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The transcriptional repressor Gfi1 prevents lupus autoimmunity by restraining TLR7 signaling

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Abbreviations: ABC, age-associated B cell; BMDC, bone marrow-derived DC; BMF, bone marrow-derived macrophage; cDC, conventional DC; DC, dendritic cell; GC,

germinal center; Gfi1, growth factor independence 1; IFN, interferon; IRF, IFN-regulatory factor; MFI, mean fluorescence intensity; pDC, plasmacytoid DC; SLE, systemic lupus erythematosus; TLR, Toll-like receptor.

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Abstract

The transcriptional repressor growth factor independence 1 (Gfi1) is important in myeloid and lymphoid differentiation. In the current study we evaluated the involvement of Gfi1 in systemic lupus erythematosus (SLE). We found that Genista mice, which carry a hypomorphic mutation in the *gfi1* gene or Gfi1-deficient (*Gfi1*^{-/-}) mice develop signs of spontaneous lupus autoimmunity, including increased serum levels of IgM and IgG2a, autoantibodies against RNA and DNA, glomerular immunodeposits and increased frequencies of plasmablasts, germinal center (GC) B cells and age-associated B cells (ABCs). On the contrary, Genista mice deprived of TLR7 did not show any of these phenotypes, suggesting that the observed lupus autoimmunity in Genista mice is TLR7-dependent. Moreover, Genista mice showed an increased activation of dendritic cells (DCs), B and T cells that was dependent on TLR7 for DCs and B cells, but not for T cells. Upon TLR7 or TLR4 stimulation Genista DCs produced increased amounts of TNF, IL-6 and IFN- β and showed increased NF- κ B phosphorylation and IRF7 nuclear translocation, suggesting that Gfi1 controls the NF- κ B and type I IFN signaling pathway downstream of TLRs. Our data reveal that Gfi1 plays a critical role in the prevention of spontaneous lupus autoimmunity by negatively regulating TLR7 signaling.

Introduction

Growth factor independence 1 (Gfi1) is a transcriptional repressor that is required for multilineage blood cell development, since it controls both the differentiation of stem and progenitor cells into lymphoid and myeloid cells, and their functions [1, 2]. Gfi1 forms a complex together with other cofactors to control histone modifications that lead to silencing of the target gene promoters [1, 2]. In humans, Gfi1 mutations cause autosomal dominant severe congenital neutropenia, and dominant non-immune chronic idiopathic neutropenia of adults, which are heterogeneous hematopoietic disorders that cause predispositions to leukemias and infections [2, 3].

The Gfi1 protein consists of a six C-terminal zinc-finger domains and an N-terminal SNAG-domain linked by an intermediary region [4]. The transcriptional repression activity of Gfi1 depends on the zinc finger motifs required for DNA binding, as well as the SNAG-domain responsible for nuclear localization [5, 6]. Mouse studies have shown that Gfi1 is implicated in the development and function of hematopoietic stem cells, granulocytes, macrophages, dendritic cells (DCs), B and T cells and the control of endotoxin-mediated innate immune responses [7]. Indeed, Gfi1-deficient ($Gfi1^{-/-}$) mice are highly susceptible to LPS-induced septic shock due to exaggerated production of proinflammatory cytokines by macrophages, suggesting a negative regulatory role for Gfi1 in the TLR4-initiated signalling pathway [7, 8]. Upon TLR4 stimulation, Gfi1 directly interacts with the p65 subunit of NF- κ B through its intermediary region to control its DNA binding activity, thereby regulating the expression of a large number of NF- κ B target genes [9]. Nevertheless, the implication of Gfi1 in the signaling pathway downstream of other TLRs has not been investigated.

Toll-like receptors (TLRs) sense conserved molecules from invading microorganisms, as well as endogenous molecules released from damaged tissue or dead cells and play crucial role in orchestrating both innate and adaptive immunity [10, 11]. Thus, TLRs must be tightly regulated to avoid excessive activation by endogenous or exogenous ligands that can disrupt immune homeostasis and result in the development of inflammatory and autoimmune diseases, including systemic lupus erythematosus (SLE). SLE is a systemic autoimmune disease that affects multiple organs and is characterized by production of autoantibodies against self-nucleic acids and associated proteins [12]. Accumulating evidence shows that TLRs and especially those detecting nucleic acids, are implicated in the pathogenesis of autoimmune diseases by recognition of self-molecules. The nucleic acid-sensing TLRs that are common between human and mouse include TLR3, which recognizes double-stranded RNA [13], TLR7 and TLR8 (human), which detect single-stranded RNA [14-16] and TLR9, which senses DNA containing CpG motifs [17]. Upon activation, TLR7, TLR8 and TLR9 recruit the adaptor molecules MyD88, while TLR3 recruits TRIF, and trigger the release of pro-inflammatory cytokines and type I interferons (IFN) through activation of NF- κ B, MAP kinases and IFN-regulatory factors (IRFs) [10].

In the current study we evaluated the involvement of Gfi1 in the development of lupus and the signaling pathways that are controlled by Gfi1 in DCs. We found that both Genista mice, which carry a hypomorphic mutation in the *gfi1* gene [18], and Gfi1^{-/-} mice [19] develop signs of spontaneous lupus autoimmunity. Our analysis revealed a previously unknown role for Gfi1 as a negative regulator of the TLR7 signaling pathway, since Genista mice deprived of TLR7 (Genista/TLR7^{-/-}) were

protected from lupus autoimmunity. At the cellular levels, Genista DCs upon TLR7 or TLR4 stimulation produced increased amounts of TNF, IL-6 and IFN- β , which was accompanied by faster and stronger NF- κ B phosphorylation and IRF7 nuclear translocation, suggesting that Gfi1 controls these signaling pathways downstream of TLR7 and TLR4. Overall, our results show that Gfi1 plays a critical role in the prevention of spontaneous lupus autoimmunity by negatively regulating TLR7 signaling.

Results

Genista and Gfi1^{-/-} mice develop spontaneous lupus autoimmunity

Previous studies have shown that Genista mice that carry a hypomorphic mutation in the Gfi1 gene and Gfi1^{-/-} mice are neutropenic and have increased numbers of monocytes, with Genista having a milder phenotype than Gfi1^{-/-} mice [7, 18]. Indeed, side-by-side flow cytometry analysis revealed the Gfi1^{-/-} mice were severely neutropenic, while Genista mice had 4 times less neutrophils than WT mice (Fig. 1A). Moreover, the increased percentage of blood monocytes was more profound in Gfi1^{-/-} than in Genista mice (Fig. 1A).

Assessment of serum levels of IgM and IgG2a isotypes revealed that Genista and Gfi1^{-/-} mice possess significantly increased levels compared to WT mice, whereas Gfi1^{-/-} mice also had significantly elevated IgG2a levels compared to Genista mice (Fig. 1B). In order to address if Gfi1 is implicated in autoimmunity the levels of IgG autoantibodies against RNA and DNA were also evaluated. Genista and Gfi1^{-/-} mice had significantly increased titers compared to WT sera, with Gfi1^{-/-} mice showing the highest increase (Fig. 1C). Immunohistological staining of kidney sections revealed

stronger glomerular deposition of IgM and IgG in Genista and Gfi1^{-/-} mice than in WT controls, with Gfi1^{-/-} mice showing also increased size of glomeruli compared to Genista mice (Fig. 1D). Thus, these data suggest that Gfi1 deficiency or dysfunction can lead to lupus-like autoimmunity.

Lupus autoimmunity in Genista mice depends on TLR7 signaling

In contrast to Gfi1^{-/-} mice that are susceptible to infections and die at early age, Genista mice have normal viability up to 12 months, they manifest no weight loss and there is no need to be kept under long-term antibiotic treatment [7, 18]. Therefore, we focused our further *in vivo* studies on Genista mice. Moreover, since there is a strong association between SLE and increased TLR7 signaling [20], we also generated Genista mice deprived of TLR7 (Genista/TLR7^{-/-}) and studied them in parallel with the Genista mice. Genista/TLR7^{-/-} mice had normal appearance, growth, fertility and normal survival rates up to 8 months of age that we followed up the mice. Assessment of IgM and IgG2a isotype levels and IgG autoantibodies against RNA, DNA and Smith ribonucleoproteins (smRNP) in sera revealed that Genista mice had significantly increased levels compared to WT controls, whereas Genista/TLR7^{-/-} mice had normal levels (Fig. 2A and B). Assessment of the frequencies of plasmablasts (B220^{lo}CD138⁺) and GCs B cells (B220⁺GL-7⁺CD38⁻) revealed that both populations were significantly increased in Genista mice compared to WT controls, whereas in Genista/TLR7^{-/-} mice these populations appeared normal (Fig. 2C and D). In agreement with the FACS results, immunohistological analysis further confirmed the presence of increased splenic GCs in Genista mice versus WT or Genista/TLR7^{-/-} samples (data not shown). Furthermore, immunostaining of kidneys revealed that glomerular deposition of IgM and IgG was substantially greater in

Genista mice than in WT controls, whereas Genista/TLR7^{-/-} mice showed a slightly diminished deposition versus WT mice (Fig. 2E). Collectively, these data indicate that Genista mice develop spontaneous lupus autoimmunity that it is TLR7-dependent.

In order to further validate the contribution of TLR7 in the lupus phenotype of Genista mice, we crossed the Genista mice with TLR8^{-/-} mice that develop spontaneous lupus due to increased TLR7 expression and signaling on DCs [21, 22]. Although Genista, TLR8^{-/-}, and Genista/TLR8^{-/-} mice showed similar increased titers of IgM, IgG2a, and IgG autoantibodies against RNA, DNA and smRNP compared to WT mice (Supporting Information Fig. 1A-B), the Genista/TLR8^{-/-} mice displayed increased percentages of splenic plasmablasts and GC B cells, as well as more profound glomerular deposits of IgM and IgG compared to Genista or TLR8^{-/-} mice (Supporting Information Fig. 1C-E), suggesting that increased expression of TLR7 (due to TLR8 deficiency [21, 22] in Genista mice) worsens the lupus phenotype observed in Genista.

