) and (B) are representative of 2-3 independent experiments (n= 4-5 per group).

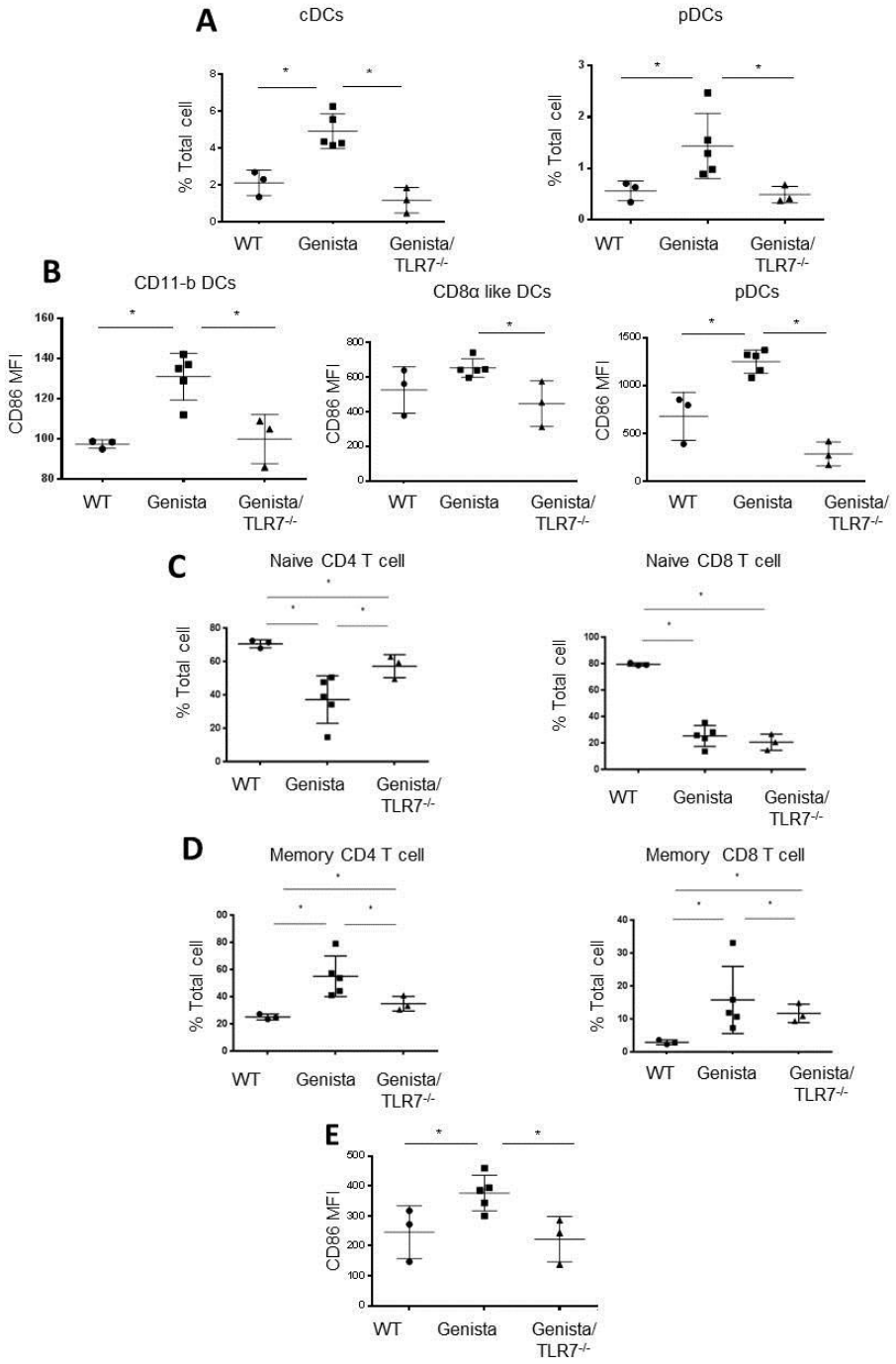

Figure 19: Increased frequencies of DCs and activated DCs, B cells and T cells in Genista spleens compared to WT or Genista/TLR7$^{-/-}$. Splenocytes from 6-7 months old male WT, Genista and Genista/TLR7$^{-/-}$ mice were analyzed by flow cytometry: (A) percentage of conventional DCs (CD11c^{hi}/IA-IE^{+}) and pDCs (CD11c^{int}/SiglecH^{+}) populations, (B) MFI of CD86 on CD11-b like DC, CD8α- DC and pDCs, (C) CD4 and CD8 T naïve cells (CD44^{lo} CD62L^{hi}), (D) CD4 and CD8 activated/memory T cells and (E) MFI of CD86 on B cells. Data are representative of 2 independent experiments (n= 3-5 per group).

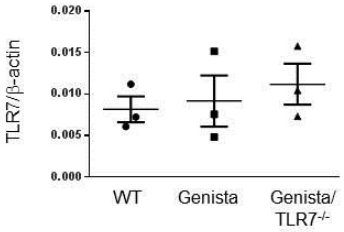

Figure 20: Normal expression of TLR7 in Genista and Gfi-1$^{-/-}$ spleens. Splenic total RNA was extracted from 4 months-old female WT, Genista and Gfi-1$^{-/-}$ mice and the expression of TLR7 was assessed by quantitative PCR. Each point represents one mouse and horizontal bars denote the median. Data are representative of two independent experiments (n=3 per group).

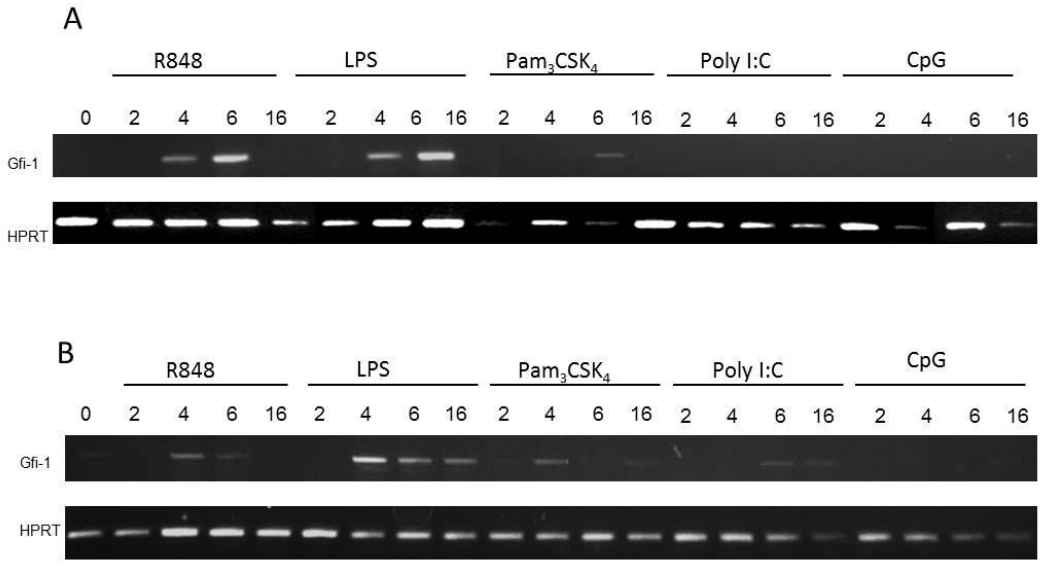

Figure 21: Induction of Gfi-1 mRNA expression by TLR ligands. BMMs (A) and BMDCs (B) from WT mice were stimulated with R848, LPS, Pam_3CSK_4, poly I:C or CpG for the indicated time-points and the mRNA expression of Gfi-1 was evaluated by semi-quantitative RT-PCR. HPRT was used as house-keeping gene. Representative data from 2 independent experiments.

