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Deficiency in Interleukin-1 receptor-associated kinase (IRAK) 4 activity attenuates manifestations of murine Lupus

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Abstract

Interleukin-1 receptor-associated kinase (IRAK) 4 mediates host defense against infections. As an active kinase, IRAK4 elicits full spectra of myeloid differentiation primary response protein (MyD) 88-dependent responses, while kinase-inactive IRAK4 induces a subset of cytokines and negative regulators whose expression is not regulated by mRNA stability. IRAK4 kinase activity is critical for resistance against *Streptococcus pneumonia*, but its involvement in autoimmunity is incompletely understood. In this study, we determined the role of IRAK4 kinase activity in murine lupus. Lupus development in BXSB mice expressing the Y chromosome autoimmunity accelerator (*Yaa*) increased basal and Toll-like receptor (TLR) 4/7-induced phosphorylation of mitogen-activated protein kinases, p65 nuclear factor-κB (NF-κB), enhanced tumor necrosis factor (TNF)-α and C-C motif chemokine ligand (CCL) 5 gene expression in splenic macrophages, but decreased levels of Toll-interacting protein and IRAK-M, without affecting IRAK4 or IRAK1 expression. Mice harboring kinase-inactive IRAK4 on the lupus-prone *Yaa* background manifested blunted TLR signaling in macrophages and reduced glomerulonephritis, splenomegaly, serum anti-nuclear antibodies, numbers of splenic macrophages, total and TNF-α$^+$ dendritic cells, activated T- and B-lymphocytes, and lower TNF-α expression in macrophages compared to lupus-prone mice with functional IRAK4. Thus, IRAK4 kinase activity contributes to murine lupus and could represent a new therapeutic target.

Keywords

Autoimmunity; IRAK4; Toll-like receptors; Innate Immunity; Macrophage

Introduction

Systemic lupus erythematosus is a devastating autoimmune disease, characterized by inflammation affecting the skin, kidney, lung and heart [1]. The role of adaptive immunity in lupus has been evidenced by production of autoreactive antibodies (Abs), deposition of immune complexes in the kidney [2], increased numbers of Th17, Tfh cells and decreased numbers and functions of Treg lymphocytes [3]. Studies over the last decade have uncovered

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the involvement of the innate immunity, macrophages (MΦs) and plasmacytoid dendritic cells (DCs) in lupus [4–8] and the importance of dysregulated TLR signaling [9]. However, the role of kinase activity of IRAK4 versus its adapter function in lupus is unclear.

TLRs are sensors of microbial structures, *e.g.* lipopolysaccharide (LPS), and endogenous "alarmins", *e.g.* ribonucleoproteins and nucleic acids [10]. Ligand recognition initiates TLR dimerization, bringing intracellular Toll-interleukin-1R (TIR) domains together to recruit adapter proteins [11]. All TLRs except TLR3 associate with MyD88 to recruit and activate IRAK4, IRAK1 and IRAK2, engaging TNFR-associated factor (TRAF) 6 and activating transforming growth factor-β-activated kinase (TAK)-1 [12]. TAK-1 activates mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB), inducing transcription of cytokines, and triggers the MK2-dependent pathway regulating mRNA stability [13]. Kinase-inactive IRAK4 adapter signals via a MAPK-extracellular regulated kinase (ERK) (MEKK) 3-dependent pathway of NF-κB activation that induces cytokines (CCL2, CCL4, CCL7) and negative regulators (*e.g.* SH_2 inositol phosphatase-1 (SHIP-1), A20) whose expression is not regulated by mRNA stability [13, 14]. Endosome-associated TLR3 and TLR4 utilize TIR domain-containing adapter inducing interferon (IFN)-β (TRIF) to activate TRAF-associated NF-κB activator-binding kinase (TBK)-1 that phosphorylates IFN-regulatory factor 3, leading to transcription of type I IFNs [12]. TRIF also acts via receptor-interacting protein-1-TAK-1 to mediate delayed activation of MAPKs and NF-κB [12].

The significance of TLRs in lupus is supported by increased expression of TLR2, TLR7, TLR9 mRNA in PBMC of lupus patients [15], association of TLR7 and TLR9 polymorphisms with lupus [16, 17], and protection of $TLR7^{-/-}$ mice from the disease [18, 19]. A lupus-enhancing Yaa locus in the Y chromosome of BXSB male mice contains an extra copy of *tlr7* [20, 21], and TLR7 transgenic mice develop lupus [22–24]. The inactivating mutation in the chaperone uncoordinated 93 homologue b1, which precludes trafficking of TLR7-9 to endosomes [25], reduces auto-Abs against DNA or RNA and increases survival in lupus-prone mice [26]. A loss of MyD88 or IRAK1 protected lupus-prone mice from the disease [27–30] while mice lacking IRAK-M, a negative TLR regulator [31], had exacerbated lupus [32]. Thus, the MyD88-IRAK4-IRAK1 axis is a critical regulator of lupus.

Despite growing appreciation of the importance of the TLR pathway, it is unknown how kinase and adapter functions of IRAK4 contribute to lupus. To address this question, we determined TLR4- and TLR7-mediated activation of MAPKs, NF-κB, inflammatory cytokines and expression of IRAK4 and IRAK1 in splenic macrophages (MΦs) from 16 week-old lupus-prone male BXSB/MpJ mice expressing the *Yaa* locus (designated BXSB/*Yaa*) compared to cells from age-matched BXSB female mice without lupus. Secondly, we engineered mice harboring kinase-deficient or kinase-sufficient IRAK4 on the lupus-prone (BXSB/*Yaa*) background and examined nephritis, splenomegaly, serum anti-nuclear Abs (ANA) and infiltration of the spleen with macrophages, dendritic cells (DCs), polymorphonuclear leukocytes (PMNs), T- and B-lymphocytes and cell activation. MΦs from lupus-prone BXSB/*Yaa* mice showed increased TLR4- and TLR7-driven activation of MAPKs, NF-κB and induction of TNF-α and CCL5 mRNAs, decreased IRAK-M and Toll-interacting protein (Tollip) expression and no changes in IRAK4 or IRAK1 levels compared

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to cells from lupus-free BXSB animals. F2 BXSB/*Yaa* x B6 mice harboring kinase-inactive IRAK4 manifested blunted TLR signaling in macrophages and had attenuated nephritis, splenomegaly, reduced levels of serum ANA and infiltration of immune cells in the spleen, compared to lupus-prone F2 animals expressing kinase-sufficient IRAK4. Expression of kinase-inactive IRAK4 adapter on the lupus-prone background lowered the number of splenic MΦs, total and TNF$^+$ DCs, reduced TNF-α expression in splenic MΦs, and suppressed the number and percentages of IFN-γ$^+$ TCRβ$^+$ T-cells and B220$^+$CD138$^+$ B-lymphocytes. These results indicate that a loss of IRAK4 kinase activity attenuates manifestations of murine lupus and suggest the potential for antagonists of IRAK4 activation for intervention in lupus.