Altered B cell compartment in Genista mice

Given that SLE is an autoimmune disease with B cell hyperactivity, and Gfi1 is implicated in B cell development, we next assessed the status of B cells. The frequencies of splenic B cells were significantly reduced in both Genista and Genista/TLR7^{-/-} mice compared to WT mice (Fig. 3A and Table 1). Staining for CD69 and MHC class II revealed that Genista splenic B cells were activated compared to WT or Genista/TLR7^{-/-} B cells (Fig. 3B), and this was accompanied by increased TLR7 expression in Genista versus WT B cells (Supporting Information Fig. 2). Moreover, since in the setting of lupus autoimmunity the populations of

marginal zone (MZ), B1 and transitional T1 and T2 B cells can be affected we also evaluated these populations. Compared to WT mice, both Genista and Genista/TLR7^{-/-} mice had similar increased frequencies of MZ B cells (WT 9.6 ± 1.6 , Genista 17.5 ± 1.8 , Genista/TLR7^{-/-} 22.4 ± 6.4) (Fig. 3C) and dramatically reduced frequencies of peritoneal B1a (WT 15.5 ± 4.9 , Genista 2.3 ± 0.7 , Genista/TLR7^{-/-} 2.9 ± 1.2) and B1b B cells (WT 18.4 ± 3.4 , Genista 7 ± 3.1 , Genista/TLR7^{-/-} 8.9 ± 1.1) (Fig. 3D). The frequencies of T1 B cells were similar between the three genotypes, however there was a tendency for reduced frequency of these cells in Genista mice compared to WT or Genista/TLR7^{-/-} mice (Supporting Information Fig. 3A). In addition, the frequencies of T2 and T3 B cells were similar in all three genotypes (Supporting Information Fig 3A). Furthermore, we also assessed the frequency of age-associated B cells (ABCs) that secrete autoantibodies and appear in autoimmune-prone mice by the time of onset of autoimmunity, through a TLR7-dependent manner [23, 24]. Genista mice had a significant increase in the frequency of ABCs in their spleens compared with age matched WT or Genista/TLR7^{-/-} mice (Supporting Information Fig. 3B), and the expression levels of T-bet was higher in Genista than in WT or Genista/TLR7^{-/-} ABCs (Supporting Information Fig. 3C).

Next, we performed *ex vivo* studies using mouse splenocytes. Genista B cells showed spontaneous increased expression of CD86 compared to WT B cells, and upon stimulation with R848, LPS or CpG, Genista B cells exhibited a stronger response than WT B cells (Fig. 3E and Supporting Information Fig. 4A). Moreover, Genista splenocytes produced significantly increased levels of IL-6 compared to WT splenocytes both in unstimulated conditions, as well as upon stimulation with R848, LPS, or CpG (Fig. 3F and Supporting Information Fig. 4B). As expected,

Genista/TLR7^{-/-} splenocytes were unresponsive to R848, while upon LPS stimulation produced similar level of IL-6 as Genista cells (Fig. 3F).

B cells can proliferate and may differentiate into antibody producing cells in response to TLR triggering [25]. Therefore, we tested these parameters and their dependency on TLR7 in Genista mice. We found that WT and Genista B cells proliferate similarly in response to R848, LPS or CpG, while as expected Genista/TLR7^{-/-} B cells did not respond to R848 (Supporting Information Fig. 4C). However, in the course of proliferation we noticed that Genista cells showed a higher ability to differentiate into plasmablasts upon R848, LPS or CpG stimulation compared to WT cells, while Genista/TLR7^{-/-} B cells did not respond to R848, but upon LPS or CpG stimulation differentiated as efficiently as Genista cells (Supporting Information Fig. 4D). Altogether, these results show that in Genista mice the B cells are more activated and respond stronger to TLR ligands.

Spontaneous activation of DCs and T cells in Genista mice

DCs and T cells are also implicated in the development and progression of lupus. Therefore, we next assessed the frequencies and activation status of these cell types. The frequency of conventional DCs (cDCs) and plasmacytoid DCs (pDCs) were significantly increased in Genista mice compared to WT mice, whereas Genista/TLR7^{-/-} mice had normal frequencies of these cell types (Fig. 4A and Table 1). Evaluation of activation markers, such as CD86 and CD40, on the splenic DC subsets (CD11b-like, CD8 α -like and pDCs), revealed that Genista pDCs (Fig. 4B and Supporting Information Table 1) and CD11b-like DCs (Supporting Information Fig.

5C and Supporting Information Table 1) were spontaneously activated compared with WT or Genista/TLR7^{-/-} cells.

Concerning the T cell compartment, the frequencies of splenic CD4 or CD8 T cells were similar in WT and Genista mice, while Genista/TLR7^{-/-} mice showed reduced frequencies of CD4 T cells and increased frequencies of CD8 T cells compared to WT or Genista mice (Table 1). Moreover, both Genista and Genista/TLR7^{-/-} mice had significantly reduced frequencies of naïve CD4 and CD8 T cells, and increased frequencies of effector memory (CD44⁺CD62L⁻) CD4 and CD8 T cells compared to WT mice (Fig. 4C). The frequencies of central memory (CD44⁺CD62L⁺) CD4 T cells were similar in WT, Genista and Genista/TLR7^{-/-} mice, while central memory CD8 T cells were significantly increased in Genista and Genista/TLR7^{-/-} mice compared to WT mice (Fig. 4C). Thus, the increased frequencies and activation status of DCs in Genista mice are TLR7-dependent, while the increased T cell activation status in Genista mice is TLR7-independent.

Increased response of Genista DCs upon TLR stimulation

In macrophages Gfi1 expression can be induced by the TLR4 ligand LPS in a TNF-independent manner [9], but its modulation by other TLRs or its expression by DCs has not been addressed. Thus, we examined the effects of TLR ligands on Gfi1 expression in WT macrophages and DCs. Stimulation with TLR2/6, TLR3, TLR4, TLR7 or TLR9 ligands upregulated Gfi1 mRNA expression both in bone marrow-derived macrophages (BMMFs) (Supporting Information Fig. 5A) and bone marrow-derived DCs (BMDCs) (Supporting Information Fig. 5B), suggesting that in macrophages and DCs the expression of Gfi1 is altered upon TLR stimulation.

It has been reported previously that *Gfi1*^{-/-} BMMFs respond to LPS with increased TNF secretion [7, 9]. Thus, we tested whether the Genista mutation also affects the responses of macrophages and DCs to TLR stimulation. Upon R848, LPS or CpG challenge, Genista BMMFs produced normal amounts of IL-6 and TNF (Supporting Information Fig. 5D), while Genista BMDCs produced significant increased amounts of IL-6 and TNF versus WT BMDCs (Fig. 5A). Moreover, upon R848 or LPS stimulation Genista BMDCs showed increased expression of IFN- β , and the type I IFN stimulated genes, *Isg15* and *IRF7* versus WT cells (Fig. 5B). Thus, upon TLR stimulation the Genista mutation causes an overproduction of cytokines by DCs, but not by macrophages.

We next assessed the implication of Genista mutation on the NF- κ B and type I IFN signaling pathways on DCs. Upon R848 or LPS stimulation, Genista BMDCs showed stronger and faster NF- κ B p65 phosphorylation than WT cells (Fig. 5C and Supporting Information Fig. 5E). In addition, we monitored the activation of the transcription factors IRF7, which resides in the cytosol of resting cells and upon cytosolic TLR stimulation undergoes serine phosphorylation, allowing it to translocate into the nucleus and induce the expression of type I IFN genes [26]. Under resting/unstimulated conditions IRF7 was already present in the nucleus of Genista, but not of WT, BMDCs (Fig. 5C and Supporting Information Fig. 5F). In addition, upon R848 stimulation, nuclear expression of IRF7 was stronger in Genista than in WT cells at 15 and 120 min, while upon LPS stimulation WT cells showed a delayed response and nuclear expression of IRF7 was stronger in Genista than in WT cells at 15 min (Fig. 5C and Supporting Information Fig. 5F). Overall, these results show that

Genista BMDCs are more responsive than WT cells and suggest that Gfi1 is a negative regulator of the TLR7 and TLR4 signaling pathways in DCs.

Discussion

Engagement of TLRs activates multiple signaling pathways that have to be tightly regulated since excessive TLR activation can disrupt immune homeostasis and may lead to the development of autoimmune and inflammatory diseases [27, 28]. In this study, we uncovered a critical role for the transcriptional repressor Gfi1 in the development of lupus autoimmunity by restraining TLR7 signaling. We demonstrated for the first time that neutropenic Gfi1^{-/-} or Genista mice develop spontaneous lupus autoimmunity. In addition, we provide evidence that the lupus phenotype in Genista mice is TLR7-dependent, since Genista mice deprived of TLR7 were protected from lupus development, while increase of TLR7 signaling (in Genista/TLR8^{-/-} mice) led to the exacerbation of the lupus phenotype. Our finding fits with the current notion that increased TLR7 signaling contributes to the initiation and/or exacerbation of SLE in mice [22, 29-31]. Interestingly, lately it has become apparent that also in humans SLE can be attributed to increased TLR7 function. Indeed, various studies revealed that the SNP rs3853839 (C-G) at the 3' untranslated region of TLR7 is associated with elevated TLR7 transcript expression and increased risk for SLE both in Asian [32-34] and Danish [35] populations.

Many different immune cell types are implicated in the development of SLE, of which DCs and B cells play a central role [36, 37]. cDCs and B cells are major antigen presenting cells that regulate T-cell mediated autoimmunity, while pDCs drive disease by type I IFN production. DCs from lupus-prone mice display a range of

alterations in their frequency and function [36, 38]. Accordingly, we showed that Genista mice had increased numbers of splenic cDCs and pDCs, along with increased activation, while in Genista/TLR7^{-/-} mice these DC populations appeared normal, indicating that in Genista mice increased TLR7 function is responsible for the observed DC alterations. Analysis of TLR7 expression in the splenic DC subsets revealed that cDCs from Genista mice overexpressed TLR7, but this was not observed in pDCs (Supporting Information Fig. 2), suggesting that Genista mutation can affect TLR7 signaling without necessarily affecting TLR7 expression.

Contrary to the increased numbers and activation of DCs in Genista mice, previous studies have shown that Gfi1 deficiency leads to global reduction of all DCs in primary and secondary lymphoid organs, as well as, incomplete DC maturation and function [39]. Moreover, Gfi1^{-/-} hematopoietic progenitors are unable to develop into DCs *in vitro* and instead they differentiate into macrophages [39], leading to the belief that Gfi1 is a unique factor governing DC versus macrophages development. However, we found that GM-CSF treated Genista hematopoietic progenitor cells were able to give rise to DCs, suggesting that the hypomorphic Genista mutation (C318Y substitution at the third zinc finger domain of Gfi1) does not affect the ability of Gfi1 to control DC development and function. Additional studies that are out of the scope of the current manuscript are needed to further clarify the implication of different Gfi1 variants in DC development.