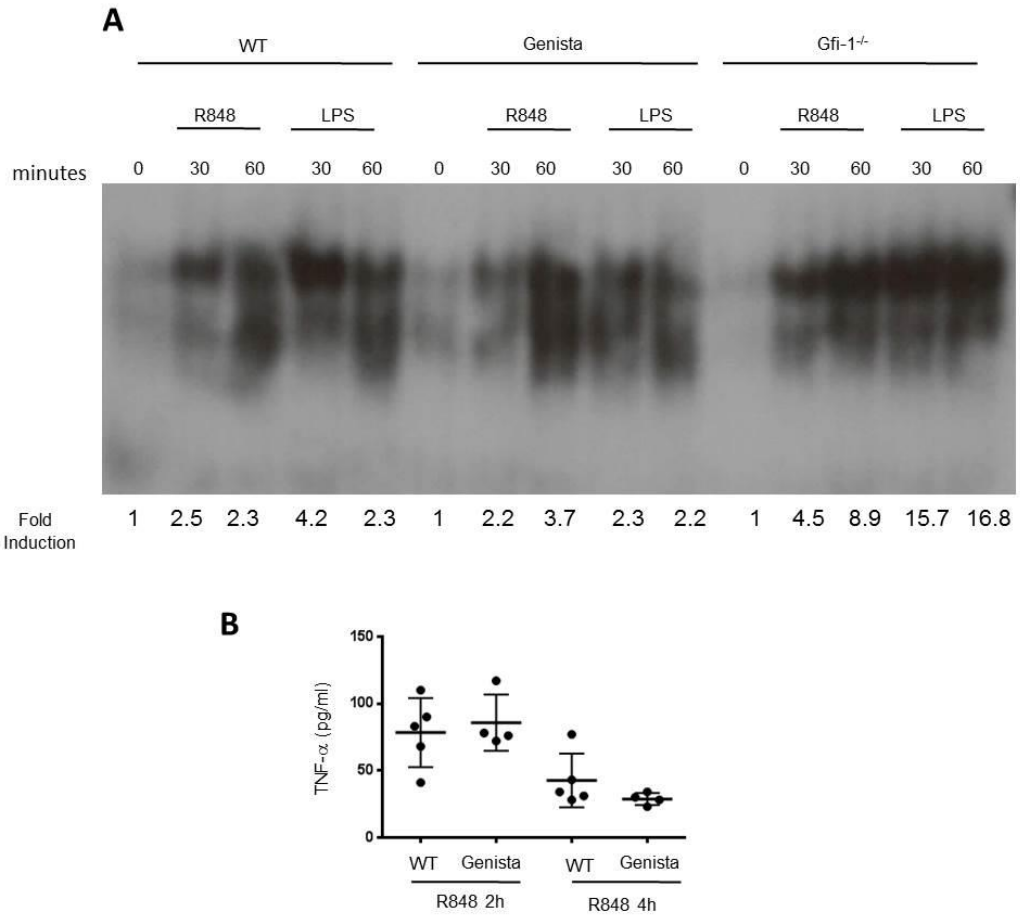

Figure 22: NF-κB DNA binding in WT, Genista and Gfi-1$^{-/-}$ macrophages upon R848 or LPS stimulation and production of TNF-α in WT and Genista sera upon R848 injection. (A) BMMs from WT, Genista and Gfi-1$^{-/-}$ mice were treated with medium or 1 ng/ml of LPS or 50nM of R848 at indicated time-points. NF-kB-DNA complex was evaluated in nuclear extracts by electrophoretic mobility shift assay (EMSA) using a radiolabeled DNA. Data are representative of two independent experiments. (B) WT and Genista mice were injected i.p. with R848 and the serum levels of TNF-α were evaluated by ELISA at 2 and 4 hours after the injection (1µg/g of body weight).

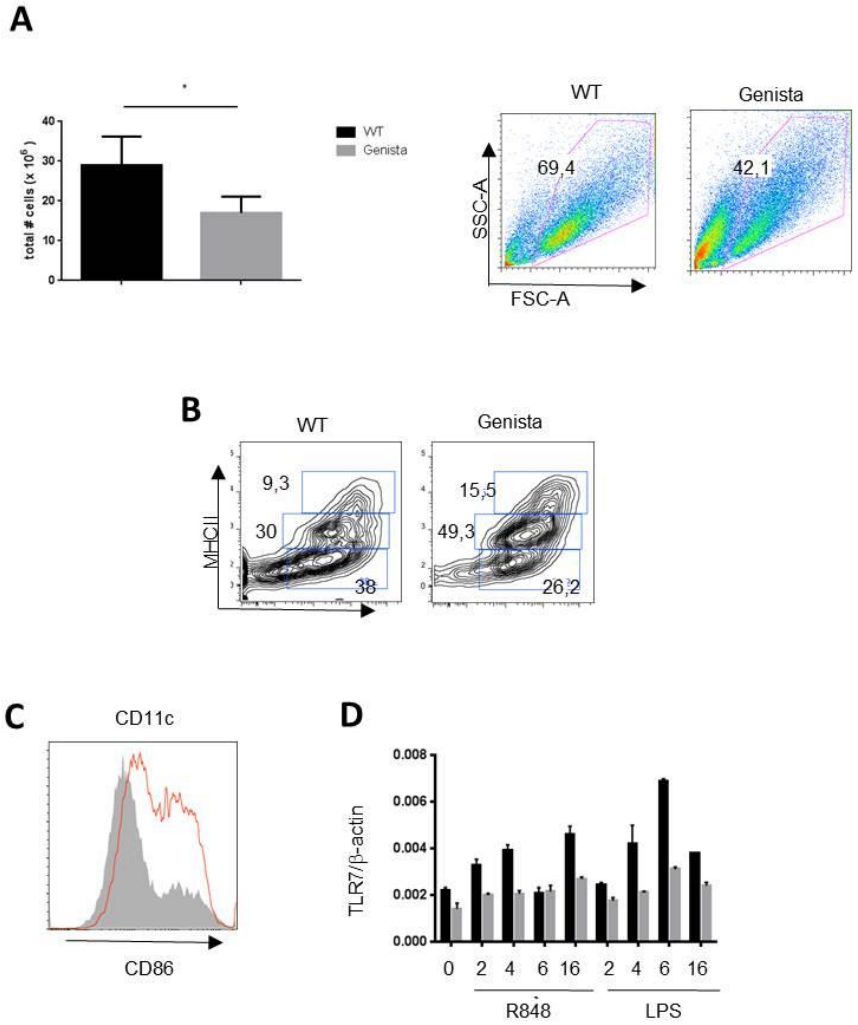

Figure 23: Reduced frequency and increased activation of BMDCs in Genista mice and and DCs expression of TLR7 upon stimulation wih R848 or LPS. BMDCs derived from WT and Genista mice in the presence of GM-CSF mice were counted and analyzed by flow cytometry to acess (A) the total number of cells and the percentage of live cells (B) surface expression of MHCII and CD11c, gates denote immature DCs ($CD11c^{+}$ $MHCII^{lo}$), intermediate ($CD11c^{+}$ $MHCII^{int}$) and mature ($CD11c^{+}MHCII^{hi}$) (C) The surface expression of CD86 was analysed on $CD11c^{+}$ cells from WT (grey color), Genista (red color) mice. (D) Total RNA was extracted from WT (black) and Genista (grey) BMDCs stimulated with R848 or LPS and the expression of TLR7 was assessed by quantitative PCR. Data are representative of two independent experiments.

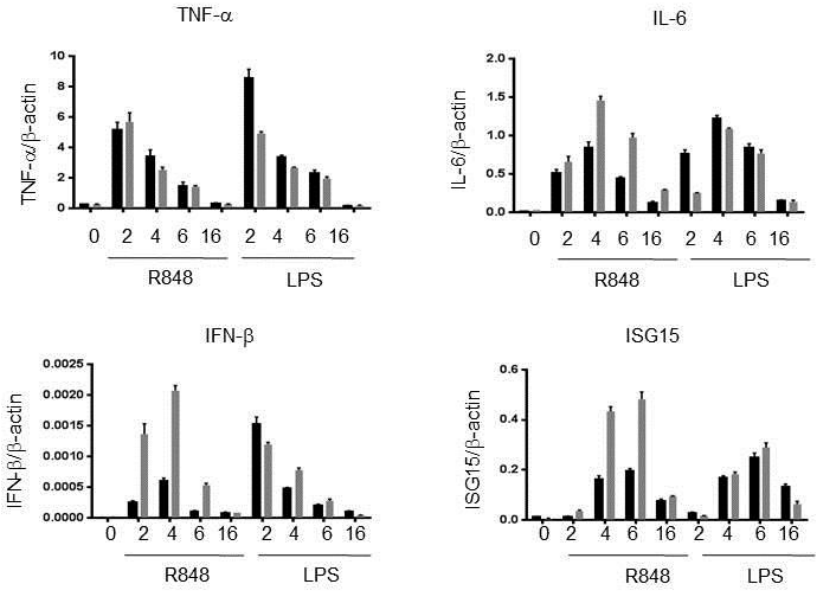

Figure 24: Cytokines and ISG-15 expression by WT and Genista BMDCs upon R848 or LPS stimulation. WT (black) and Genista (grey) BMDCs were stimulated with 50nM R848 or 1ng/ml LPS for the indicated time-points. Equal amounts of RNA were used for cDNA synthesis and analysis of cytokine expression was done by Q-PCR. Data are representative of 2-3 independent experiments.