Results

Macrophages from lupus-prone mice exhibit increased TNF-α and CCL5 but decreased Tollip and IRAK-M

First, we studied the impact of lupus development on expression of TLR-inducible, disease-associated TNF-α and CCL5 [33–35] in MΦs, cells regulating manifestation of lupus [4, 5, 9, 36]. BXSB/MpJ male mice express the *Yaa* locus conferring TLR7 duplication (BXSB/*Yaa* mice) and develop disease by 16 weeks [20, 22], displaying high levels of serum ANA (A), proteinuria (B), enlargement of renal glomeruli (C) and increased spleen weight and splenocyte numbers (D, E). Control female BXSB mice express normal levels of TLR7and lack lupus manifestations at 16 weeks (Fig. 1). Splenic MΦs from 16 week-old BXSB/*Yaa* mice responded to loxoribin (Lxrb, a TLR7 agonist) by 2–5 fold higher up-regulation of TNF-α and CCL5 mRNA compared to the responses of BXSB-derived control cells (Fig. 1F, G). LPS-induced levels of TNF-α or CCL5 mRNA in splenic MΦs from BXSB/*Yaa* mice were also higher, albeit these differences did not reach statistical significance (Fig. 1 F, G and Fig. 2 A). MΦs from 4 weeks-old, lupus-free male BXSB/*Yaa* mice had no statistically significant differences in LPS- or Lxrb-driven induction of TNF-α mRNA compared to cells from aged-matched female BXSB mice (Fig. 2C). Thus, differences in TLR-induced cytokines are lupus-specific and not due to sex differences.

Since TLR responses are shaped by positive (Pellino-1) and negative (Pellino-3, Tollip, IRAK-M) regulators [31, 37–42], we determined the levels of these molecules in in MΦs from lupus-prone and lupus-resistant mice. As shown in Figure 3, splenic MΦs from BXSB/*Yaa* mice had lower basal and LPS-driven levels of Tollip and IRAK-M mRNA (A, B) and IRAK-M protein (C, D) compared to cells obtained from BXSB mice, while showing comparable expression of Pellino-1 (E) or Pellino-3 (F) mRNA. Similar data was obtained for Lxrb-mediated regulation of IRAK-M (Fig. 3C, D), Tollip, Pellino-1 and Pellino-3 (data not shown). Thus, MΦs from lupus-prone BXSB/*Yaa* mice exhibit increased TLR7-induced TNF-α and CCL5 gene expression, a tendency to enhanced TLR4-driven expression of these cytokine genes, but lower Tollip and IRAK-M levels, correlating with the development of glomerulonephritis, splenomegaly and appearance of serum ANA.

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Lupus development enhances phosphorylation of ERK, JNK, p38 MAPK and p65 NF-κB in splenic macrophages but does not affect IRAK4/1 expression

Due to the role of MAPK and NF-κB in eliciting inflammatory cytokines [12], we determined their activation in MΦs from lupus-prone and control mice. MΦs from 16 weeks-old mice on the lupus-prone BXSB/*Yaa* background exhibited increased levels of basal and LPS- or Lxr-inducible phosphorylation of ERK1/2, JNK1/2, p38 MAPKs and p65 NF-κB compared to cells from animals on the control BXSB background (Fig. 4A, B, Fig. S1). MΦs from 4 weeks-old BXSB/*Yaa* mice (that had not developed the disease by this time) and BXSB mice showed comparable LPS- and Lxrb-induced p38 phosphorylation (Fig. 2D, Fig. S1C), indicating that enhanced TLR-induced activation of MAPK is lupus-specific. Since IRAK kinases regulate inflammatory responses [43, 44], we determined IRAK4 and IRAK1 levels in splenic MΦs derived from 16 weeks-old BXSB/*Yaa* or BXSB mice. Figure 4 shows comparable levels of IRAK4 or IRAK1 (C, D) mRNA and proteins (B) in splenic MΦs from lupus-prone or control mice treated with medium, LPS or Lxrb. Thus, MΦs from BXSB/*Yaa* mice with lupus have increased activation of ERK1/2, JNK1/2 and p38 MAPKs and p65 NF-κB, which was not due to modulations in IRAK4 or IRAK1 expression.

Lupus-prone mice with kinase-inactive IRAK4 have reduced serum ANA, proteinuria and splenomegaly

In addition to expression, induction of kinase activity of IRAK4 is a critical determinant for regulation of TLR-driven, MyD88-dependent responses [13]. To delineate the role of IRAK4 kinase activity in lupus, we engineered mice harboring kinase-inactive or WT IRAK4 on the lupus-prone (BXSB/*Yaa*) or control (BXSB) backgrounds. Female mice expressing kinase-inactive (KI) IRAK4 (C57/BL6J/IRAK4 KI mice) were crossed with male BXSB/*Yaa* mice to obtain F1 heterozygous males (Yaa^+) and females (Yaa^-) expressing WT/KI IRAK4. F1 mice were intercrossed to obtain F2 lupus-prone male (Yaa^+) mice homozygously or heterozygously expressing KI or WT IRAK4, yielding Yaa^+/IRAK4$^{KI/KI}$, Yaa^+/IRAK4$^{WT/KI}$, and Yaa^+/IRAK4$^{WT/WT}$ mice. After 16 weeks, lupus manifestations were determined in these F2 mice compared to *Yaa*-/IRAK4$^{WT/WT}$ females, and in BXSB/*Yaa* males and BXSB females, as judged by levels of serum ANA, proteinuria and splenomegaly. Figure 5 shows lower spleen weight (A) and the number of splenocytes (B), reduced proteinuria (C) and serum ANA (D) in Yaa^+/IRAK4$^{KI/KI}$ mice compared to Yaa^+/IRAK4$^{WT/WT}$ mice, and similar differences between lupus-prone BXSB/*Yaa* mice vs. control BXSB mice. B6 x BXSB/*Yaa* mice (designated Yaa^+/IRAK4$^{WT/WT}$, the lupus-prone background) expressing WT IRAK4 showed statistically significant differences in the extent of splenomegaly, proteinuria and serum ANA compared to B6 x BXSB (designated *Yaa*-/IRAK4$^{WT/WT}$, the control strain lacking *Yaa*) (Fig. 5). Splenic MΦs from mice harboring kinase-inactive IRAK4 had lower basal and LPS-induced phosphorylation of p38 MAPK (Fig. 4B, Fig. S2) and reduced TNF-α levels (Fig. 6B), consistent with the role for the IRAK4-IRAK1 axis in activating downstream signaling [13]. Thus, the presence of kinase-inactive IRAK4 in lupus-prone BXSB/*Yaa* mice attenuates disease development and inhibits p38 MAPK activation and TNF-α expression.