B cells contribute to lupus pathogenesis by autoantibody secretion and GCs are an important B cell tolerance checkpoint in the periphery. Thus, increased autoantibodies and spontaneous GCs are characteristic of several autoimmune prone mice [40].

Accordingly, in Genista mice we found an augmentation of autoantibody titers for RNA, DNA and smRNP, as well as frequencies of spontaneous GCs, which were due to increased TLR7 signaling since these phenotypes were absent in Genista/TLR7^{-/-} mice. Our findings further confirm recent observations that B cell-intrinsic TLR7 signaling, through detection of self-nucleic acids and/or retroviral elements, is essential for the development of spontaneous GCs, and that in the presence of autoimmune conditions TLR7-dependent spontaneous GCs produce pathogenic autoantibodies [41]. In addition, we observed that the B cell subset of ABCs that is driven by TLR7 signaling, secretes autoantibodies and arise during autoimmune disease both in mouse models and humans [23, 24, 42, 43], was also increased in Genista mice through a TLR7-dependent manner. Nevertheless, we noticed normal B cell proliferation in Genista mice, which is in accordance with previous studies on Gfi1^{-/-} B cells [44]. However, stimulation of Genista splenocytes with TLR7, TLR9 or TLR4 agonists promoted increased B cell differentiation to plasmablasts. Accordingly, Genista mice showed increased percentage of plasmablasts, while in Genista/TLR7^{-/-} mice this cellular compartment was normal, suggesting that plasmablasts differentiation is disturbed in Genista mice, due to increased TLR7 function.

Marginal zone and B1 B cells have the capacity to respond to foreign antigens more rapidly than conventional B cells, providing early immune responses to blood-borne pathogens, and both subsets are often altered in lupus prone mice [37]. In various transgenic mice in the C57BL/6 background that develop lupus due to increased TLR7 signaling, such as mice that carry the Yaa locus [45], transgenic mice that overexpress TLR7 [29], mice that carry the Unc93B1-D34A mutation [46], or TLR8-deficient mice [21, 22], the increased TLR7 signaling leads to reduced MZ B cell

compartment, probably due to increased activation of the MZ B cells and their exit from this compartment [47]. Surprisingly, the MZ B cell population was markedly expanded in Genista mice, and this phenotype was not altered in the absence of TLR7 signaling, suggesting that Gfi1 independently of TLR7 might act as a transcriptional regulator responsible for controlling the differentiation and/or lifespan and survival of the MZ B cells.

B1 B cells are located mainly in the peritoneal and pleural cavities and play critical roles in immediate defense against viruses and bacteria, as well as housekeeping removal of cellular debris, through the spontaneous and constitutive secretion of polyreactive IgM antibodies [48]. B1 B cells express various TLRs and upon detection of microbial components TLR signaling induce a massive egress of B1 B cells from the peritoneal cavity, which is associated with coordinated down-regulation of integrins and CD9 [49]. Thus, the dramatic reduced numbers of peritoneal B1 B cells in Genista and Genista/TLR7^{-/-} mice could be due to increased TLR signaling (other than TLR7) in B1 B cells that leads to rapid mobilization of these cells out of the peritoneal cavity or the Genista mutation leads to defects of the factors that are critical for the development and migration of B1 B cells and/or precursors in the peritoneal cavity.

To avoid inappropriate inflammatory responses, many negative TLR regulators exist that act at different levels in the active TLR signaling pathways and use different mechanisms such as dissociation of adaptor complexes, degradation of signal proteins or transcriptional regulation [28]. Based on its biochemical function as transcriptional repressor and recruiter of histone modifiers, Gfi1 is considered as an epigenetic

regulator that modify chromatin structure mostly to repress transcription [50]. Thus, Gfi1 in contrast to most known negative regulators of TLRs, does not affect the proximal cytoplasmic components of TLR signaling pathways but rather acts at the downstream end of the pathway [9]. Indeed, in macrophages Gfi1 negatively controls the TLR4-mediated inflammatory response by antagonizing NF- κ B p65 [9], but its implication to the signaling pathway downstream of other TLRs and in DCs has not been addressed. Our data clearly show that upon TLR7 or TLR4 stimulation the Genista mutation leads to increased production of cytokines (TNF, IL-6, IFN- β) and IFN stimulated genes (Isg15 and IRF7) by DCs, which is accompanied by increased NF- κ B p65 and type I IFN signaling. Thus, Gfi1 seems to be a general negative regulator of the TLR signaling pathway.

The functional contribution of neutrophils in SLE pathogenesis versus protection remains controversial. In SLE patients, impaired neutrophil extracellular trap (NET) clearance and/or aberrant NETosis has been suggested to play a prominent role in the perpetuation of autoimmunity and the exacerbation of disease, as well as the induction of end-organ manifestation [51]. However, several recent studies revealed that neutrophils can inhibit systemic autoimmunity [52, 53]. Our results show that the neutropenic Gfi1^{-/-} and Genista mice develop spontaneous lupus-like autoimmunity, rendering these mice as new mouse models of lupus autoimmunity that is accompanied by neutropenia.

In conclusion, our findings demonstrate the critical role of Gfi1 in the regulation of myeloid cells, and prevention of spontaneous lupus autoimmunity by negatively

controlling TLR7 signaling. It still remains to be seen if cases of human SLE can be attributed to Gfi1 gene deficiency or modifications.

Materials and Methods

Mice

Genista, Gfi1^{-/-}, TLR7^{-/-} and TLR8^{-/-} mice were generated as described previously [16, 18, 19, 21]. Genista/TLR7^{-/-} and Genista/TLR8^{-/-} mice were generated by intercrossing Genista with TLR7^{-/-} or TLR8^{-/-} mice. All mice were backcrossed to the C57BL/6 background for more than 10 generations. Wild-type (WT) C57BL/6 mice were purchased from the Charles River Laboratories. Mice were housed under specific pathogen-free conditions. All animal experiments have been approved by the local Ethical Committee of Marseille for animal experimentation (04364.03).

Reagents

R848, LPS from *E. coli* 0111-B4, Pam₂CSK₄, CpG ODN 1826 and poly I:C were purchased from Invivogen.

Cell culture

Bone marrow cells were extracted from mouse femur and tibias and cultured in the presence of GM-CSF or M-CSF for the production of bone marrow-derived DCs (BMDCs) or bone marrow-derived macrophages (BMMFs) as described previously [13, 21]. Erythrocyte-depleted splenocytes were cultured in RPMI medium supplemented with 10% FCS, 1% MEM Non-Essential Amino Acids, and 50 μM β-mercaptoethanol.

Serological analysis and ELISA

Evaluation of IgM and IgG2a, and IgG autoantibodies against DNA, RNA and smRNP in serum samples was performed as described previously [21]. Cell culture supernatants were assayed for IL-6 by ELISA (eBioscience) according to manufacturer's instructions.

Flow cytometry

Cell suspensions were incubated with 24G2 hybridoma supernatant (to block Fc receptors) and stained using immunofluorescence-labeled antibodies against the following antigens: B220/CD45R, CD3, CD19, CD11b, Ly6G, CD21, CD5, CD44, CD86, CD38, CD138, CD24, CD172a (SIRP α), CD69, CD43, CD40, CD45.2, CD93, IgM, TLR7 and CD62L from BD Biosciences, IA/IE (MHC class II), GL7, CD23, T-bet, CD4 and CD8 from eBioscience and CD11c and SiglecH from Biolegend. For intracellular TLR7 staining, cell suspensions were stained for the indicated extracellular antigens, before fixation with Cytotfix (BD Biosciences). Cells were then permeabilized with saponin 0.1% and stained for TLR7. Intracellular T-bet staining was performed using the FoxP3 Fixation/Permeabilization buffer (eBioscience). Flow cytometry was conducted using an LSR2 (BD Biosciences) and data were analyzed with FlowJo (Tree Star). The full gating strategy that used for flow cytometry analysis is presented in Supporting Information Fig. 6. For splenocyte proliferation experiments, erythrocyte-depleted splenocytes were labeled with CellTrace Violet (Life technologies) according to manufacturer's recommendations. After 4 days of stimulation, cells were harvested and stained with antibodies for analysis by flow cytometry.

RNA isolation and quantitative real-time PCR

Total RNA was isolated by TRIzol reagent (Gibco, Invitrogen) and reversed transcribed with SuperScript II reverse transcriptase (Invivogen). Q-PCR was performed with the SYBR Premix Ex Taq (Takara) using the primers shown on Supporting Information Table 2, on an Applied Biosystems PRISM 7700 Sequence Detection System, and the amount of target was calculated relative to the calibrator by $2^{-\Delta CT}$.

Immunohistology

Murine kidneys or spleens were embedded in OCT compound and frozen in liquid nitrogen. Cryostat sections were rehydrated in PBS, incubated with blocking solution (2% BSA in PBS) for 2h at room temperature. Kidneys were stained with anti-IgG Alexa Fluor 488 (Invitrogen) and IgM PE (BD Biosciences) and fluorescence images were acquired using a Zeiss LSM 780 laser scanning confocal microscope and analyzed with the Zen software.

Western blot

For whole cell protein extracts BMDCs were washed with ice-cold PBS and cells were lysed in RIPA buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1% Triton-X100, pH 7.5), while for isolation of cytosolic or nuclear protein extracts BMDCs were treated in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA) or buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl 1 mM EDTA), respectively. Protein samples were then loaded onto 10% SDS-PAGE gels, electrophoresed and

transferred onto Immobilon P membrane (Millipore). Blotting was performed using the following antibodies: p65 and phospho-p65 S311 (Santa Cruz), IRF7 (OriGene) and beta-actin (Sigma). The bound antibodies were detected using the Pierce ECL Western Blotting substrate (Thermo Scientific).

Statistics

Statistics were calculated using Prism 5 (GraphPad Software) by the Mann-Whitney U test with p values indicated throughout as *p < 0.05, **p < 0.01, and ***p < 0.001.

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Conflict of interest

The authors declare no commercial or financial conflict of interest.

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Table 1. Body weight, spleen weight, total cell count, and major splenic cell populations of WT, Genista and Genista/TLR7^{-/-} mice ^{a)}.