Table 3: Frequencies of plasmablasts and germinal centers of WT, Genista and Genista/TLR7$^{-/-}$ mice

Genotype	Plasmablasts	Germinal Centers
WT	1.27 ± 0.14	0.8 ± 0.32
Genista	2.7± 0.4 [1]	3.6 ± 1.07 [3]
Genista/TLR7$^{-/-}$	1.3 ± 0.15 [2]	0.8 ± 0.3 [4]

Data are from 4- to 5-months-old male mice. Values shown are average ± SD.

[1] $P < 0.005$ vs. WT
[2] $P < 0.005$ vs. Genista
[3] $P < 0.005$ vs. WT
[4] $P < 0.05$ vs. Genista

Table 4: Frequencies of of splenic marginal zone (MZ) B cells and peritoneal B1a and B1b B cells of WT, Genista and Genista/TLR7$^{-/-}$ mice

Genotype	MZ B cells	B1a B cells	B1b B cells
WT	9.3 ± 1.5	22 ± 5.3	13.5 ±2.3
Genista	17.3 ± 1.9 [5]	1.8 ± 0.6 [1]	3.9 ±1.3 [1]
Genista/TLR7$^{-/-}$	22.4 ± 6.4 [5]	2.9 ± 0.8 [1 4]	7.9 ±1.5 [1 2]

Data are from 4- to 5-months old male mice. Values shown are average ± SD.

[1] $P < 0.005$ vs. WT
[2] $P < 0.005$ vs. Genista
[4] $P < 0.05$ vs. Genista
[5] $P < 0.05$ vs. WT

Materials and Methods

Mice. Genista mice were generated as described previously (Ordonez-Rueda et al., 2012). Double Genista/TLR7$^{-/-}$ and Genista/TLR8$^{-/-}$ mice were generated by intercrossing Genista with TLR7$^{-/-}$ (Lund et al., 2004) or TLR8$^{-/-}$ mice (Demaria et al., 2010). Mice were housed under specific pathogen-free conditions at the Centre d'Immunologie de Marseille-Luminy and experiments were conducted in accordance with institutional guidelines for animal care and use and European directives.
Gfi-1$^{-/-}$ mice were kindly provided by Pierre Brhuns from Département d'Immunologie, Laboratoire Anticorps en Thérapie et Pathologie, Institut Pasteur, Paris, France

Reagents. R848, LPS from Escherichia coli 0111-B4, Pam_3CSK_4, CpG ODN 1826, and poly I:C were purchased from Invivogen.

Preparation of Cells. Bone marrow cells from mice were extracted from femur and tibias and cultured with GM-CSF or M-CSF for the production of bone marrow dendritic cells (BM-DCs) or BM-macrophages, respectively as previously described (Demaria et al., 2010).

***In vivo* injection of R848**. WT and Genista mice were injected *i.p*. with 1µg/g of body weight with R848 and sera were collected after 2 and 4 hours for evaluation of levels of TNF-α by ELISA.

Serological Analysis. Evaluation of IgM and IgG2a, and IgG autoantibodies against DNA, ribonucleoprotein (RNP) and Smith ribonucleoprotein (smRNP) on serum samples were performed as described previously (Demaria et al,2010). For anti-RNA, plates were coated with add 50 µl /well of 5µg/ml mouse RNA in PBS and incubated overnight at 4°C. Next day, the plates were incubated with 1:50 diluted serum samples in working solution (PBS + 0.05% Tween20+1%BSA), and the assay was developed with HRP-labeled goat anti-mouse IgG (Southern Biotech).

Flow Cytometric Analysis. Cell suspensions were incubated with 24G2 hybridoma supernatant and then stained using immunofluorescence-labeled antibodies against the following antigens: B220, CD3, CD4, CD8, NK1.1, CD19, CD11b, CD11c, Ly6G, Ly6C, CD21, CD23, CD5, CD44, CD86, CD38, CD138, from BD Biosciences, IA/IE (MHC class II), GL7 from eBioscience. Flow cytometry was conducted using an LSR2 (BD Biosciences) and data were analyzed with FlowJo (Tree Star).

RNA isolation, RT- PCR and Q- PCR.

Total RNA was isolated with Trizol (Invitrogen, Carlsbad, USA), contaminant DNA was removed using DNA I (Ambion) and first-strand cDNA was synthesized using 3 μg total RNA and SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR primer pairs specific for Gfi-1 and hypoxanthinephosphoribosyltransferase (HPRT) which sequences are shown below:

Gfi-1 5'- GTGGCAAAAGATTCCACCAG -3'
5'- TGAAGCCTGTGTGCTTTCTG -3'

HPRT 5′-GTTGGATACAGGCCAGACTTTGTTG-3′
5′ GAGGGTAGGCTGGCCTATAGGCT-3′

Taq polymerase (Invitrogen) was 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 following primers (shown below) for TLR7, IL-6, TNF-α, ISG15 and β-actin. 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–ΔCT, resulting in data expressing a target copy number ratio (target gene /β-actin).

TLR7	5′-TGGCTCCCTTCTCAGGATGA-3′
	5′-CCGTGTCCACATCGAAAACA-3
IFN-β	5′-CGTTCCTGCTGTGCTTCTCC-3
	5′-TCTTGGAGCTGGAGCTGCTT-3
IL-6	5’-TGTTCTCTGGGAAATCGTGGA-3 ’
	5’-TTTCTGCAAGTGCATCATCGTT-3’
TNF-α	5’-TTCTATGGCCCAGACCCTCA-3’
	5’-CAGCTGCTCCTCCACTTGGT-3’
ISG15	5’-ACCCTTTCCAGTCTGGGTCT-3’
	5’-TCGCTGCAGTTCTGTACCAC3’
β-actin	5′-CCTGAACCCTAAGGCCAAC-3′
	5′-GACAGCACAGCCTGGATGG-3′

Electrophoretic mobility shift assay (EMSA). For nuclear protein extracts, bone-marrow macrophages were stimulated with 10ng/ml LPS 50nM R848. After 0, 30 and 60 min, cells were lysed in buffer NAR A [10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA (pH 8), 0.1 mM DTT, 1x Complete inhibitor cocktail (Roche, Cat. 1697498), 0.04 M β-glycerophosphate, 1 mM NAF and 0.5 mM PMSF]. Samples were incubated on ice for 10 minutes. 11μl of 1% NP-40 (Ipegal CA-630, Sigma I-3021) was added and samples were incubated for 5 minutes at RT, vortexed and centrifugated for 1.5 minutes at 4.000 rpm at 4^{o}C. The pellet was washed with buffer NAR A and incubated in buffer NAR C [20mM HEPES, 0.4M NaCl, 1mM EDTA, 0.1 mM DTT, 1x Complete inhibitor cocktail (Roche, Cat. 1697498), 0.04 M β-glycerophosphate and 1 mM NAF and 1mM PMSF]. Samples were incubated at 4^{o}C on a shaker for 30 min, centrifugated for 10 minutes at 13.000 rpm at 4^{o}C and supernatants containing nuclear extra were transferred to a fresh tube. Protein concentrations were determined using Quickstart Bradfort Protein Assay (Bio-Rad laboratories).The NF-κB consensus oligonucleotide (5'-AGTTGAGGGGACTTTC CCAGGC-3') (Promega. Oligonucleotide) was end labeled with [ϒ-^{32}P] ATP (Perkin-

Elmer) using T4 polynucleotide kinase (Promega). Binding reactions were prepared using 3 μg of nuclear protein extracts in binding buffer (5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, 0.5 μg poly dIdC (Becton Dickinson), 2.5% (v/v) glycerol and 2% (v/v) Ficoll) to a final volume of 20 μl. Reactions were incubated on ice for 10 min, 1.75 pmol of ^{32}P-labeled NF-κB oligonucleotide was added and the reactions were incubated for 15 min at room temperature. Binding reactions were electrophoresed on a 5% non-denaturing polyacrylamide gel and analyzed by autoradiography.