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BXSB/Yaa mice expressing KI IRAK4 have reduced infiltration and activation of splenic MΦs, DCs, T and B cells

Infiltration of MΦs, PMNs, DCs, T- and B-lymphocytes to the spleen and their activation orchestrates innate and adaptive responses involved in lupus pathogenesis [4–6, 45–49]. Thus, we determined the impact of IRAK4 kinase activity on leukocyte infiltration to the spleen and their activation statuses in F2 BXSB/*Yaa* x B6 mice expressing KI or WT IRAK4 compared to these parameters in control mice. Figure 6 shows lower numbers of F4/80$^+$ MΦs expressing decreased intracellular TNF-α (A, B), reduced numbers of CD11c$^+$ DCs and lower percentages of CD11c$^+$TNF-α$^+$ DCs (C, D) in the spleen of male BXSB/*Yaa* x B6 mice expressing kinase-inactive IRAK4 (*Yaa*$^+$/IRAK4$^{KI/KI}$) compared to lupus-prone BXSB/*Yaa* x B6 mice with active IRAK4 (*Yaa*+/IRAK4$^{WT/WT}$). No differences in the number (Fig. 6E) or percentages (Fig. 6F) of splenic Ly6G$^+$ PMNs were observed between BXSB/*Yaa* x B6 animals expressing kinase-sufficient or deficient IRAK4, while there was higher numbers and percentages of PMNs in lupus-prone BXSB/*Yaa* mice vs control BXSB mice (Fig. 6E, F). Lupus-prone mice harboring KI IRAK4 had lower numbers and percentages of TCR-β$^+$IFN-γ$^+$ activated T cells in the spleen (Fig. 6I, J), and decreased numbers of splenic B220$^+$ total B-lymphocytes (Fig. 6G) and CD138$^+$ (syndecan-1) B220$^+$ activated plasma cells (Fig. 6H) compared to lupus-prone mice expressing WT IRAK4. 16 week-old BXSB/*Yaa* lupus-prone mice used as positive controls had higher numbers of splenic MΦs, DCs, activated T- and B-lymphocytes compared to control BXSB mice (Fig. 6A–J). These results suggest that expression of kinase-inactive IRAK4 lowers infiltration of the spleen with MΦs, DCs, T- and B-lymphocytes and reduces their activation, ameliorating lupus manifestations.

Discussion

This paper unveils a critical role of IRAK4 kinase activity in promoting murine lupus. Along with DCs, MΦs play a key role in modulating nephritis manifestations during SLE [4–8]. Depletion of MΦs in lupus-prone mice by GW2580, a selective inhibitor of the colony stimulating factor-1 receptor kinase, reduced nephritis caused by anti-glomerular Abs, as judged by decreased proteinuria, serum creatinine, blood urea nitrogen, and CCL5 [4]. Lupus-prone mice depleted for folate-expressing MΦs exhibited reduced immune complex deposition, diminished tissue damage and prolonged survival [50]. MΦ depletion decreased anti-DNA Ab production by (NZB x NZW) F1 splenocytes via eliminating the main source of IL-6 [51]. Furthermore, glomerulonephritis development in lupus-prone NZW/B mice was associated with increased MΦ infiltration expressing INF-inducible genes [4, 5]. Thus, we first examined the impact of lupus development on TLR signaling in splenic MΦs, complementing this approach with analyses of the impact of genetic deficiency in IRAK4 kinase activity on lupus manifestations, infiltration leukocytes to the spleen and activation of splenic macrophages, dendritic cells, T- and B-lymphocytes. BXSB/*Yaa* mice with developed lupus (as evidenced by serum ANA, proteinuria, glomerulonephritis, and splenomegaly) exhibited increased TLR4- and TLR7-driven phosphorylation of ERK1/2, JNK1/2, p38 MAPKs and p65 NF-κB in splenic MΦs. Consistent with the role of MAPKs and NF-κB in regulation of cytokine expression [12], we found increased levels of TLR4- and TLR7-inducible TNF-α and CCL5 mRNA in splenic MΦs from lupus-prone mice.

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These enhanced responses were not due to elevated IRAK4 or IRAK1 expression, a known mechanism responsible for enhanced TLR signaling [43, 44], as similar levels of IRAK4 and IRAK1 mRNA were seen in MΦs derived from lupus-prone or lupus-resistant mice. Instead, enhanced TLR4- or TLR7-driven IRAK4-mediated activation of MAPKs, NF-κB and increased TNF-α and CCL5 gene expression in MΦs from mice with lupus correlated with lower expression of Tollip and IRAK-M, negative regulators of TLR signaling [31, 41]. In contrast, no significant changes in the mRNA levels of Pellino-1 (positive) or Pellino-3 (negative) regulators [37–39, 42] were observed, indicating the selective effect of lupus on expression of TLR regulators. IRAK-M acts by preventing dissociation of IRAK-1 from MyD88 or inducing other negative regulators A20, SHIP-1, inhibitor of NF-κB-α [31, 52], whereas Tollip suppresses IRAK1 activation [41]. Thus, lower levels of IRAK-M and Tollip could underlie heightened TLR-driven activation of IRAK4 during lupus development in mice, increasing expression of lupus-promoting cytokines, e.g. TNF-α and CCL5, and exaggerating disease manifestations. Interestingly, IRAK-M was also reported to facilitate MΦ differentiation into anti-inflammatory, wound-healing M2-like MΦs [53]. It is possible that lower levels of IRAK-M and Tollip facilitate MΦ reprogramming into the M1 phenotype, promoting inflammation and autoimmunity.

IRAK4 acting as an active kinase triggers the classical TAK1-dependent pathway involving activation of MAPKs and inhibitor of NF-κB kinase (IKK)-β, nuclear translocation of p50/p65 NF-κB, and activation of inflammatory cytokines[13]. Furthermore, kinase-competent IRAK4 plays a critical role in regulating intermediates that control stability of cytokine mRNAs, and drives TLR7-9-inducible type I IFNs [13]. Notably, kinase-inactive IRAK4 acts as an adapter, mediating a non-classical NF-κB activation pathway via MEKK3 and IKK-α/IKK-γ that induces a subset of cytokines and negative regulators not regulated by mRNA stability, such as CCL2, CCL4, CCL7, suppressor of cytokine signaling-1, SHIP-1, A20 [13, 54–56]. Previous studies in mice have established that IRAK4 kinase activity is required for protection against *Streptococcus pneumoniae* [57] and for the development of methylated serum albumin-induced arthritis [58]. In addition, despite accumulating auto-reactive B cells, IRAK4-deficient human patients do not display autoreactive Abs or develop autoimmune disease [59], suggesting that the IRAK4 pathway is essential for the development of lupus. However, it has been unclear how IRAK4 kinase activity vs. IRAK4 adapter functions regulates lupus development. Having established relationships between increased IRAK4-mediated IRAK1 activation and lupus manifestations, we sought to address the role of IRAK4 kinase activity in murine lupus. To this end, we bred lupus-prone male BXSB/*Yaa* mice with female mice expressing kinase-inactive IRAK4 (IRAK4 KI) to obtain F2 mice lupus-prone (*Yaa*+) males homozygously expressing KI/KI IRAK4, WT/WT IRAK4 or heterozygously expressing WT/KI IRAK4. Lupus manifestations and TLR signaling in these mice were examined to compare them to each other and to control females expressing WT/WT IRAK4. Mice on the lupus-prone BXSB/*Yaa* background expressing kinase-inactive IRAK4 had decreased serum ANA, proteinuria, and splenomegaly. The degree of attenuation was comparable to that in classical lupus-prone BXSB/*Yaa* male and control BXSB female mice, indicating that IRAK4 kinase activity contributes to lupus. A loss of IRAK4 kinase activity led to significantly attenuated