		WT	Genista	Genista/TLR7 ^{-/-}
Body weight (g)		27.8 ± 0.6	32.5 ± 1.3 ^a	33.3 ± 0.6 ^a
Spleen weight (mg)		86.4 ± 9.5	102.2 ± 22.1	80.6 ± 2.3 ^b
Total cell count (x10 ⁶)		65.2 ± 3.4	59.9 ± 5.1	64.4 ± 3.2
Cell type	Surface markers	Frequency		
T cells	CD3 ⁺	26.7 ± 1.4	27.4 ± 2.0	26.9 ± 2.3
CD4 (% T cells)	CD3 ⁺ CD4 ⁺	54.6 ± 1.6	56.7 ± 2.9	49.3 ± 3.4 ^{a,b}
CD8 (% T cells)	CD3 ⁺ CD8 ⁺	32.3 ± 0.9	29.2 ± 2.8	38.5 ± 2.8 ^{a,b}
B cells	B220 ⁺	64.7 ± 2.8	56.5 ± 3.3 ^a	57.5 ± 3.2 ^a
cDCs	CD11c ^{hi} MH CII ^{hi}	1.6 ± 0.8	3.7 ± 1.6 ^a	1.9 ± 0.2 ^b
pDC	CD11c ^{int} Sig IecH ⁺	0.4 ± 0.2	1.0 ± 0.6 ^a	0.4 ± 0.1 ^b
Monocytes*	CD11b ^{hi} M HCII ^{lo/-}	3.2 ± 0.8	11.2 ± 3.9 ^a	9.6 ± 2.9 ^a

a) Data are from 5 or 6-7 (*) months old male mice. Values shown are average of at least four mice per genotype ± SD. ^a p < 0.05 versus WT and ^b p < 0.05 versus Genista determined by the Mann-Whitney U test.

Figure legends

Figure 1. Genista and *Gfi1*^{-/-} mice develop spontaneous autoimmunity. (A) Representative FACS profiles of blood cells from WT, Genista and *Gfi1*^{-/-} mice stained with anti-CD45.2, -CD5, -B220, -CD11b and -Ly6G mAb. Percentage of neutrophils (CD11b^{high}Ly6G^{high}) and monocytes (CD11b^{high}Ly6G⁻) are indicated. Sera from WT (n=8), Genista (n=8) and *Gfi1*^{-/-} (n=6) mice were used for the evaluation of (B) IgM and IgG2a levels and (C) anti-RNA and -DNA autoantibody production by ELISA. Each point represents value from one mouse and horizontal bars denote the mean. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 determined by the Mann-Whitney U test. (a. u., arbitrary units). (D) Kidney sections from WT, Genista and *Gfi1*^{-/-} mice were stained with anti-IgM or anti-IgG (scale bar = 50 μm, original magnification 100×). (A-D) Data are from 3-4 months old male mice and are representative of two independent experiments, with (A and D) n = 3 mice/group.

Figure 2. Genista mice develop TLR7-dependent lupus autoimmunity. Sera from 9 weeks old male WT (n=10), *Genista* (n=7) and *Genista/TLR7*^{-/-} (n=5) mice were used for the evaluation of (A) of IgM and IgG2a levels and (B) anti-RNA, anti-dsDNA and anti-smRNP autoantibodies by ELISA. Representative flow cytometry plots and graphical plots of the percentages of (C) B220^{lo}CD138⁺ plasmablasts and (D) B220⁺GL7⁺CD38⁻ GC B cells in splenocytes derived from 5 months old male WT, *Genista* and *Genista/TLR7*^{-/-} mice (n= 3–5 mice/group) **p* < 0.05 determined by the Mann-Whitney U test. (E) Kidney sections from male WT, *Genista* and *Genista/TLR7*^{-/-} mice (n = 3 mice per group) were stained with immunofluorescence anti-IgM or anti-IgG antibodies (scale bar = 50 μm, original magnification x100).

Images are representative of two independent experiments. (A-D) Each point represents value from one mouse and horizontal bars denote the mean. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ determined by the Mann-Whitney U test. Data in C and D are representative of two independent experiments.

Figure 3. Spontaneous B cell activation and enhanced B cell responses to TLR agonists in Genista mice. (A-B) Total splenocytes from 3 months old female WT, Genista and Genista/TLR7^{-/-} mice were analyzed for the expression of CD19, CD69 and MHC-II. (A) Percentages of CD19-positive cells were evaluated and (B) mean fluorescence intensity (MFI) was calculated for CD69 and MHC-II on CD19-gated cells. (C) Splenocytes from 7 months old male WT, Genista and Genista/TLR7^{-/-} mice were analyzed by flow cytometry for the expression of CD19, CD21, and CD23. Numbers denote the percentage of marginal zone (CD19⁺CD21^{hi}CD23^{lo/-}), follicular (CD19⁺CD21^{int}CD23^{hi}), and immature (CD19⁺CD21⁻CD23⁻) B cells in the indicated gates. (D) FACS analysis of CD43 and CD5 expression on CD19-gated cells of the peritoneal cavity from 5 months old male WT, Genista and Genista/TLR7^{-/-} mice shows the percentage of B1a (CD19⁺CD43⁺CD5^{int}), B1b (CD19⁺CD43⁺CD5⁻), and B2 (CD19⁺CD43⁻CD5⁻) B cells. (E) Total splenocytes from 6 months old female WT (dotted line), Genista (black line) mice were stimulated with 10 nM R848 or 500 ng/ml LPS. After 24 h, cells were stained for CD19 and CD86. Plots represent expression of CD86 on CD19-gated cells. (F) Total splenocytes from WT, Genista or Genista/TLR7^{-/-} mice were left untreated or stimulated with the indicated doses of R848 and LPS for 16 h. IL-6 production in culture supernatants was measured by ELISA. (A-F) Data are shown as mean \pm SD (n = 3-5 per group) and are

representative of (A-B) 3, (C) 4, (D) 2 and (E-F) 4 independent experiments. $*p < 0.05$, $**p < 0.01$ determined by the Mann-Whitney U test.

Figure 4. Immune cells and activation status in WT, *Genista* and *Genista/TLR7^{-/-}* mice. (A) Dot plots representing the percentage of splenic conventional (CD19⁻CD11c^{hi}MHCII^{hi}) and plasmacytoid (CD19⁻CD11c^{int} SiglecH⁺) DCs in 5-7 months old male WT (n = 8), *Genista* (n = 9) and *Genista/TLR7^{-/-}* (n = 7) mice. Each point represents value from one mouse and horizontal bars denote the mean. (B) MFI was calculated for CD86 and CD40 on pDCs. Data are shown as mean \pm SD (n = 7-9 per group) and are pooled from two independent experiments. (C) Representative FACS profiles and graphical analysis of splenocytes from 5 months old male WT, *Genista* and *Genista/TLR7^{-/-}* mice, analyzed for the expression of CD3, CD4, CD8, CD44 and CD62L. CD62L CD44 staining profiles of gated CD4⁺ (upper) and CD8⁺ (lower) T cells were used to identify naïve (CD62L^{hi} CD44^{lo/-}), central memory (CD62L^{hi} CD44^{hi}) and effector memory (CD62L^{lo} CD44^{hi}) subpopulations. Plots represent mean \pm SD, data are representative of two independent experiments (n = 3-5 per group). $*p < 0.05$, $**p < 0.01$ determined by the Mann-Whitney U test.

Figure 5. Increased responses of *Genista* DCs to TLR agonists. (A-B) BMDCs from WT (n=3) and *Genista* (n=3) mice were stimulated with R848, LPS or CpG. (A) After 8 h, the concentrations of IL-6 and TNF in the culture supernatants were assessed by ELISA. $*p < 0.05$ determined by the Mann-Whitney U test. (B) BMDCs were stimulated with 50 nM R848 or 1 ng/ml LPS for the indicated time points. Expression of IFN- β , Isg15 and IRF7 were assessed by quantitative PCR and normalized with the $2^{-\Delta\text{CT}}$ method to the β -actin internal control after extraction of total RNA. (A and B)

Data are shown as mean \pm SD (n = 3 mice/group). (C) BMDCs were stimulated with 50 nM R848 or 10 ng/ml LPS for the indicated time points. Phospho-p65 (S311) was analyzed by immunoblotting on whole cell lysates. IRF7 translocation was evaluated by immunoblotting on nuclear extracts. Blots were stripped reprobed for p65 or β -actin as loading controls. (A-C) Data are representative of 3 independent experiments.

Figure 1.

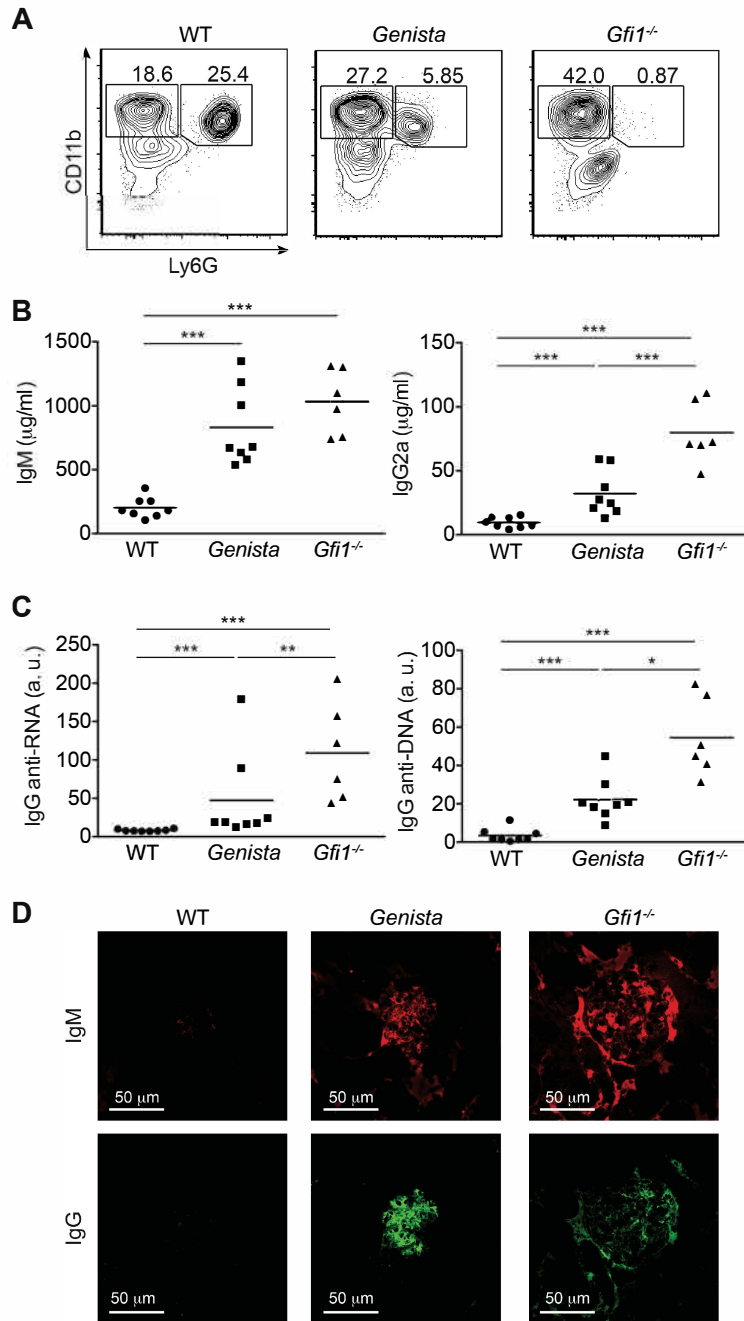


Figure 2.