Statistical Analysis. Statistics were calculated using Prism5 (GraphPad Software) by Mann–Whitney U test with P values indicated throughout as *P < 0.05, **P < 0.01, and ***P < 0.001.

Discussion and Perspectives

SLE is a complex chronic autoimmune disease that arises spontaneously with divers clinical manifestations and characterized by the presence of autoantibodies against nuclear antigens (Tan et al., 1982). The pathophysiology of SLE remains incompletely understood and there is currently no cure for lupus (Xiong and Lahita, 2014). However, recent studies indicate a role for Toll-like receptors (TLRs) and their signaling pathways in the initiation and establishment of SLE (Khandanpour et al., 2010; Person et al., 2003). My thesis had two aims: The first was to study the cooperation of TLR8 and TLR9 in controlling TLR7-mediated lupus, and the second was to investigate the implication of Gfi-1 in lupus development and TLR signaling by studying Genista mice. The discussion and perspectives of my two aims are presented below:

Aim 1: Cooperation of TLR8 and TLR9 in controlling TLR7-mediated lupus.

Nucleic-acid TLRs play an important role in SLE not only through their direct signaling, but also by regulating the expression and function of each other. TLR7, TLR8, and TLR9 sense microbial or endogenous nucleic acids and are implicated in the development of SLE, where both TLR8 and TLR9 control TLR7 function and TLR7-mediated lupus (Demaria et al., 2010; Nickerson et al., 2010; Santiago-Raber et al., 2010). However, it was unknown if TLR8 and TLR9 have an additive effect on controlling TLR7. The first aim of my aim was to investigate whether TLR8 and TLR9 act in parallel or in series in the same or different cell types in controlling TLR7-mediated lupus. We demonstrated that double TLR8/9$^{-/-}$ mice on the C57BL/6 background had increased abnormalities characteristic of SLE compared to single TLR8$^{-/-}$ or TLR9$^{-/-}$ mice, suggesting that TLR8 and TLR9 have an additive effect on controlling TLR7. Moreover, we found that the additive effect of TLR8 and TLR9 originates from the fact that they act on different cell types, where TLR8 controls TLR7 function on DCs, while TLR9 restrains TLR7 response on B cells (Desnues et al., 2014). These results have been already discussed in our paper (Desnues et al., 2014), so below I extended the discussion of our findings by focusing on studies that were reported lately and after the publication of our paper.

In murine lupus models, deletion of TLR7 limits autoimmune inflammation, whereas deletion of TLR9 exacerbates disease, however, whether TLR7 and/or TLR9 signaling on B cells or myeloid cells is responsible for these effects has not been fully addressed. To address this question a recent study used chimeric mice to evaluate the effect of B cell-intrinsic deletion of TLR7 versus TLR9 using the WASp lupus mouse model (Jackson et al., 2014). WASp mice carry a mutation that abolishes expression of Wiskott-Aldrich syndrome protein on B cells, but not on other hematopoietic lineages. WASp B cells drive the development of lupus that is characterized by spontaneous GCs, class-switched IgG2c autoantibodies and glomerulonephritis. By studying chimeric mice that were generated by transferring WT, $WAS^{-/-}$, $WAS^{-/-}/TLR7^{-/-}$ or $WAS^{-/-}/TLR9^{-/-}$ B cells in B cell deficient mice, it was shown that chimeric mice that were carrying $WAS^{-/-}/TLR7^{-/-}$ B cells had reduced autoimmunity compared to chimeric mice that received $WAS^{-/-}$ B cells. In contrast, transfer of $WAS^{-/-}/TLR9^{-/-}$ B cells resulted in decreased DNA-reactive antibodies, but increased antibodies targeting a broad range of systemic autoantigens and more severe autoimmunity than chimeric mice that received $WAS^{-/-}$ B cells. These data suggest that B cell-intrinsic TLR7 and TLR9 control the autoantibody repertoire and systemic inflammation, but have opposing impact, whereas TLR9 signaling on B cells is protective and TLR7 signaling on B cells is harmful for SLE.

In accordance with these findings, we demonstrated that $TLR9^{-/-}$ and $TLR8/9^{-/-}$ splenic B cells show similar and increased activation upon TLR7 stimulation compared to $TLR8^{-/-}$ or WT cells, however this capacity is lost in isolated B-cell cultures. We asked if this effect is B cell intrinsic and to do so, we added WT or $TLR9^{-/-}$ B cells in $TLR9^{-/-}$ or WT splenocytes cultures depleted of B cells, respectively. We found that $TLR9^{-/-}$ B cells in WT B cell depleted splenocytes showed increased activation and response compared to WT B cells in $TLR9^{-/-}$ B cell depleted splenocytes (**Figure 1** in Annexes). This information confirms the B-cell intrinsic TLR9 deletion effect. The fact that we could not see this effect in isolated B cell cultures could be due to the fact that B cells require additional signals from other cell types either by direct cell to cell contact or as soluble factors.

Regulatory B cells is a subset of B cells that is now recognized as an essential component of the immune system that shares surface markers with MZ B and B1a cells and its function depends on IL-10 (Sang et al., 2013). It has been demonstrated that regulatory B cells are important for disease suppression in lupus, since they inhibit the disease onset in NZB/W F1

mice (Watanabe et al., 2010) and suppress the disease manifestation in MRL-Fas (lpr) mice by IL-10 dependent mechanisms (Blair et al., 2009). Moreover, regulatory B cells isolated from lupus patients produced less IL-10, and lacked the suppressive capacity of their healthy counterparts (Blair et al., 2010). Thus, altered cellular function within regulatory B cells may impact effector immune responses in SLE and other autoimmune diseases (Rosser et al., 2014). Based on the implication of regulatory B cells in SLE it would be interesting to check if the regulatory B cells in TLR8$^{-/-}$, TLR8/9$^{-/-}$ and TLR9$^{-/-}$ mice have impaired function and if there is a correlation with the disease phenotype.

We have shown that TLR8 controls TLR7 expression and function and that TLR8$^{-/-}$ mice in the C57BL/6 background develop TLR7-dependent autoimmunity (Demaria et al., 2010; Desnues et al., 2014), but a recent study challenged our findings (Umiker et al., 2014). Umiker et al. investigated how the expression of specific TLRs contributes to SLE pathogenesis using the 564Igi SLE mouse strain (Umiker et al., 2014). The 564Igi is an SLE mouse strain carrying gene-targeted heavy and light chain antibody genes encoding an anti-RNA autoantibody in the C57BL/6 background (Berland et al., 2006). By crossing 564Igi mice with TLR7$^{-/-}$, TLR8$^{-/-}$, TLR9$^{-/-}$, TLR7/9$^{-/-}$ or TLR7/8$^{-/-}$ mice (all mice in the C57BL/6 background) and evaluating IgG anti-RNA autoantibodies in mouse sera, it was shown that 564Igi/TLR7$^{-/-}$ mice had lower levels of autoantibodies in their sera compared to 564IgI mice, but higher than WT mice, whereas 564Igi/TLR8$^{-/-}$ or 564Igi/TLR9$^{-/-}$ mice had similar IgG anti-RNA levels like 564Igi mice. Moreover, 564Igi/TLR7/TLR9$^{-/-}$ mice had decreased autoantibody levels compared to 564Igi mice, while in 564Igi/TLR7/TLR8$^{-/-}$ sera no anti-RNA autoantibodies were detected. Based on these data, the authors concluded that TLR8 can drive the production of anti-RNA autoantibodies by the fact that double deficiency for TLR7/TLR8, but not for TLR7/TLR9, leads to absence of autoantibodies in the 564Igi mouse sera. However, only by studying 564IgI/TLR8$^{-/-}$ mice conclusions can be driven regarding the role of TLR8, but the authors provided very limited data, which makes difficult to really conclude what is the role of TLR8 in the 564IgI mouse model. In addition, by examining TLR8$^{-/-}$, 564IgI and WT mice in the C57BL/6 background and evaluating IgG2b levels, and anti-RNA and anti-DNA autoantibodies, Umiker et al. showed that TLR8$^{-/-}$ mice had reduced autoantibodies compared to 564Igi mice, something that is not surprising since we have shown before that TLR8$^{-/-}$ mice on the C57BL/6 background present only mild lupus disease.