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basal and LPS-driven activation of p38 MAPK in splenic MΦs and inhibited TNF-α protein expression, indicating that IRAK4 kinase activity plays a critical role in promoting lupus.

Migration of MΦs and DCs to the spleen regulates innate immunity and promotes activation of B- and T-lymphocytes [45–47]. Since lupus is associated with increased infiltration of the spleen with MΦs, PMNs, DCs, T- and B-lymphocytes and their increased activation [1, 9, 45–49], we studied whether expression of kinase-inactive IRAK4 in lupus-prone mice affects these parameters. BXSB/*Yaa* mice expressing kinase-sufficient IRAK4 developed exaggerated lupus and exhibited higher numbers of F4/80$^+$ splenic MΦs that were activated, judged by their higher levels of TNF-α, compared to BXSB/*Yaa* mice harboring kinase-inactive IRAK4. The loss of IRAK4 activity resulted in decreased numbers of CD11c$^+$ DCs, B220+ and B220$^+$ CD138$^+$ B-lymphocytes, and lower numbers and percentages of IFN-γ^+ T-lymphocytes. Previously, B-lymphocytes obtained from IRAK4-deficient patients and IRAK4 KI mice in infection settings were shown to have impaired IgM production [60, 61]. Furthermore, one report demonstrated deficient T cell activation associated with impaired protein kinase C activation and NF-κB induction in IRAK4$^{-/-}$ mice [62], and there was impaired Th17 functions in IRAK4 KI mice [63]. Our results support a positive regulatory role of IRAK4 in lupus associated with increased activation of macrophages, T- and B-lymphocytes.

It is plausible that the lack of auto-Abs and deficient activation of plasma cells in lupus-prone mice harboring kinase-inactive IRAK4 could be B cell-intrinsic. This is consistent with the direct involvement of TLR2, TLR7 and TLR9 in B cell activation in lupus and deficiencies in auto-Ab production in lupus-prone mice with B cell-specific ablations of MyD88 or TLRs [64–67]. B cell-extrinsic role of IRAK4 is also possible., MΦs and DCs produce type I IFNs, IL-6, BAFF, IL-10, IL-13 involved in B cell activation, survival, differentiation and Ab isotype switching [68–71], and DCs can activate Tfhs [72]. Depletion experiments in lupus-prone mice showed a critical role of MΦs and pDCs in B cell activation and nephritis [4, 5, 50, 51, 73]. Deficiencies in MΦs can impair B cell proliferation, survival and Ab class switching due to reduced production of IL-6 [51, 74, 75] and BAFF [70, 76]. BAFF mediates Ab isotype switching [76], survival and proliferation of B-cells [70, 71], and MΦ-derived IL-6 exacerbates lupus by promoting production of anti-DNA IgG [51, 74]. Thus, a loss of IRAK4 kinase activity in MΦs and DCs could indirectly contribute to deficient B cell activation by reducing their expression of B cell-activating cytokines or co-stimulatory molecules, impairing production of auto-Abs. Unravelling the specific role of IRAK4 kinase activity in MΦs, DCs or B-lymphocytes will require cell-specific knock-in of kinase-inactive IRAK4 and will be addressed in our future studies.

IRAK4 activity is prerequisite for expression of chemokines and chemokine receptors [13, 14, 54], and their deficient induction in IRAK4 KI mice could account for impaired migration and/or activation of MΦs and DCs in the spleen. Decreased numbers and activation of splenic MΦs and DCs could in turn account for lower numbers and activation of T- and B-lymphocytes, due to deficient expression of T- and B-cell-guiding chemokines, activating cytokines and/or possible defects in Ag presentation, expression of MHC molecules and cytokines. On the other hand, we cannot exclude T cell- intrinsic role for

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IRAK4 that might determine the extent of T cell migration and/or activation, due to a reported role of IRAK4 kinase activity in T-lymphocyte activation [62].

To the best of our knowledge, this paper is the first demonstration that IRAK4 kinase activity positively regulates lupus development in a model of genetically-predisposed murine lupus. Our data highlight the importance of the MyD88-IRAK4-IRAK1 axis for murine lupus, consistent with earlier results on the protective role of MyD88 and IRAK1 deficiency against disease development in lupus-prone mice [27–30]. They also extend an earlier report showing that inhibition of kinase activity of IRAK4 and IRAK1 with a small molecule inhibitor attenuates expression of lupus-associated cytokines in monocytes and plasmacytoid DCs from lupus patients [77]. These results suggest a potential for inhibitors of IRAK4 activation to attenuate development of lupus.

Materials and methods

Reagents and cell culture

Highly purified LPS from *Escherichia coli* O111:B4 and loxoribine (Lxrb) were from Invivogen (San Diego, CA). The following Abs were used: anti-phospho (p)-p38 and anti-p38, anti-p-IRAK1, anti-p-p65 and anti-p65 (Cell Signaling Technology, Danvers, MA), anti-IRAK1, anti-IRAK-M, anti-Tollip, anti-pERK1/2 and anti-ERK1/2, anti-pJNK1/2 and anti-JNK1/2, and anti-tubulin (Santa Cruz Biotechnology, Dallas, Texas). Splenic MΦs were obtained by differentiation of splenocytes with CSF-1-containing L929-conditioned medium [78]. This procedure led to a significant enrichment of $CD11b^{+}F4/80^{+}$ splenic MΦs (Fig. S3) [78]. Splenocytes were plated into 10-cm tissue culture dishes and cultured for 7 days in RPMI medium supplemented with 2 mM L-glutamine, 100 u/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 5×10^{-5} M β-mercaptoethanol (complete RPMI), and 25% L929-conditioned medium. Following trypsin treatment (0.5%), cells were resuspended in complete RPMI and used for experiments.