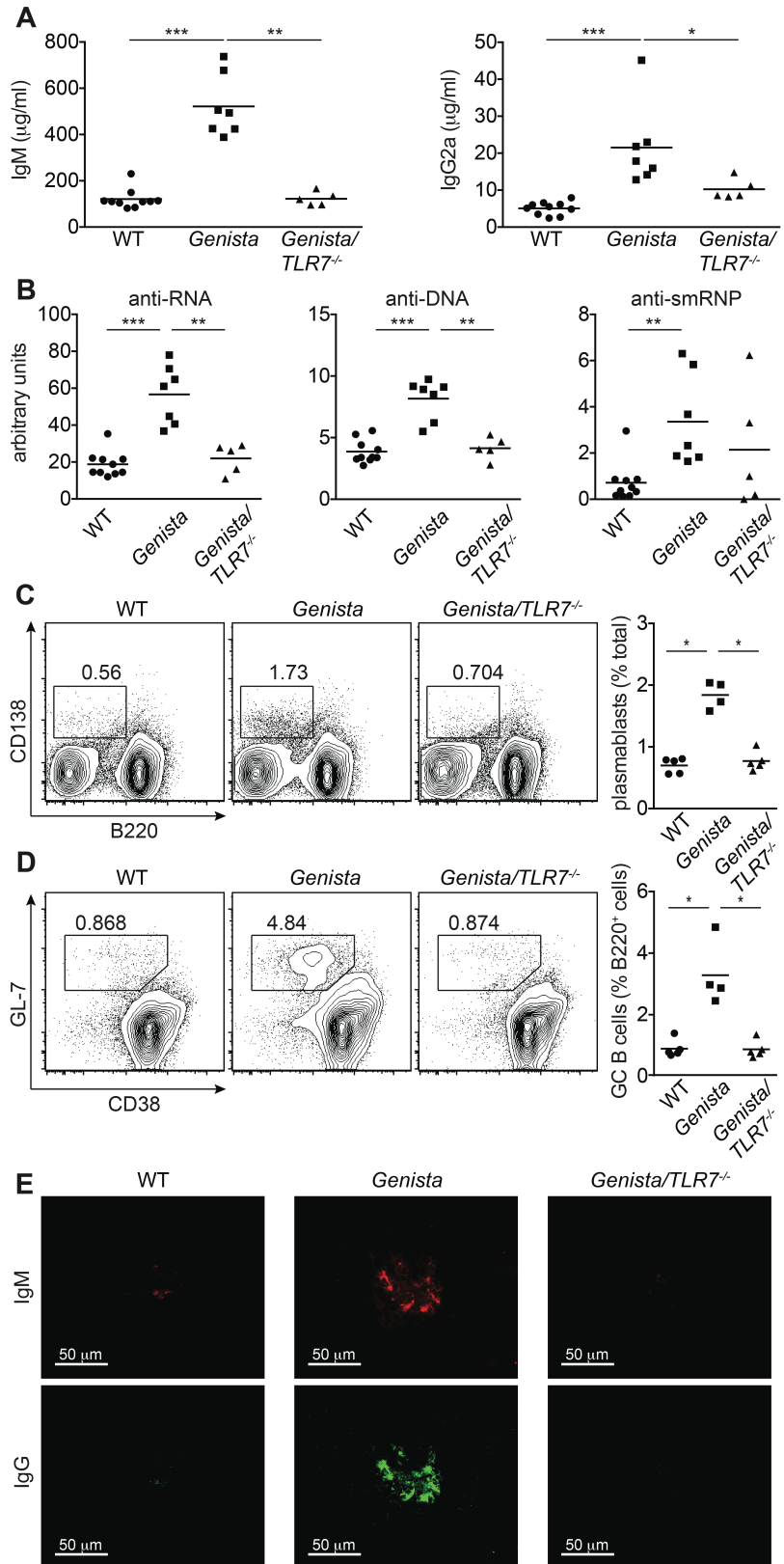


Figure 3.

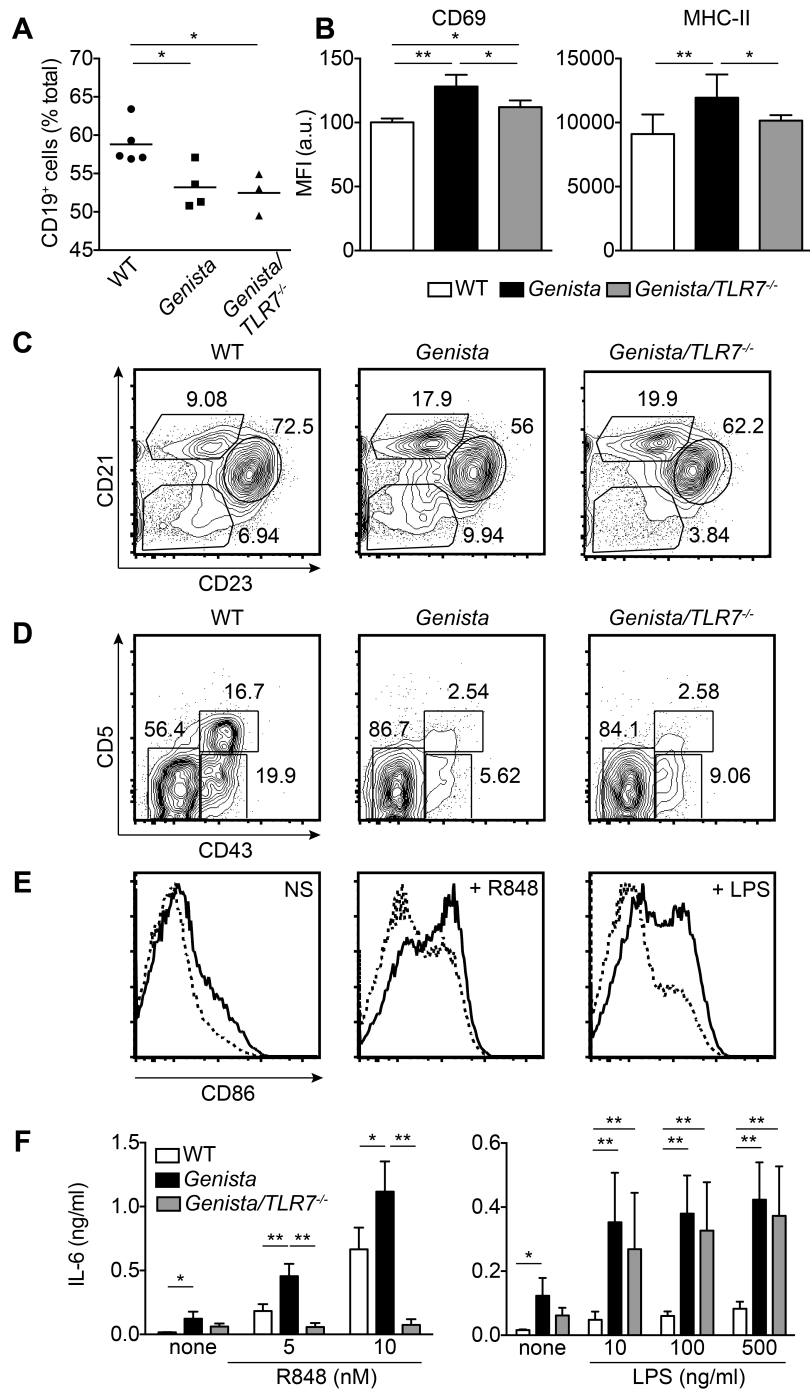


Figure 4.