Moreover, similar to our findings [supplementary Fig. 4B (Umiker et al., 2014), see bellow] and in the 1/30 sera dilution TLR8$^{-/-}$ had increased anti-RNA IgM compared to WT mice. Finally, the authors claim an absence of ANA staining in TLR8$^{-/-}$ mice by comparing them to 564Igi mice (that show severe lupus disease), but no WT mice were included for direct comparison.

In our case, by studying TLR8$^{-/-}$ and TLR9$^{-/-}$ mice on the C57BL/6 background we showed that lack of TLR8 or TLR9 leads to increased autoantibody production compared to WT (C57BL/6) mice, while absence of TLR7 leads to lower levels of immunoglobulins IgM and IgG2a and absence of ANA staining compared to WT mice (Demaria et al., 2010; Desnues et al., 2014). Thus, TLR8$^{-/-}$ mice show signs of lupus when compared to WT mice, and milder lupus in comparison to 564Igi mice.

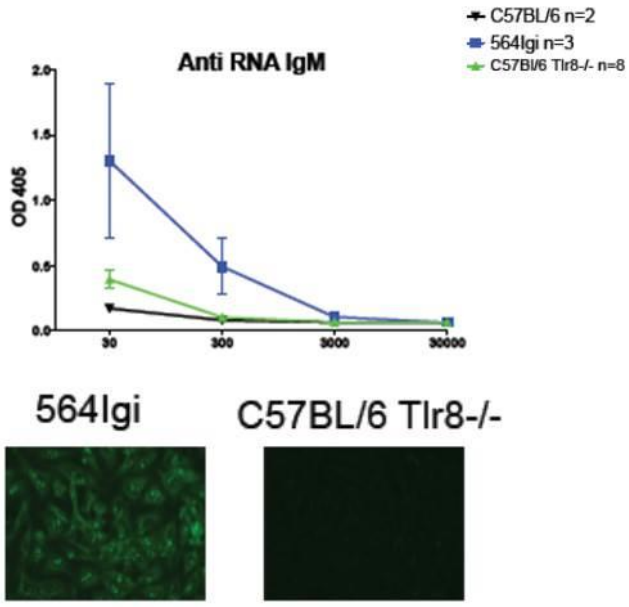

Figure paper Umiker et al.,2014: Anti- RNA IgM antibody detected in the sera of 3 months-old mice using yeast RNA as capture antigen and IgG ANA staining of HEP-2 cells using sera from C57Bl/6 mice (Umiker et al., 2014, Supplementary Figure 4).

Studies on the role of RNA receptor TLR8 in inflammation have been limited by its different function in human versus rodents. Mouse TLR8 does not recognize ssRNA ligands, RNA viruses, or small molecules that are potent agonists for human TLR8 (Heil et al., 2003; Hemmi et al., 2002) a difference caused by the absence of 5 amino acids that are necessary for RNA recognition by human TLR8 (Liu et al., 2010). Thus, in order to study the role of human TLR8 in small animal, Guiducci et al. generated multiple transgenic mouse lines that express human TLR8. Mice expressing high levels of TLR8 mRNA developed a devastating disease, with severe inflammation in the pancreas, salivary glands and joints and failed to breed. However, mice expressing lower levels of TLR8 survived normally and when tested for the collagen-induced arthritis model, showed increased susceptibility and the levels of huTLR8 correlated with proinflammatory cytokines in the joints. In same study, a pathogenic role for TLR8 in humans was suggested by its increased expression in patients with systemic arthritis and a correlation of TLR8 expression with the elevation of IL-1β levels and disease status (Guiducci et al., 2013). Thus, self-recognition via human TLR8 results in a constellation of diseases, strikingly distinct from those related to TLR7 signaling, and points to specific inflammatory diseases that may benefit from inhibition of TLR8 activation and signaling in humans. However, inhibition of human TLR8 might lead to increased TLR7 function since we and others have shown cross-regulatory interaction between TLR7 and TLR8, whereas TLR8 keep under control TLR7 expression and function (Demaria et al., 2010; Desnues et al., 2014; Wang et al., 2006). In accordance, Guiducci et al. revealed that in huTLR8 transgenic mice the expression of mouse TLR7 was significantly decreased both in spleens and purified cellular subsets (Guiducci et al., 2013).

In our work, we demonstrated that TLR7 is regulated by TLR8 and TLR9 in a cell-specific manner, where TLR8 controls TLR7 expression and signaling in DCs and TLR9 controls TLR7 function in B cells. The reasons for this TLR-interaction cell type specificity need to be clarified. We can speculate that depending on the cell type TLR7, TLR8 and TLR9 can be located in the same or distinct intracellular compartments, and/or their trafficking to endosomes is mastered by different molecules. Studies that deal with the location and trafficking of the individual TLRs in primary cells will be crucial for answering these issues.

Aim 2: Investigate the implication of Gfi-1 in lupus development and TLR signaling by studying Genista mice

TLR signaling pathways must be tightly regulated because inappropriate TLR stimulation may disrupt the fine balance between pro- and anti-inflammatory responses (Kondo et al., 2012). Such disruptions may harm the host through the development of inflammatory and autoimmune diseases such as SLE and rheumatoid arthritis. Several pathways and molecules can act individually or in combination to regulate TLR responses. These molecules maintain the balance between activation and inhibition and ensure balancing of the beneficial and adverse effects of antigen recognition (Kondo et al., 2012). The transcriptional repressor Gfi-1 has been shown to negatively regulate TLR4 signaling (Sharif-Askari et al., 2010) and Gfi-1$^{-/-}$ mice show signs of lupus autoimmunity (Igwe et al., 2008). Based on the fact that Gfi-1 seems to be implicated in autoimmunity and that TLR7 plays a central role in the immunopathology of SLE, the second aim of my aim was to study the role of Gfi-1 in lupus development and TLR signaling. To do so, we studied Genista mice that carry a hypomorphic mutation of the Gfi-1 gene that leads to a novel model of neutropenia (Ordonez-Rueda et al., 2012). We found that Genista mice in the C57BL/6 background develop spontaneous lupus that is TLR7-dependent and Gfi-1 acts as a transcriptional repressor downstream of TLR7 and controls type-I IFN expression.

MZ B cells are the first subset of B cells to encounter blood-borne antigens (Mebius and Kraal, 2005). Antigen activated MZ B cells migrate toward the follicle where they can either receive CD4^{+} T cell help to become plasma cells or they can activate CD4^{+} T cells, which in turn activate follicular B cells. A weak affinity for self-antigens suggests that MZ B cells can become pathogenic in the context of lupus. We demonstrated that Genista mice have increased numbers of MZ B cells compared to WT mice and this phenotype does not depend on TLR7. Several lupus models have correlated the disease progression with higher expansion of MZ B cells, including New Zealand Black (NZB) mouse and its F1 cross with New Zealand White mouse (NZB/W) (Wither et al., 2000). It would be interesting to check if in Genista mice there is also a correlation of disease progression and MZ B cell expansion.

Another important cell-type correlated with lupus are the B1 B cells, which are a separate lineage of B lymphocytes found mostly in the pleural and peritoneal cavities, and in lower numbers in the spleen. We found a dramatic reduction in both Genista and Genista/TLR7$^{-/-}$ mice compared to WT mice, however Genista/TLR7$^{-/-}$ presented a significant increase of B1 population compared to Genista mice. It was showed that LPS injection *in vivo* leads to decreased numbers of B1 cells in the peritoneal cavity in WT mice suggesting that B1 B cells actively migrate out of the peritoneal cavity in WT mice (Moon et al., 2012). Moreover, a 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 (Ha et al., 2006). Thus, we can speculate that the drastically reduced numbers of B1 B cells in Genista mice could be due to a certain degree to increased TLR activation, since Genista/TLR7$^{-/-}$ mice show significant increase of this population compared to Genista mice.