Mice

BXSB/MpJ lupus-prone male mice that express the *Yaa* locus predisposing to lupus (termed here BXSB/*Yaa*), BXSB female mice, and C57BL/6J mice were from Jackson Laboratories (Bar Harbor, ME). Mice expressing kinase-inactive IRAK4 ($IRAK4^{KI}$ mice) were described [25, 54] and kindly provided by Drs. Kirk Staschke, Eli Lilly, Indianapolis, IN, and Stefanie N. Vogel, University of Maryland School of Medicine, Baltimore, MD. Bxsb/Yaa male mice were bred with $IRAK4^{KI}$ female mice to produce F1 heterozygotes expressing $IRAK4^{WT/KI}$ species. F1 mice were intercrossed to produce F2 mice expressing the $IRAK4^{WT/WT}$, $IRAK4^{WT/KI}$ or $IRAK4^{KI/KI}$ variants on the lupus-accelerating (*Yaa*$^{+}$, males) or control (*Yaa*$^{-}$, females) backgrounds. Genotypes were confirmed by PCR with primers distinguishing WT and KI IRAK4: forward, 5′-AGGGCTGTGTGAACAACACC-3′; reverse, 5′-GGCTTCCTAACGTCACCTGG-3′. Splenomegaly, proteinuria, glomerulonephritis, serum ANA) and infiltration of leukocytes in the spleen were subsequently examined. All animal studies have been approved by the Animal Use and Care Committee, University of Connecticut Health Center.

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Determination of lupus manifestations

The extent of glomerulonephritis was determined by measuring proteinuria and histological H&E staining of kidney sections. Proteinuria was determined by urinalysis by multisticks, using Roche Diagnostics™ POC Chemstrip™ Urine Test Strips (Fisher Scientific). To examine a glomerular and interstitial inflammation, kidneys were fixed in formaldehyde, embedded in paraffin, sections were stained with H&E and visualized by light microscopy. ANA levels in serum samples (1:50 dilution) were determined by ELISA, using mouse ANA ELISA kit (Alpha Diagnostic Intl., San Antonio, TX), according to the manufacturer's recommendations.

Flow Cytometry

Live and dead cells were distinguished using Live-Dead Aqua (Invitrogen) and Zombie UV™ Fixable Viability Kit (Biolegend, San Diego, CA). The following conjugated anti-mouse Abs (Biolegend) were utilized: LEAF™ anti-CD16/32, FITC anti-TCR β (H57-597), FITC anti-CD11b, FITC Armenian hamster IgG isotype control, PE anti-CD138 (Syndecan-1) (281-2), PE rat IgG2a, κ isotype control, PE anti-TNF-α (MP6-XT22), PE Rat IgG1, κ isotype control, PE Rat IgG2a, κ isotype control, APC anti-Ly-6C (HK1.4), APC rat IgG2c, κ Isotype control, APC/Cy7 anti-Ly-6G (1A8), APC/Cy7 rat IgG2a, κ isotype control, Pacific Blue™ anti-CD45R/B220 (RA3-6B2), Pacific Blue™ anti-F4/80 (BM8), Pacific Blue™ rat IgG2a, κ isotype control, APC anti-CD44 (IM7), APC rat IgG2b, κ isotype control, PE/Dazzle™ 594 anti-IFN-γ (XMG1.2), PE/Dazzle™ 594 rat IgG1, κ isotype control,, PE/Dazzle™ 594 anti-CD11c (N418), PE/Dazzle™ 594 Armenian Hamster IgG Isotype control. Splenic single-cell suspensions were pre-incubated with Fc block (anti-CD16/32) for 15 min in ice-cold PBS containing 3% FBS, and incubated with Abs for cell surface staining, followed by live and dead stain. Cells were washed, fixed and permeabilized with BD Cytofix-Cytoperm buffer, incubated with the respective Abs for intracellular staining, washed and analyzed by FACS. Cells were acquired on a LSR II (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (FlowJ LLC, Ashland, OR). Single live cells were analyzed by forward and side scatter, followed by gating on live cells using Live-Dead Aqua or Zombie UV™ Fixable Viability dyes and finally FSC pulse width by FSC pulse height. Plasma B cells were identified as $B220^+$ $CD138^+$, IFN-γ-expressing T cells were identified with the $TCR\beta^+$ population, $TNF\text{-}\alpha^+$ MΦs were identified within the $F4/80^+$ $CD11c^-$ population, DCs were identified as $CD11c^+$ $F4/80^-$ population, PMNs were identified as $Ly\text{-}6C^{hi}$ $Ly\text{-}6G^{hi}$ population. MFI (mean fluorescence intensity) values of TNF-α in macrophages were calculated in the $F4/80^+$ $CD11c^-$ population, and expressed as FMO (fluorescence minus one – to its isotype control) for comparison between replicates.

Analyses of gene expression

RNA was isolated with Trizol (ThermoFisher Scientific, Waltham, MA), treated with DNase, and re-purified, as recommended by the manufacturer. cDNA was synthesized from 1 μg total RNA, using a reverse transcription system (Promega, Madison, WI), and subjected to real-time-PCR, combining 5 μl of cDNA, 0.3 μM gene-specific primers, and SYBR Green Supermix (Bio-Rad, Hercules, CA) on a MyIQ Real-Time PCR machine (Bio-Rad). The following primers were used: mouse *Hprt*: forward, 5′-

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GCTGACCTGCTGGATTACATT-3′, reverse: 5′-GTTGAGAGATCATCTCCACCA-3′; *Gapdh*: forward, 5′-GGCATTGCTCTCAATGACAA-3′, reverse, 5′-ATGTAGGCCATGAGGTCCAC-3′; *Tnf-α*: forward, 5′-ACCCTCACACTCAGATCATCT-3′, reverse, 5′-TTGTCTTTGAGATCCATGCCGT-3′; *Ccl5*: forward, 5′-GAGTGACAAACACGACTGCAA GAT-3′, reverse: 5′-CTGCTTTGCCTACCTCTCCCT-3′; *Pellino-1*: forward, 5′-CTTTATCTCGAGCCCAGACG-3′, reverse: 5′-CTGACTGCGTGTCGGAATTA-3′; *Pellino-3*: forward, 5′-ACATGCCAACGGAGTGAAGC-3′, reverse, 5′-AGCGGCCAATCTGGAACAT-3′; *Tollip*: forward, 5′-TGTGGTACAGGCAAAATTGG-3′, reverse, 5′-GGCACTGTGCACTGAATGAC-3′; *Irak-m*: forward, 5′-CATCAACTATGGAGTAAGCTGGAC-3′, reverse, 5′-GTCCAGGGTCGTTTTCTCTG-3′; *Irak4*: forward, 5′-GTAGAGTCCAGCGACACTCG-3′, reverse, 5′-GCTGACACGTTGCCATTACT-3′, *Irak1*: forward, 5′-CCTTCAGAGAGGCTAGCTGTACC-3′, reverse, 5′-ACTTTGACCTCTGAGTCTGAGGG-3′. Gel-purified PCR products validated by sequencing were used as standards, and data were presented as quantities of specific mRNAs normalized to house-keeping *gapdh* or *hprt*.