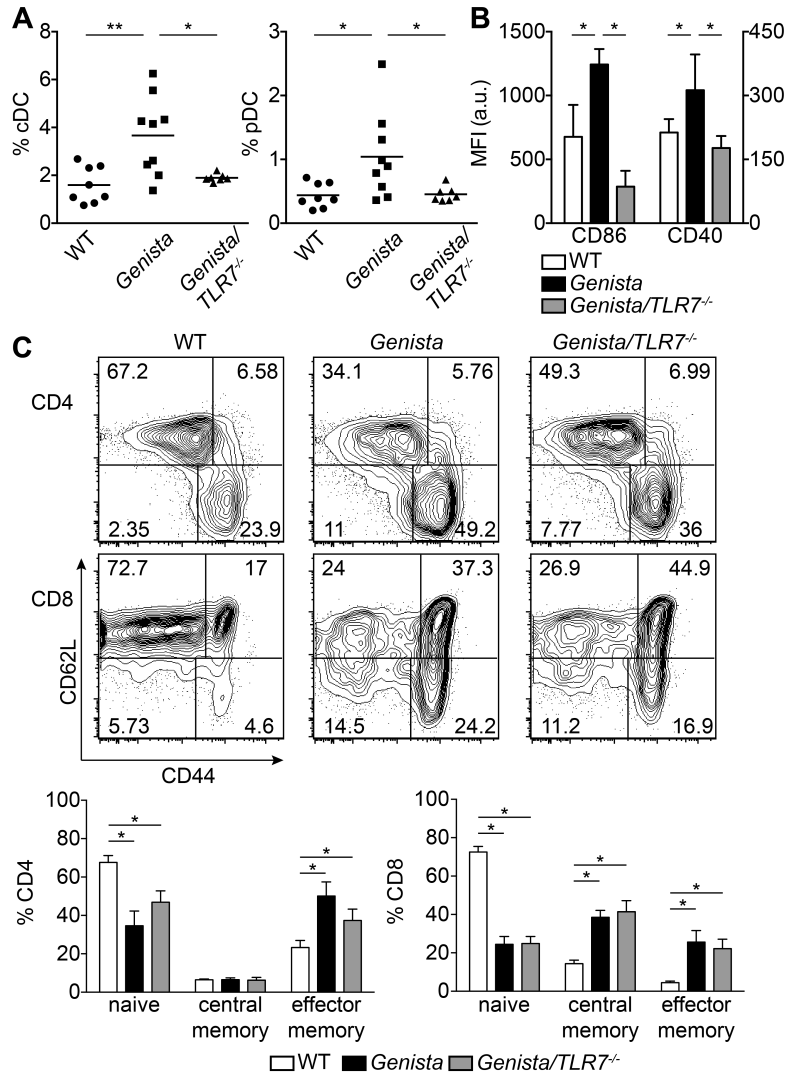
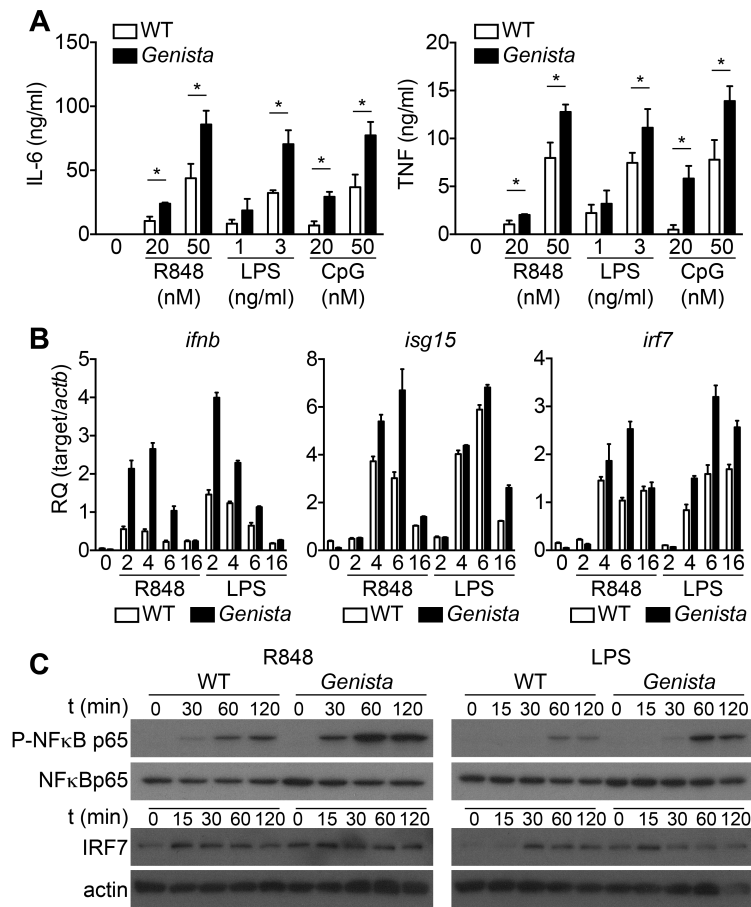


Figure 5.



Supporting Information Table 1. Activation status of WT, Genista and Genista/TLR7^{-/-} splenic DC subtypes

DC subtype	Activation marker	WT	Genista	Genista/TLR7 ^{-/-}
CD11b-like	CD86	236.0 ± 102.1	375.0 ± 59.8	188.3 ± 104.4 ^b
	CD40	302.8 ± 20.4	365.0 ± 10.6 ^a	330.3 ± 14.8 ^b
CD8α-like	CD86	482.3 ± 207.7	654.2 ± 52.5	354.7 ± 205.3 ^b
	CD40	247.0 ± 33.4	372.3 ± 26.0 ^a	310.8 ± 37.8
pDC	CD86	677.0 ± 249.9	1243.0 ± 121.5 ^a	286.7 ± 123.7 ^b
	CD40	213 ± 31.6	312.5 ± 84.1 ^a	176.8 ± 28.3 ^b

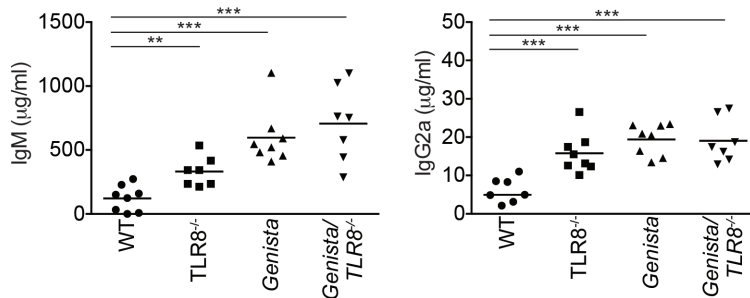
^a, $P < 0.05$ versus WT and ^b, $P < 0.05$ versus Genista determined by the Mann-Whitney U test. Values shown are average of at least three mice per genotype ± SD.

Supporting Information Table 2. Primers used for qPCR

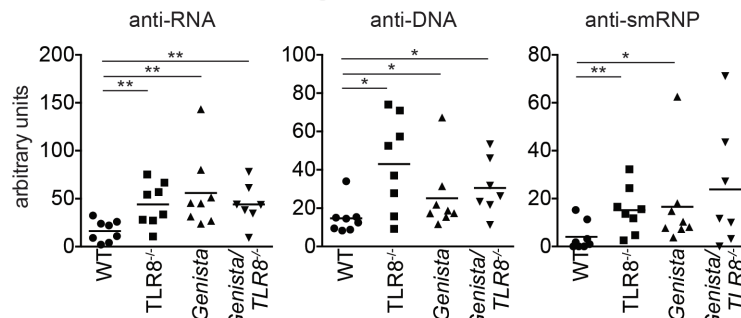
Target	Primer sequence (5'→3')
Ifn- β	forward: CGTTCCTGCTGTGCTTCTCC
	reverse: TCTTGGAGCTGGAGCTGCTT
Isg15	forward: ACCCTTTCCAGTCTGGGTCT
	reverse: TCGCTGCAGTTCTGTACCAC
IRF7	forward: CGCCACACCCCATCTT
	reverse: AGCCCGAAACTCCTCCAGTT
Gfi1	forward: CCCTTTGCGTGCGAGATGT
	reverse: CAGCGTGGATGACCTCTTGAA
β -actin	forward: TGGAATCCTGTGGCATCCATGAAACC
	reverse: TAAACGCAGCTCAGTAACAGTCCG

Supporting Information Figures and legends

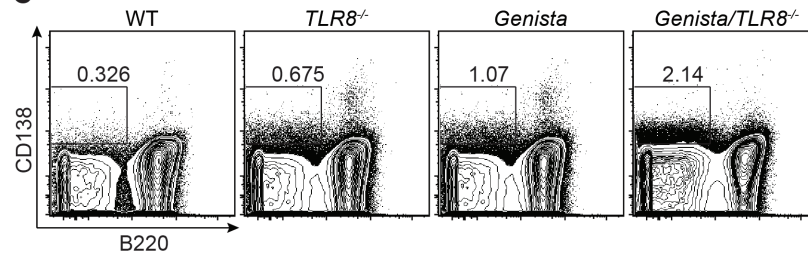
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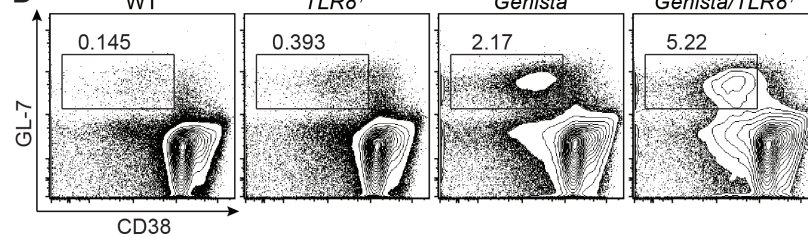
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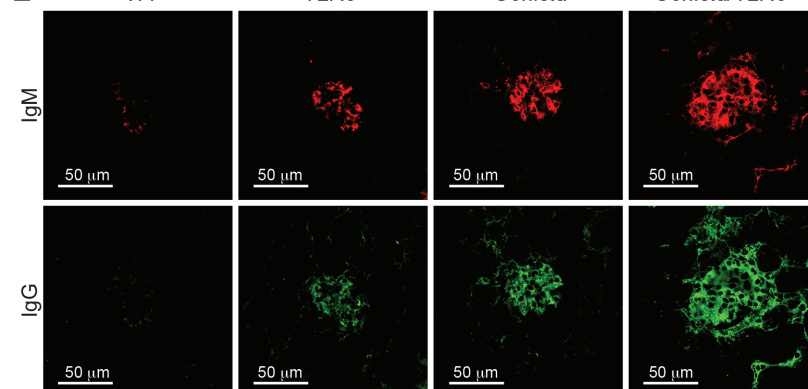
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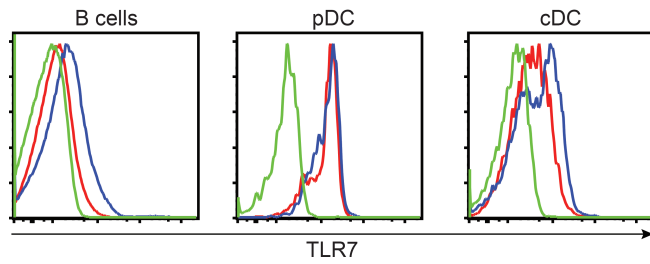