Gfi-1 was suggested as a general TLR-regulator, based on the fact that upon stimulation of macrophages with TLR4 ligand Gfi-1 expression is induced (Karsunky et al., 2002a; Sharif-Askari et al., 2010). Since TLR7 is important for the development of lupus in Genista mice, we wondered if Gfi-1 expression is induced upon TLR7 activation and if this expression is restricted to macrophages or not. In order to do that, we generated BMMs and BMDCs from WT mice and we stimulated them with TLR4 (LPS) and TLR7 (R848) ligands. We observed that Gfi-1 mRNA expression was induced by both TLR ligands and in both cell types. Moreover, we found that Gfi-1 expression was also induced upon TLR3 or TLR2 stimulation suggesting that transcriptional repressor Gfi-1 might be a general TLR-regulator.

Since Genista lupus phenotype depends on TLR7, we investigated if TLR7 is overexpressed in cells or tissues, but we found normal expression of TLR7, suggesting that the disease pathogenesis is not due to overexpression of TLR7. It is possible that Genista mutation leads to increased TLR7 signaling to uncontrolled Gfi-1 repression, or there is more availability of endosomal TLR ligands that leads to increased TLR signaling.

NF-κB is one of the elements of TLR-mediated response in cells of the innate immune system and the physiological consequences of TLR stimulation is the release of inflammatory

cytokines such as TNF-α, IL-1β and IL-6. In response to LPS stimulation, Gfi-1$^{-/-}$ BMMs show increased induction of NF-κB complexes and increased expression of TNF-α compared to WT BMMs, suggesting that Gfi-1 negative regulates the TLR4 signaling pathway by antagonizing NF-κB DNA binding activity (Sharif-Askari et al., 2010). Moreover, Gfi-1$^{-/-}$ mice succumb to LPS-induced septic shock due to increased TNF-α production (Karsunky et al., 2002a). We revealed that Gfi-1$^{-/-}$ BMMs show increased induction of NF-κB complexes not only upon LPS, but also upon R848 stimulation, indicating that Gfi-1 participates in NF-κB DNA binding activity upon TLR4 or TLR7 stimulation. To our surprises, we observed that NF-κB binding activity in Genista macrophages was normal and sera from Genista mice produced normal levels of TNF-α upon R848 injection, indicating that NF-κB activity was not affected in Genista mice. Based on the fact that Genista mice develop lupus that is TLR7-dependent and that TLR7 activation can trigger strong type-I IFN responses, we wondered if Genista mutation leads to impaired type I IFN response. Upon R848 or LPS stimulation Genista BMDCs expressed increased levels of IFN-β, as well as IFN-stimulated gene like ISG15, versus WT cells suggesting that the IFN pathway was affected by the Genista mutation. Gfi-1 exerts its role as transcriptional repressor by interacting and forming a complex with histone modifying enzymes, as histone deacetylases (HDAC) and lysine methyltransferase (G9a) that act together to repress the transcription of genes (Duan et al., 2005; Saleque et al., 2007). Two recent studies showed that inhibition of HDAC3 and G9 leads to increased type I IFN gene expression (Fang et al., 2012; Genin et al., 2012). So, we can hypothesize that Genista mutation leads to the development of lupus due to uncontrolled IFN expression. Further studies have to be done to clarify how the Genista mutation affects IFN expression and whether Gfi1-deficiency mirrors the phenotype of Genista mutation. One approach will be by checking the activation of signaling molecules involved in TLR7 signaling pathway. It would be also important to access if Gfi-1 induces transcriptional repression in a direct way or indirectly through induction of other molecules by blocking protein synthesis.

We should not misprize the fact that Genista is a neutropenic mouse model and that neutrophils are the most abundant effector cells of innate immunity. The role of neutrophils in the pathogenesis of SLE is still not very clear. Lately several studies revealed that neutrophils

can play a role in the perpetuation and exacerbation of the disease by the generation of neutrophils extracellular traps (NETs) (Knight and Kaplan, 2012; Yu and Su, 2013). NETs are web-like structures composed of chromatin and granular molecules released by activated neutrophils through a process called NETosis and can thus provide a source of autoantigens (Brinkmann et al., 2004; Christen and von Herrath, 2005). Sera from SLE patients have a decreased ability to degrade NETs (Hakkim et al., 2010; Leffler et al., 2012)and the NETs can trigger pDCs to produce type-I interferons, which in turn can prime neutrophils for additional NETosis leading to a self-amplifying feedback loop (Garcia-Romo et al., 2011; Lande et al., 2011)(Lande et al., 2011; Garcia-Romo et al., 2011). Moreover, anti-nucleoprotein (RNP) immune complexes could activate neutrophils from SLE patients to release NETs and this process requires TLR7 activation (Garcia-Romo et al., 2011). So far, the information obtained from NETs and lupus patients were based by *in vitro* studies and the pathogenic role in mouse models of lupus remains to be clarified (Garcia-Romo et al., 2011; Hakkim et al., 2010; Lande et al., 2011; Leffler et al., 2012).

Based on the information that neutrophils can play a role on the exacerbation of lupus disease, we asked whether the lack of neutrophils would affect the development of lupus in TLR8$^{-/-}$ mice. In order to answer this question, we crossed TLR8$^{-/-}$ mice with Genista mice to generate TLR8$^{-/-}$/Genista mice. We found that TLR8$^{-/-}$/Genista mice presented a dramatic increase of both plasmablasts and GCs frequencies in the spleen and also had increased deposition of IgG in kidneys compared to Genista, TLR8$^{-/-}$ or WT mice (**Figure 2** in Annexes). As we showed and discussed above, Genista and TLR8$^{-/-}$ mice develop lupus so it was not surprising that TLR8$^{-/-}$/Genista mice developed more severe lupus phenotype than the single transgenic mice. However, it is not possible to conclude if this is due as a result of an additive effect or the fact that absence of neutrophils worsens the lupus phenotype in TLR8$^{-/-}$ mice.

Gfi-1 and other genes as ELA2, HAX-1 and WASP are genes implicated in severe congenital neutropenia in humans, which is a primary immunodeficiency disease with bone marrow maturation arrest of granulocytic differentiation (Dale et al., 2000; Devriendt et al., 2001; Ishikawa et al., 2008; Person et al., 2003). Among these genes, WASP has been well investigated and correlated with increased susceptibility to autoimmunity in humans and lupus in mice. The mutation in the gene coding for the WASP leads to a X-linked primary

immunodeficiency disorder named Wiskott-Aldrich syndrome that can affect many cellular processes in immunity, as impairment in adhesion, migration, respiratory burst and degranulation of neutrophils (Zhang et al., 2006). Wiskott-Aldrich is marked by a very high (up to 70%) incidence of autoimmunity, and patients can manifest complications as vasculitis, arthritis and nephropathy (Dupuis-Girod et al., 2003; Imai et al., 2004). It was found that $WASP^{-/-}$ mice develop proliferative glomerulonephritis, anti-nuclear and anti-dsDNA antibodies at much higher levels than control mice, with titers approaching autoimmune-prone-mouse strains such as MRL/lpr and NZB/NZWF1 (Humblet-Baron et al., 2007; Nikolov et al., 2010). Our data with Genista mice and these above strongly indicate that neutropenia can contribute to the pathogenesis of lupus. It is important to keep in mind that normal numbers of neutrophils are crucial for immune homeostasis and the proof is that chronic neutropenia is often associated with impairment in the function of several immune cell types. It would be interesting to check if in other neutropenic mouse models such as $HCLS1^{-/-}$ (Skokowa et al., 2012) and $G\text{-}CSF^{-/-}$ (Lieschke et al., 1994), neutropenia leads to lupus development.