Preparation of cell extracts and Western blot analysis

Cells were lysed for 30 min in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM PMSF, 1 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 20 mM β-glycerol phosphate and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). After centrifugation, supernatants were collected and protein concentration determined using the DC Protein Assay Kit (Bio-Rad). Samples were re-suspended in Laemmli sample buffer and boiled for 10 min. Proteins (20 μg) were separated on 4–20% polyacrylamide gels (Life Technologies) and electrotransferred to Immobilon-P membranes (Bio-Rad). Membranes were blocked in TBS/5% milk, probed with Abs, washed, incubated with secondary IgG-HRP, and bands were visualized by ECL. The protein bands were quantified using the NIH Image-J software package and expressed as values of phosphorylated proteins normalized to total species or to β-actin or tubulin, as specified in the Figure legends.

Statistical analysis

Data were processed with the GraphPad Prism 5 statistical software package (GraphPad Software, San Diego, CA). For pairwise comparisons, Student t-test was employed. For comparison of multiple groups, a one-way ANOVA with repeated measures was used, followed by post-hoc comparisons with Tukey s multiple paired comparison test, with the confidence interval set at 95%. The results were expressed as mean SD values.

Supplementary Material

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Acknowledgments

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Abbreviations

IRAK	interleukin-1 receptor-associated kinase
MyD	myeloid differentiation primary response protein
Yaa	Y chromosome autoimmunity accelerator
TLR	Toll-like receptor
MΦs	macrophages
TNF	tumor necrosis factor
CCL	C-C motif chemokine ligand
Ab	antibody
TIR	Toll-interleukin-1R
TRAF	TNFR-associated factor
TAK	transforming growth factor-β-activated kinase
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor-κB
ERK	extracellular regulated kinase
MEKK	MAPK-ERK kinase
SHIP	SH_2 inositol phosphatase
IFN	interferon
TRIF	TIR domain-containing adapter inducing IFN-β
TBK	TRAF-associated NF-κ B activator-binding kinase
IRF	IFN-regulatory factor
IKK	inhibitor of NF-κB kinase
ANA	anti-nuclear Abs

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DCs	dendritic cells
PMNs	polymorphonuclear leukocytes
Tollip	Toll-interacting protein
LPS	lipopolysaccharide
Lxrb	loxoribine
KI	kinase-inactive
p	phospho
FMO	fluorescence minus one

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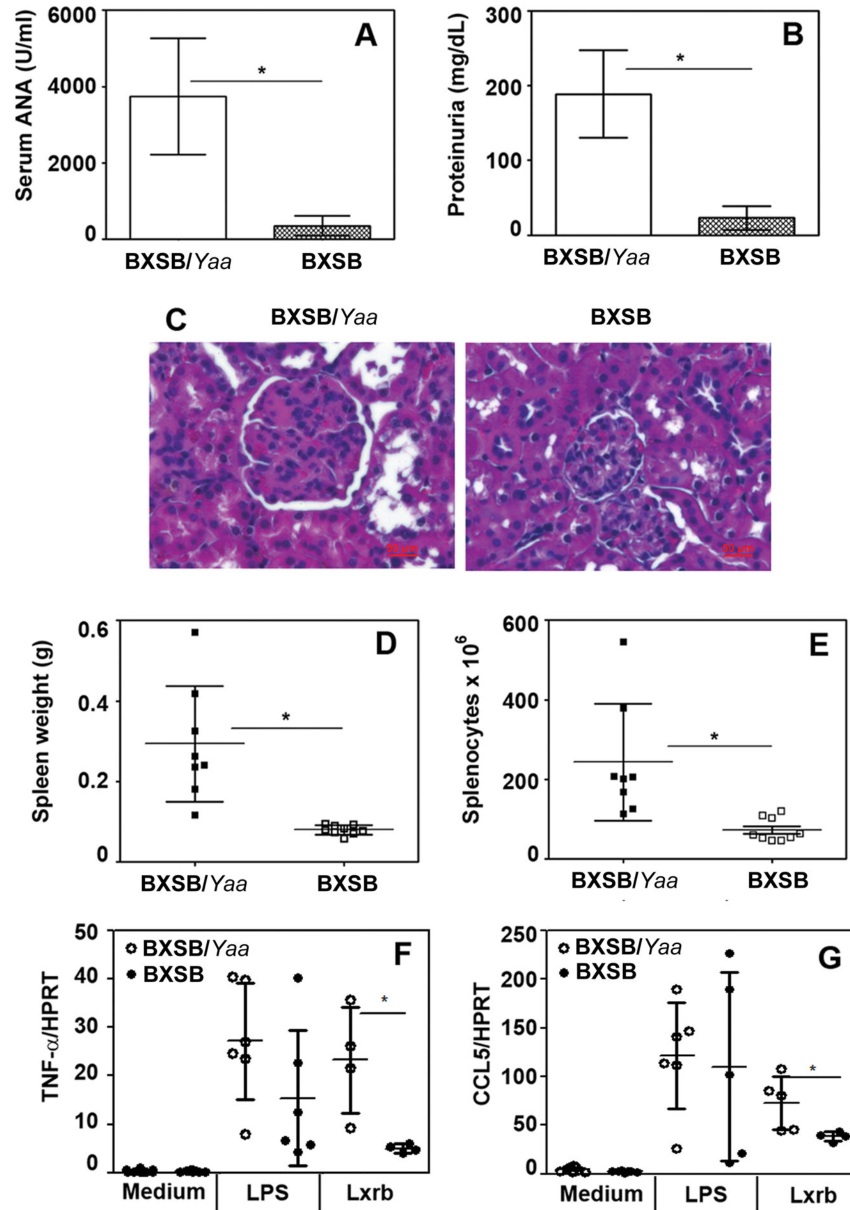

Figure 1. BXSB/*Yaa* mice develop glomerulonephritis, serum ANA, splenomegaly, and their splenic MΦs show increased TLR7-driven TNF-α and CCL5 mRNA compared to cells from Bxsb animals

Sixteen week-old BXSB/ *Yaa* and BXSB mice were used to obtain serum samples for ELISA-based analyses of ANA (A), urine samples for multistick determination of proteinuria (B), kidney sections were subjected to H&E staining and histological analysis (C), and splenomegaly was determined by weighing the spleens (D) and calculating total number of splenocytes (E). (F and G) Splenic MΦs obtained from sixteen week-old BXSB/ *Yaa* and BXSB mice were treated for 3 h with medium, 100 ng/ml LPS or 1 mM Lxrb (Lxrb), RNA was isolated, reverse transcribed and analyzed by real-time PCR to determine TNF-α (F) and CCL5 (G) mRNA levels. (A, B) Data are shown as mean ± SD (n= 9 BXSB/ *Yaa* and 6 BXSB mice) and are pooled from 3 independent experiments. (C)

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Images are representative of at least three independent experiments (scale bar: 50 μm;.magnification x 20). (D–G) Data are shown as mean ± SD and are pooled from three independent experiments. *$p<0.05$ (Student t-test).