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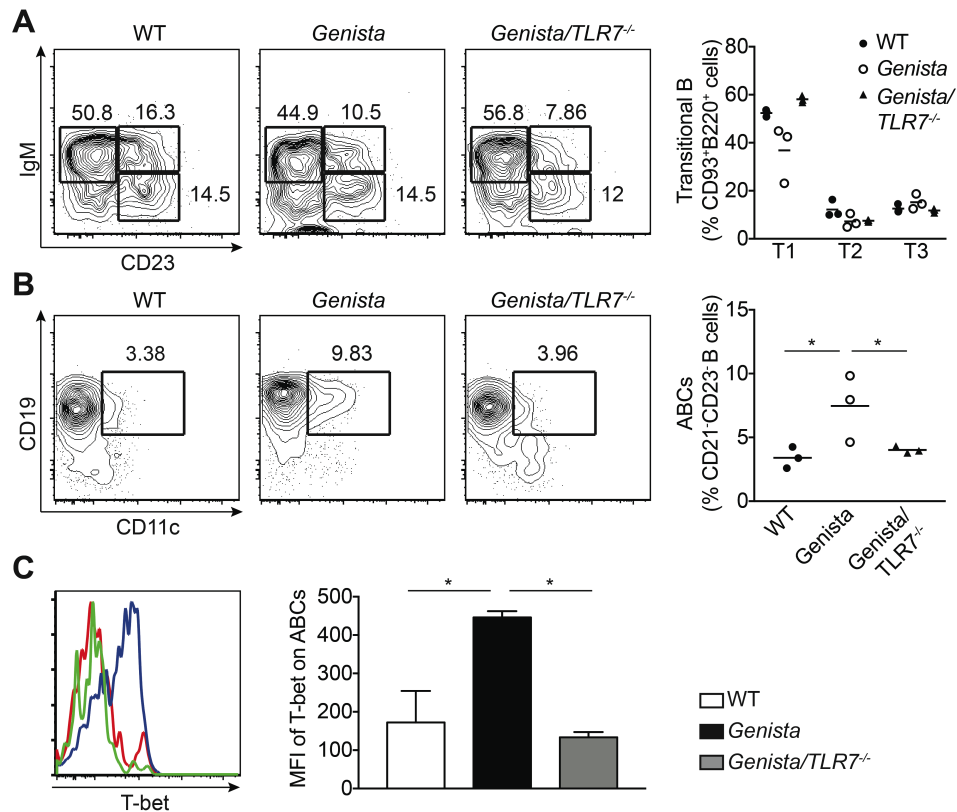


Supporting Information Figure 1. Increased lupus phenotype in Genista/TLR8^{-/-} versus Genista or TLR8^{-/-} mice. (A and B) Sera from 3 to 4 months old male WT (n = 8), TLR8^{-/-} (n=7), Genista (n = 8) and Genista/TLR8^{-/-} (n = 7) mice were used for the evaluation of (A) serum levels of IgM and IgG2a and (B) IgG autoantibodies against RNA, DNA and smRNP

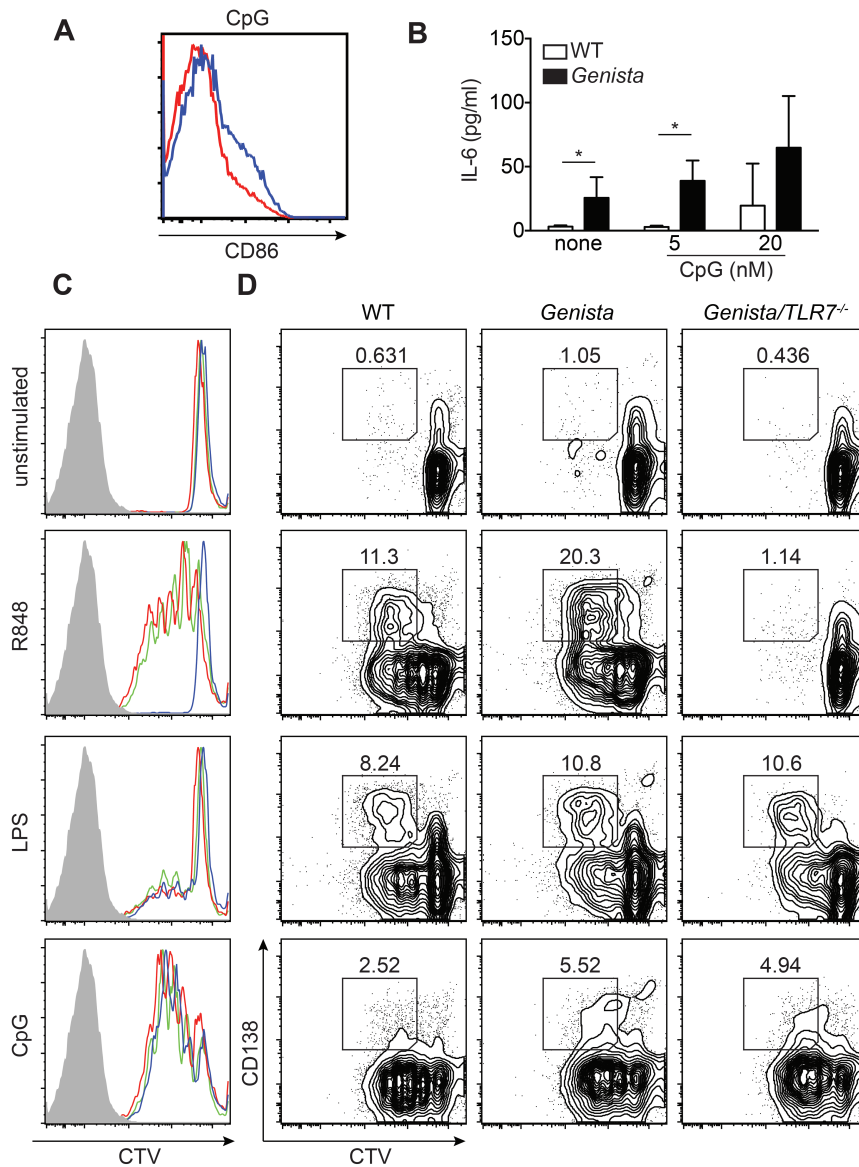
by ELISA (a. u., arbitrary units). Each point represents value from one mouse and horizontal bars denote the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ determined by the Mann-Whitney U test. (C and D) Representative FACS profiles of (C) B220^{lo}CD138⁺ plasmablasts and (D) B220⁺GL7⁺CD38⁻ GC B cells from 4 months old female WT, TLR8^{-/-}, Genista and Genista/TLR8^{-/-} mice. (E) Kidney sections from 2 to 3 months old female WT, TLR8^{-/-}, Genista and Genista/TLR8^{-/-} mice were stained with anti-IgM (top) and anti-IgG and acquired on Zeiss LSM780 microscope (scale bar = 50 μ m, original magnification 100 \times ; numerical aperture of the objective oil lenses, 1.4). Images were analyzed with the Zen software. Data in C-E are representative of two independent experiments (n = 3 per group).



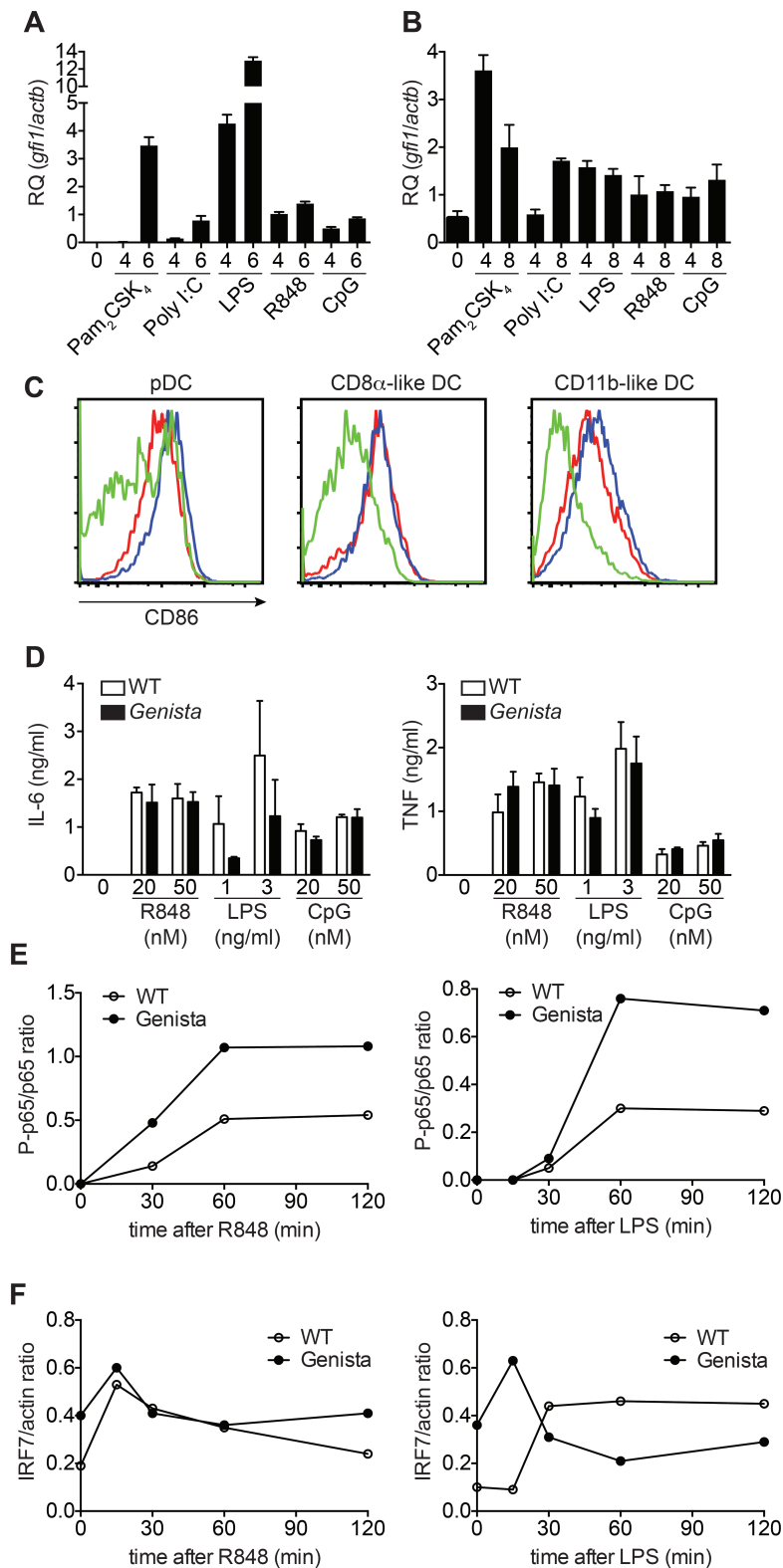
Supporting Information Figure 2. Expression of TLR7 by splenic B cells and DCs at steady state. Splenocytes from 6 months old female WT (red line), Genista (blue line) and Genista/TLR7^{-/-} (green line) mice were stained for B220, CD11c, SiglecH, MHCII and TLR7. Plots represent the intracellular expression of TLR7 after gating on B cells (B220⁺SiglecH⁻), pDC (B220⁺CD11c^{int}SiglecH⁺) or cDC (B220⁻CD11c^{hi}MHC II^{hi}).