Other possible mechanism by which neutropenia contributes to lupus pathogenesis is the fact that arrested maturation of neutrophils at final stages of differentiation can lead to impaired neutrophil function due to structural perturbations and malfunction of the neutrophil elastase protein and increased cell death, leading to release of autoantigens. This subsequently leads to accumulation of misfolded neutrophil elastase in the endoplasmic reticulum, which triggers apoptosis of neutrophil precursors (Grenda et al., 2007). Moreover, impairment in neutrophils function by blocking NETosis can increase the disease in lupus prone mouse (Campbell et al., 2012). Based on the observation that NET formation relies on activity of the enzyme NADPH oxidase 2 (Nox2) of neutrophils in both humans and mice, generation and characterization of MRL/lpr mice deficient for Nox2 was performed in order to test if inhibition of NETs play a role in SLE pathogenesis (Campbell et al., 2012). Surprisingly, Nox2-deficient MRL/lpr mice had increased spleen weight, proteinuria levels and renal disease compared to MRL/lpr mice. This mutation led to neutrophils with impaired function and cell-death independent of NETosis, implicating that neutrophil cell death is important determinant for lupus development (Campbell et al., 2012). We cannot ignore the fact that atypical $CD11b^{+}Ly\text{-}6G^{int}$ neutrophils are present in Genista mice, although they represent a small population, they can

be a source of autoantigens and contribute to trigger lupus. It may be complicated to access cell death by these atypical neutrophils due to their small numbers, but it would be interesting to check if there is recognition and reaction against them by accessing the sera antibodies against components from neutrophils, such as MPO and other cytoplasmic components.
The contribution of neutrophils to SLE and autoimmunity is an expanding field of research and there are still many questions unanswered. Studies should be done in order to clarify the mechanisms that make the neutrophils change from protectors to pathogenic. Moreover, the pathogenic role of NETs that was described so far, by in vitro studies, should be clarified by *in vivo* studies.

In this study we have shown a contribution of Gfi-1 in development of lupus in mice. Studies with patients presenting variants in the Gfi-1 allele have been published (Khandanpour et al., 2010; Person et al., 2003), but there are no information if any of these patients developed autoimmunity. It would be of interest to identify if lupus patients present mutations in the Gfi-1 gene, and whether in neutropenic patients due to mutation in Gfi-1 gene there are alterations in TLR signaling pathways.

Concluding remarks

SLE is a complex disease with a multifactorial etiology in which many cell-types are involved, including B-cells, DCs, T-cells and their subtypes. In the last years it has been documented that TLRs and their signaling pathways are important in the pathogenesis of SLE (Christensen and Shlomchik, 2007; Theofilopoulos et al., 2010). Among the endosomal TLRs, it has been shown that TLR7 is the most pathogenic regarding lupus both in mice and humans. In our studies, we showed that both TLR8 and TLR9 keep under control TLR7 expression and function in different cell types. We also demonstrated the contribution of the transcription repressor Gfi-1 in lupus development on the control of TLR7 signaling and type-I IFN production. These interactions among TLRs and signaling regulation should be taken in account in order to target lupus disease. The challenge is to modulate immune signaling without exaggerated suppressing innate immune signaling and deregulation of other signaling pathways. Therefore, it is important to find a balance between the suppression of disease-inducing inflammation while keeping the beneficial host immune response.

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Annexes

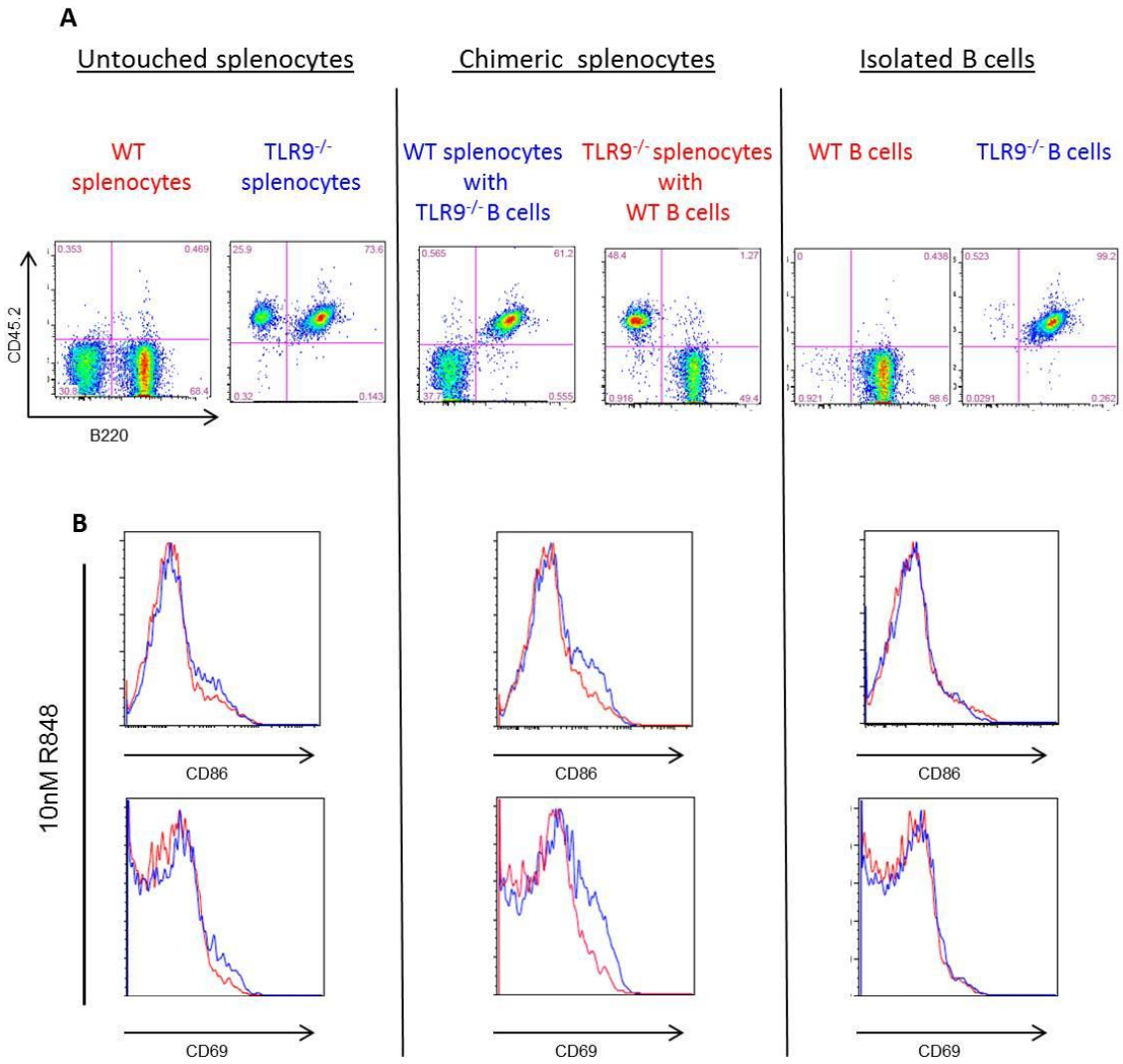

Figure 1 : Increased activation of TLR9$^{-/-}$ splenic B cells upon R848 stimulation depends on the microenvironment of the spleen. (A) Flow cytometry plots representing the following groups: «Untouched splenocytes» that correspond to the gated B cell population from WT ($B220^{+}CD45.2^{-}$) or TLR9$^{-/-}$ ($B220^{+}CD45.2^{+}$) splenocytes; «Chimeric splenocytes» that correspond to splenocyte cultures from WT or TLR9$^{-/-}$ mice that were previously depleted from B cells and received TLR9$^{-/-}$ and WT B cells, respectively; and «Purified B cells» corresponding to B cells isolated from WT or TLR9$^{-/-}$ splenocytes. (B) Flow cytometry histograms of CD86 (upper) or CD69 (lower) expression upon R848 stimulation for 16 hours on gated $B220^{+}$cells derived from WT or TLR9$^{-/-}$ «Untouched splenocytes», «Chimeric splenocytes» or «Purified B cells».