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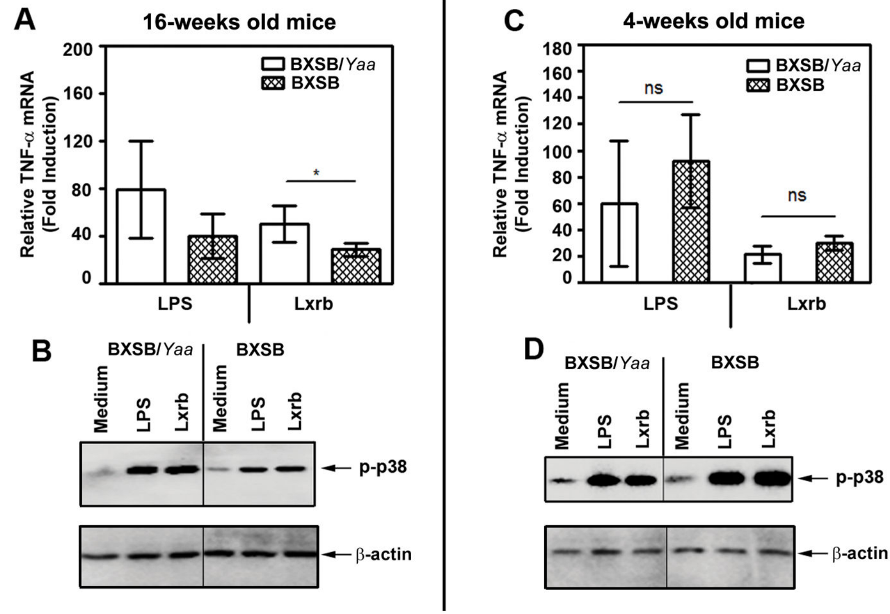

Figure 2. TNF-α gene expression and phosphorylation of p38 MAPK in MΦs from 16 week- and 4 week-old BXSB/*Yaa* or BXSB mice

Splenic MΦs from 16 week-old (A, B) or 4 week-old (C, D) BXSB/ *Yaa* and BXSB mice were treated for 3 h (A, C) or 20 min (C, D) with medium, 100 ng/ml LPS or 1 mM Lxrb. A, C: RNA was isolated, converted to cDNA and analyzed by real-time PCR with the respective gene-specific primers. B, D: Cell extracts were subjected to Western blot analyses with Abs against phosphorylated and total species of p38 MAPK. A, C: The summary data of three experiments are presented. B, D: Shown are the representative results for MΦs from one BXSB/ *Yaa* vs one BXSB mice. Similar results were observed for cells obtained from other two mice of each strain.

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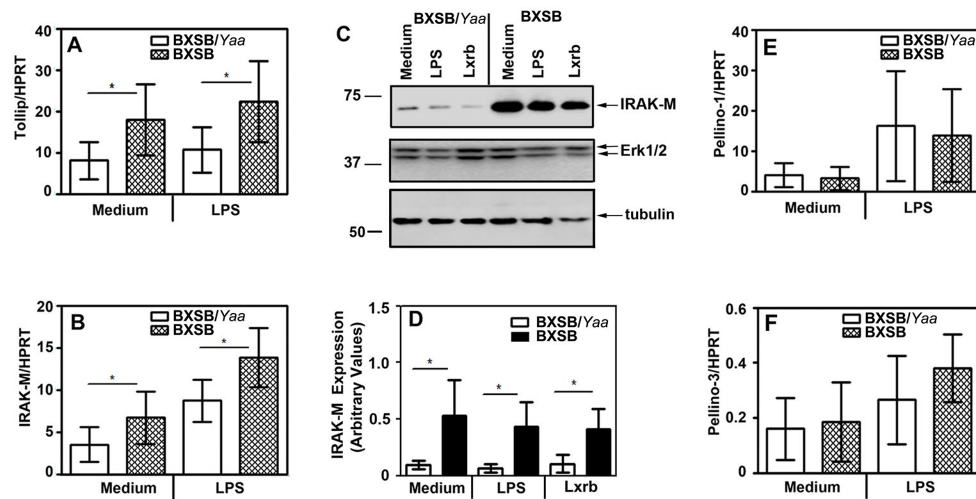

Figure 3. The impact of lupus development on Pellino-1, Pellino-3, Tollip and IRAK-M gene expression

Splenic MΦs from 16 weeks-old BXSB/*Yaa* (n=14) or BXSB (n=9) mice were treated for 3 h (A, B, E, F) or 20 min (C, D) with medium, 100 ng/ml LPS, or 1 mM Lxrb, cell cultures from each individual mice were processed. RNA was isolated, reverse-transcribed and subjected to real-time PCR analyses of Tollip (A), IRAK-M (B), Pellino-1 (D), or Pellino-3 (E) gene expression relative to HPRT. *p<0.05. Protein expression of IRAK-M relative to tubulin (C) was examined by Western blot analyses. Shown are data from representative mice. Western blot results from all mice from three experiments were quantified and shown in (F) as arbitrary values (mean ± SD) of IRAK-M expression (calculated by normalizing the intensity of the respective bands to those of total Erk1/2 and tubulin bands). *p<0.05 (one-way Anova with Tukey post-hoc test).

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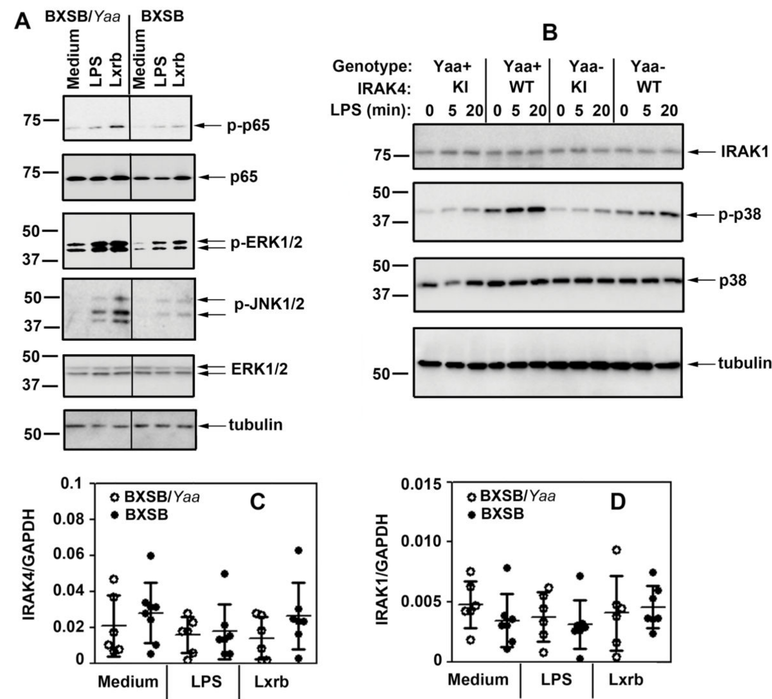