Supporting Information Figure 3. Genista mice have normal frequencies of transitional T1, T2 and T3 B cells and increased frequencies of ABCs and T-bet expression in ABCs. Splenocytes from 4 months old female WT, Genista and Genista/TLR7^{-/-} mice (n=3 per group) were stained with CD3, CD19, B220, CD93, IgM, CD21, CD23 and T-bet antibodies. Transitional B cells were gated in the immature CD93⁺B220⁺ gate and divided into T1 (IgM⁺CD23⁻), T2 (IgM⁺CD23⁺) and T3 (IgM^{lo}CD23⁺) cells. (A) Representative flow cytometry plots and graphical plot of the percentages of T1, T2 and T3 B cells. Each point represents value from one mouse and horizontal bars denote the mean. (B) Representative flow cytometry plots and graphical plot of the percentages of ABCs (CD3⁺B220⁺CD21⁻CD23⁻CD19⁺CD11c⁺). Each point represents value from one mouse and horizontal bars denote the mean. (C) Mean fluorescence intensity (MFI) of T-bet expression on ABCs of WT (red), Genista (blue) and Genista/TLR7^{-/-} (green) mice. Data are shown as mean ± SD. *p < 0.05 determined by the Mann-Whitney U test. ABCs were determined in the CD3⁺B220⁺CD21⁻CD23⁻ gate as CD19⁺CD11c⁺.

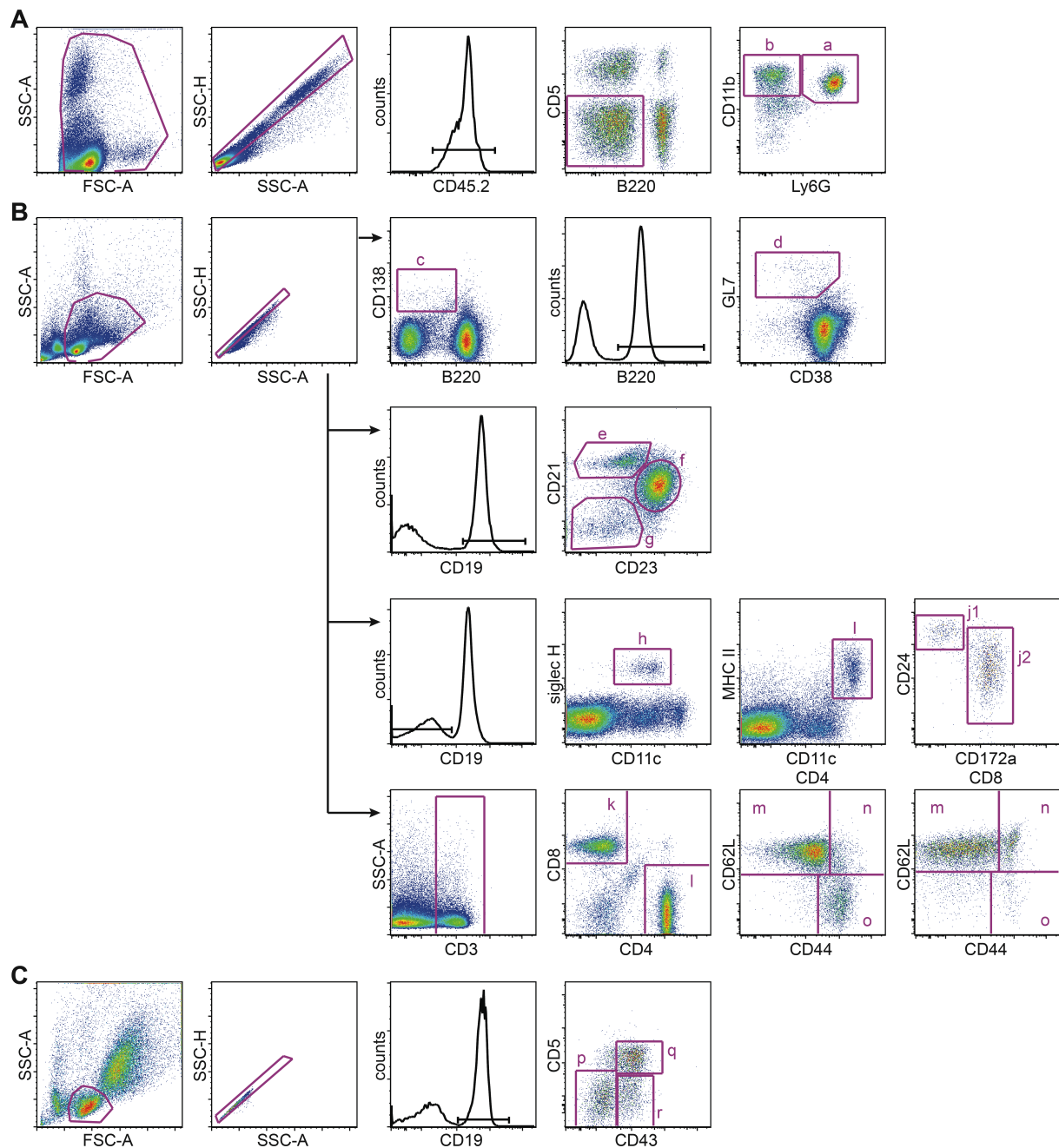


Supporting Information Figure 4. Enhanced B cell response to TLR agonists in Genista mice. (A) Total splenocytes from 6 months old female WT (red) and Genista (blue) mice were stimulated with 20 nM CpG. After 24 h, cells were stained for CD19 and CD86. Plots represent expression of CD86 on CD19-gated cells. Data are shown as mean \pm SD and are representative of at least 2 independent experiments (n = 3 per group). (B) Total splenocytes from WT and Genista were stimulated with indicated doses of CpG for 16 h (n = 3 per group). IL-6 production in culture supernatants was measured by ELISA. * $p < 0.05$ determined by the Mann-Whitney U test. (C and D) Total splenocytes from 3 months old female WT, Genista, or Genista/TLR7^{-/-} mice were labeled with CellTrace Violet (CTV) and stimulated with 100 nM R848, 2 μ g/ml LPS or 100 nM CpG. After 3 days, cells were stained for CD19, CD138. (C) Proliferation of WT (green), Genista (red) and Genista/TLR7^{-/-} (blue) CD19-gated B cells was assessed by dilution of CTV fluorescence and (D) differentiation into plasmablasts by the expression of CD138 in CD19-gated B cells. Data are representative of at least 2 independent experiments (n = 3-5 per group).



Supporting Information Figure 5. Expression of Gfi1 by WT macrophages and DCs, DC activation status and BMMFs cytokine production upon TLR stimulation and DC responses upon TLR activation. (A) BMMFs or (B) BMDCs from WT mice were left untreated or stimulated with Pam₂CSK₄ (TLR2/6 ligand) (30 ng/ml for BMMFs and 50 ng/ml for BMDCs), 50 μ g/ml poly I:C (TLR3 ligand), 10 ng/ml LPS (TLR4 ligand), 50 nM R848 (TLR7 ligand) or CpG (TLR9 ligand) (30 nM for BMMFs and 50 nM for BMDCs) for the indicated time points. Total RNA was extracted from the cells and the expression of Gfi1 was assessed and

normalized with the $2^{-\Delta CT}$ method to the β -actin internal control by Q-PCR. Data are from a single representative experiment of 3 independent experiments. (C) Increased activation status of Genista splenic DC subsets at steady state. Splenocytes from 7 months old male WT (red line), Genista (blue line) and Genista/TLR7^{-/-} (green line) mice were stained for CD19, CD11c, SiglecH, CD11b, CD8, MHC-II and CD86. Plots represent the expression of CD86 after gating on pDC (CD19⁻CD11c^{int}SiglecH⁺), CD8-like cDC (CD19⁻CD11c^{hi}MHC-II^{hi}CD11b⁻CD8 α ⁺) and CD11b-like cDC (CD19⁻CD11c^{hi}MHC-II^{hi}CD11b⁺CD8 α ⁻). (D) BMMFs from WT (n=3) and Genista (n=3) mice were left untreated or stimulated with R848, LPS or CpG. After 8 h, the concentrations of IL-6 and TNF in the culture supernatants were assessed by ELISA. (E-F) Densitometry quantification related to figure 5C. Densitometry was determined for the P-NF- κ B p65, NF- κ B p65, IRF7 and β -actin bands. Graphs represent the ratio of (E) P-NF- κ B p65 to NF- κ B p65 and (F) IRF7 to β actin band intensities, respectively. Data (A-F) are representative of at least 2 independent experiments (n = 3-5 per group).



Supporting Information Figure 6. Gating strategies used for immunophenotyping cell populations in different compartments. Cells from (A) blood, (B) spleen or (C) peritoneal lavage were gated via FSC/SSC on live cells and debris and doublets were excluded. (A) In the blood, T cells ($CD5^+$) and B cells ($B220^+$) were excluded from $CD45.2^+$ hematopoietic cells and the remaining cells were separated into neutrophils ($Ly6G^+CD11b^+$, fraction a) and monocytes ($Ly6G^-CD11b^+$, fraction b). (B) In the spleen, plasmablasts were determined as $CD138^+B220^{lo/-}$ cells (fraction c), while GC B cells were determined as $B220^+GL7^+CD38^-$ cells (fraction d). $CD19^+$ cells were divided into MZ ($CD21^+CD23^{-/lo}$, fraction e), follicular ($CD21^+CD23^+$, fraction f) and immature ($CD21^-CD23^-$, fraction g) B cells. For the analysis of DCs, $CD19^+$ cells were gated out, pDCs were then defined as $CD19^-SiglecH^+CD11c^{int}$ (fraction h) and cDCs as $CD19^-MHCII^{hi}CD11c^{hi}$ cells (fraction i). cDCs were further stained with CD24 and CD172a to obtain CD8-like ($CD19^-MHCII^{hi}CD11c^{hi}CD24^+CD172a^-$, fraction j1) and CD11b-like ($CD19^-MHCII^{hi}CD11c^{hi}CD24^-CD172a^+$, fraction j2) DC cells. Moreover, splenocytes were stained for CD3 and separated into CD8 T cells (fraction k) and CD4 T cells (fraction l), which were further divided into naïve ($CD62L^{hi}CD44^{lo/-}$, fraction m), central

memory (CD62L^{hi} CD44^{hi}, fraction n) and effector memory (CD62L^{lo} CD44^{hi}, fraction o) subpopulations. (C) For peritoneal cells, CD19⁺ B cells were stained with CD5 and CD43 to obtain B2 (CD43⁻CD5⁻, fraction p), B1a (CD43⁺CD5⁺, fraction q) and B1b (CD43⁺CD5⁻, fraction r) cells.