Genista/TLR8$^{-/-}$ present aggravated lupus phenotype

We showed previously that TLR8$^{-/-}$ mice develop lupus that is TLR7-dependent and our current studies revealed that Genista mice also develop a TLR7-dependent lupus phenotype. So wondered if the combination of these two mutations would aggravate lupus disease. To address this point, we crossed Genista mice with TLR8$^{-/-}$ on the C57BL/6 background, in order to generate Genista/TLR8$^{-/-}$ mice. Double Genista/TLR8$^{-/-}$ did not present any phenotypical abnormalities up to 6 months of age. We tested in serum from 9 weeks old female WT, TLR8$^{-/-}$, Genista and Genista/TLR8$^{-/-}$ the levels of IgM and anti-RNA autoantibodies by ELISA. Sera from TLR8$^{-/-}$, Genista and Genista/TLR8$^{-/-}$ showed similar and significantly increased IgM and anti-RNA autoantibody compared to WT (**Figure 2A**). Next, we evaluated by flow cytometry the percentages of plasmablasts (B220^{lo}CD138^{+}) and spontaneous splenic germinal centers in 4 months old female WT, TLR8$^{-/-}$, Genista and Genista/TLR8$^{-/-}$. We found that both plasmablasts and germinal centerswere increased in all three genotypes compared to WT controls, where Genista/TLR8$^{-/-}$ mice showed the highest increase, followed by Genista and TLR8$^{-/-}$ mice (**Figure 2B and C**). Next, we accessed the deposition of IgG in the kidneys and we noticed that Genista/TLR8$^{-/-}$ mice presented the strongest deposition and also biggest glomeruli size, followed by Genista and TLR8$^{-/-}$ mice (**Figure 2D**). So, the deficiency of TLR8 leads to aggravated lupus phenotype in Genista mice.

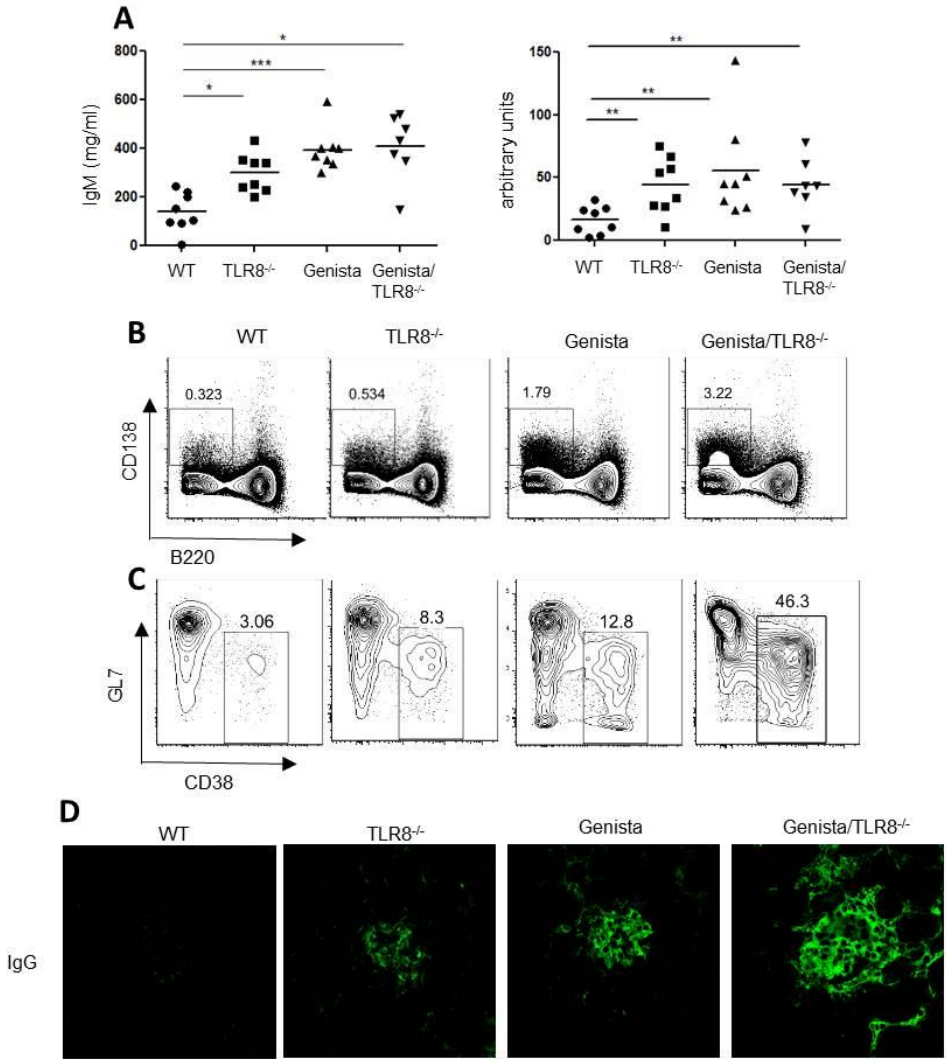

Figure 2 : Increased lupus in Genista/TLR8$^{-/-}$ mice versus Genista or TLR8$^{-/-}$ mice. (A) Sera from 9 weeks-old female mice WT, TLR8$^{-/-}$, Genista and Genista/TLR8 $^{-/-}$ were used for the evaluation of serum levels of IgM and and RNA- specific autoantibody production by ELISA. Each point represents one mouse and horizontal bars denote the median. WT, TLR8$^{-/-}$, Genista and Genista/TLR8 $^{-/-}$ mice were used to evaluate (B) splenic B220^{lo}CD138^{+} plasmablasts and (C) splenic B220^{+}GL7^{+}CD38^{-} germinal center B cells in 4 months old female mice. (D) Kidney sections stained with immunofluorescence anti-IgG antibody. Data represent one mouse from 3-4 per group.

Resumé

Le lupus érythémateux disseminé (LED) est une maladie chronique auto-immune caractérisée par la production d'autoanticorps dirigés contre les antigènes nucléaires. Des nombreuses études indiquent un rôle des récepteurs Toll-like (TLR). Des études antérieures de notre laboratoire ont révélé que le TLR8 murin contrôle la fonction de TLR7 dans les cellules dendritiques et est aussi impliqué dans le lupus. TLR9 contrôle également le lupus dépendant de TLR7. Mon projet de thèse avait deux objectifs dont le premier était de comprendre comment le TLR8 et TLR9 contribuent au lupus dépendant de TLR7. En outre, nous avons révélé que TLR8 contrôle l'expression de TLR7 dans les cellules dendritiques, tandis que le TLR9 contrôle la fonction de TLR7 dans les cellules B. Le deuxième objectif était d'étudier l'implication du répresseur transcriptionnel Gfi-1 dans la signalisation des TLR et le développement de lupus en utilisant des souris Genista qui portent une mutation ponctuelle dans le gène Gfi-1. Nous avons constaté que les souris Genista développent un lupus dépendant de TLR7 et que Gfi-1 agit comme un répresseur de la transcription en aval de TLR7 et contrôle l'expression d'Interféron de type I dépendante des TLR. Ainsi, le déséquilibre des interactions entre TLR ainsi que les facteurs transcriptionnels en aval de ces TLR peuvent conduire à des mécanismes d'inflammation et d'auto-immunité qu'il est important de prendre en compte dans le développement d'approches thérapeutiques nouvelles ciblant les TLRs.

Mots-clef: récepteur Toll-like, auto-immunité, Gfi-1, knock out mice, LED

Summary

Systemic lupus erythematosus is a chronic autoimmune disease characterized by production of autoantibodies against nuclear antigens. Many studies indicate a role for Toll-like receptors (TLRs) in the initiation and establishment of systemic lupus erythematosus (SLE). Previous studies in the lab revealed that murine TLR8 controls TLR7 function in dendritic cells (DCs) and is implicated in SLE. TLR9 also controls TLR7-mediated lupus. My thesis had two aims: the first was to understand how the cooperation of TLR8 and TLR9 contributes to TLR7-mediated lupus. By studying double TLR8/9-deficient mice we found that TLR8 and TLR9 have an additive effect on controlling TLR7-mediated lupus, where TLR8 controls TLR7 function on DCs, while TLR9 restrains TLR7 responses in B cells. The second aim of my thesis was to investigate the implication of Gfi-1 in lupus and TLR signaling by studying Genista mice that carry a hypomorphic mutation of Gfi-1. We found that Genista mice develop TLR7-dependent lupus and that Gfi-1 acts as a transcriptional repressor downstream of TLR7 and controls type-I IFN expression. Thus, unbalancing TLR-interactions and transcription factors downstream of TLRs can lead to inflammation and autoimmunity and these mechanisms have to be taken into account when novel therapeutic approaches are developed that target TLRs.

Key words: Toll-like receptors, autoimmunity, Gfi-1, knock out mice, SLE
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