Figure 4. Increased phosphorylation of Erk1/2, JNK1/2, p38 MAPKs and p65 NF-κB but comparable IRAK4 and IRAK1 gene expression in MΦs from lupus-prone and control mice

Splenic MΦs from 16 weeks-old BXSB/ *Yaa* or BXSB mice (A, C, D) or F2 (B6 x BXSB/ *Yaa*) or (B6 x BXSB) mice expressing kinase-sufficient or kinase-inactive IRAK4 (B) were treated for 20 min (A), as indicated (B) or for 3 h (C, D) with medium, 100 ng/ml LPS or 1 mM Lxrb. A, B: Cell extracts were subjected to Western blot analyses with the respective Abs against IRAK1, phosphorylated and total species of IRAK1, p38, p65 and Erk1/2. C, D: RNA was isolated, converted to cDNA and analyzed by real-time PCR with the respective gene-specific primers. A, B: the results of a representative experiment are shown. Similar data were obtained in two other experiments. C, D: The data (mean ± SD) of the indicated number of mice (shown in dot plots) from 3 experiments are depicted.

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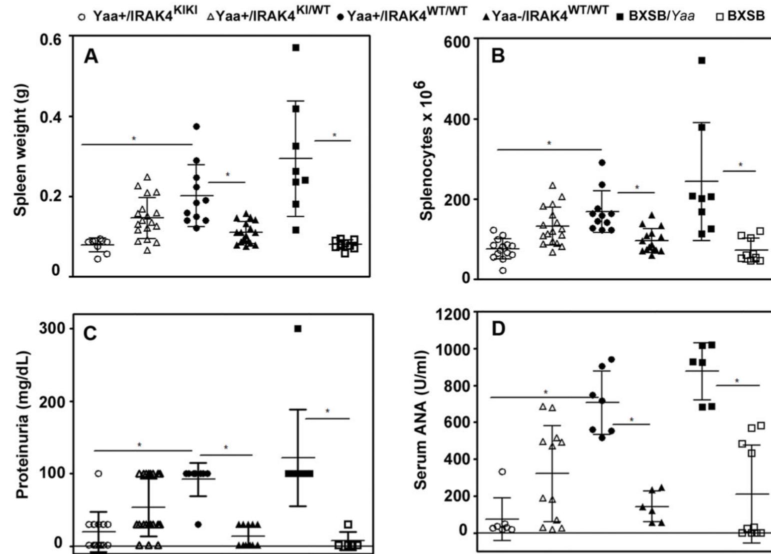

Figure 5. IRAK4 kinase deficiency ameliorates manifestations of autoimmunity in lupus-prone BXSB/*Yaa* mice

Sixteen weeks-old F2 mice homozygoulsy or heterozygously expressing kinase-inactive IRAK4 or WT IRAK4 on the lupus-prone background (Yaa+/IRAK4$^{KI/KI}$, n=8; Yaa+/IRAK4$^{WT/KI}$, n=15; or Yaa+/IRAK4$^{WT/WT}$, n=7), F2 mice expressing WT IRAK4 on the control background (Yaa-/IRAK4$^{WT/WT}$, n=8), or BXSB/ *Yaa* and BXSB mice (n=9) were used. Splenomegaly was judged by the spleen weight (A) and the number of splenocytes (B), urine analyses with multisticks was employed to determine proteinuria (C), and serum ANA levels were examined by ELISA. *p<0.05 (one-way Anova with Tukey post-hoc test). Data (mean ± SD) are pooled from three independent experiments.

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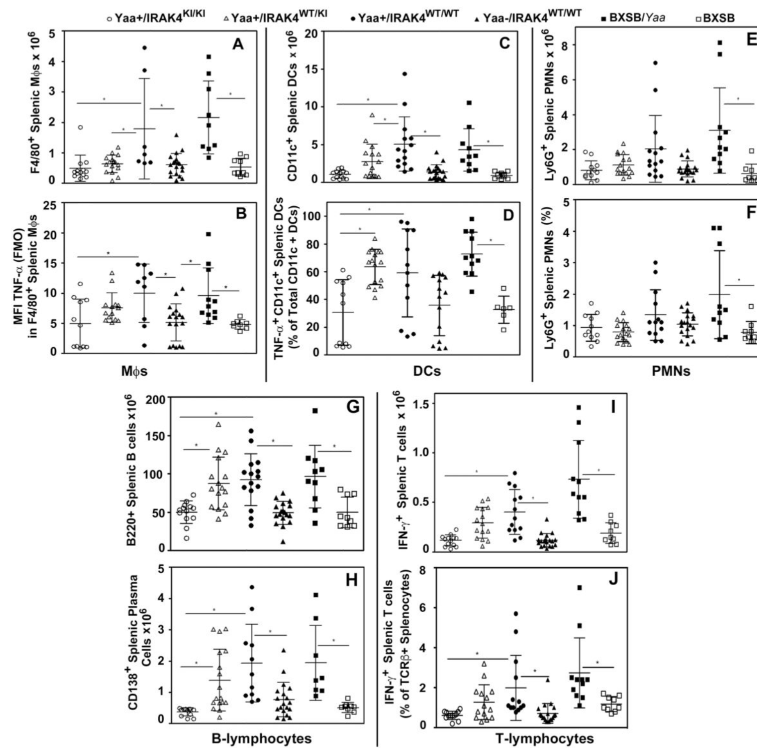

Figure 6. The impact of IRAK4 kinase deficiency on recruitment and activation of MΦs, DCs, T- and B-lymphocytes into the spleen

Sixteen weeks-old F2 mice Yaa^{+}/IRAK4$^{KI/KI}$ (n=11), Yaa^{+}/IRAK4$^{WT/KI}$ (n=14), Yaa^{+}/IRAK4$^{WT/WT}$ (n=7–14), Yaa^{-}/IRAK4$^{WT/WT}$ (n=16), as well as BXSB/*Yaa* (n=10) or BXSB mice (n=11) were used to isolate splenocytes. Cells were stained with the respective fluorescent Abs or isotype controls, as indicated in Materials and Methods, and analyzed by FACS. *p<0.05 (one-way Anova with Tukey post-hoc test). The results (mean ± SD) of three experiments are shown.