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Loss of STAT6 promotes autoimmune disease and atopy on a susceptible genetic background

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ABSTRACT

Atopy and autoimmunity are usually considered opposed immunological manifestations. $Lyn^{-/-}$ mice develop lupus-like autoimmune disease yet have coexistent intrinsic allergic traits and are prone to severe, persistent asthma induced exogenously. Recently it has been proposed that the Th2 environment and IgE auto-Abs promotes autoimmune disease in $Lyn^{-/-}$ mice. To examine this apparent contradiction, we derived $Lyn^{-/-}$ mice with a null mutation in STAT6, a regulator of Th2 immunity that integrates signaling from the IL-4/IL-13 receptor complex. Atopy and spontaneous peritoneal eosinophilia, characteristic of $Lyn^{-/-}$ mice, were lost in young $Lyn^{-/-}$ STAT6 $^{-/-}$ mice; however, autoimmune disease was markedly exacerbated. At a time-point where $Lyn^{-/-}$ mice showed only mild autoimmune disease, $Lyn^{-/-}$ STAT6 $^{-/-}$ mice had maximal titres of IgG and IgA auto-Abs, impaired renal function, myeloid expansion and a highly activated T cell compartment. Remarkably, low level IgE auto-Abs but not IgG1 auto-Abs were a feature of some aged $Lyn^{-/-}$ STAT6 $^{-/-}$ mice. Furthermore, aged $Lyn^{-/-}$ STAT6 $^{-/-}$ mice showed dramatically increased levels of serum IgE but minimal IgG1, suggesting that class-switching to IgE can occur in the absence of an IgG1 intermediate. The results show that Lyn -deficient mice can overcome the effects of disabling Th2 immunity, highlighting the importance of Lyn in controlling Th2 responses. Our data also indicates that, under certain conditions, STAT6-independent factors can promote IgE class-switching. This work has important clinical implications as many experimental therapies designed for the treatment of asthma or atopy are based on targeting the STAT6 axis, which could potentially reveal life endangering autoimmunity or promote atopy in susceptible individuals.

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1. Introduction

Epidemiological studies suggest that Th1 inflammation (associated with autoimmune disease including rheumatoid arthritis, multiple sclerosis and type I diabetes) may suppress the development of atopy, while reciprocally, atopy may suppress the severity but not necessarily the onset of autoimmunity [1]. Indeed it has been proposed that atopy and autoimmunity are mutually exclusive disorders [2–4]. Understanding the molecular regulation of this balance has proven challenging. Previous studies have shown that

the Lyn tyrosine kinase is an important regulator of antibody-mediated autoimmune disease, with $Lyn^{-/-}$ mice developing a disease reminiscent of systemic lupus erythematosus (SLE) [5,6]. This suggests that the mice are more Th1-prone and in support of this, it has recently been reported that levels of the proinflammatory cytokines IL-6 and IFN- γ are elevated in aged $Lyn^{-/-}$ mice, while $Lyn^{-/-}$ IL-6 $^{-/-}$ and $Lyn^{-/-}$ IFN- γ $^{-/-}$ mice are protected from autoimmune disease [7,8]. In seemingly direct contradiction to these findings, it has also been shown that $Lyn^{-/-}$ mice are impaired in their ability to counter the Th1 cell-inducing pathogen *Toxoplasma gondii* and show a less robust IFN- γ response [9]. Reduced Th1 immunity is also supported by studies of $Lyn^{-/-}$ dendritic cells, which produce less IL-12, are inefficient at inducing Th1 cell priming and promote Th2 responses more readily [10,11].

While $Lyn^{-/-}$ mice are autoimmune-prone, they show simultaneous enhancement of Th2 immunity. They are atopic, expressing

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10-fold higher levels of IgE in serum, their B cells are hyper-responsive to IL-4 and more readily class-switch from IgG1 to IgE [12]. They also have several allergy-associated traits in addition to increased serum IgE, including elevated circulating histamine, augmented numbers of eosinophils and mast cells and increased expression of mast cell FcεRI [13]. Their allergic phenotype is further confirmed by the fact that antigen-challenged *Lyn*^{-/-} mice develop an intense eosinophilic inflammatory response in airways, with increased numbers of activated CD4⁺ T cells, elevated Th2 cytokines, mucus cell metaplasia, elevated IgE titers and hyper-degranulation of mast cells and eosinophils in the airway mucosa [10]. More recent studies have shown that *Lyn* is expressed in basophils, where it has been found to control the expression of the Th2 transcription factor GATA-3 [9]. Loss of *Lyn* leads to increased GATA-3 expression in basophils and increased IL-4 and IgE production [9], suggesting that one function of *Lyn* is to suppress the development of Th2 responses. GATA3 expression is regulated by the transcription factor STAT6, which in turn is regulated by IL-4/IL-13 receptor activation [14,15]. In studies that might provide a rationalization of these contradictory findings, it has recently been proposed that the Th2 phenotype of *Lyn*^{-/-} mice is, itself, a major contributory factor to their autoimmune susceptibility [16]. Autoreactive IgE antibodies were detected in *Lyn*^{-/-} mice, where they could effectively activate basophils leading to enhanced IL-4 production. Furthermore, genetic ablation of IL-4 or IgE in *Lyn*^{-/-} mice diminished anti-nuclear antibodies (ANAs) and nephritis [16].

To probe and better understand these disparities we generated compound mutant mice combining *Lyn* deficiency with STAT6 deficiency, where Th2 immune deviation is disabled. We show that loss of STAT6 on a *Lyn*-deficient background leads to a reduction of Th2 traits in young mice and early onset autoimmune disease. However, aged *Lyn*^{-/-} STAT6^{-/-} mice overcome the deficit of STAT6 and are able to produce high levels of IgE. As well as helping to understand the regulation of autoimmunity and allergy, our findings may have important implications for the asthma and allergy clinic, as therapies centred on targeting the STAT6 axis may markedly increase an individual's risk of developing atopy or precipitate underlying subclinical autoimmune disease.

2. Materials and methods

2.1. Mice

BALB/c *Lyn*^{-/-} and STAT6^{-/-} mice [5,17] were used. *Lyn*^{-/-} STAT6^{-/-} (DKO) mice were generated by intercrossing and genotyping, and were healthy and fertile. Experiments were performed in accord with NHMRC guidelines for animal experimentation, with approval from the Animal Ethics Committees of the Ludwig Institute for Cancer Research/Department of Surgery and Baker IDI Heart and Diabetes Institute, Melbourne.

2.2. Flow cytometry

Peritoneal lavage cells or single cell suspensions of spleen were stained and analysed by flow cytometry [18]. Flow cytometry and cell counting was used to determine absolute cell numbers defined as follows: B cells, B220⁺CD138^{lo}; plasma cells, B220^{lo}CD138^{hi}; regulatory T cells (Tregs), CD4⁺CD25⁺; macrophages, Mac-1⁺c-fms⁺; neutrophils, Mac-1⁺Gr-1^{hi}; eosinophils, F4/80⁺CCR3⁺Mac-1⁻; basophils, Mac-1⁺FcεRI⁺CD49b⁺c-kit⁻.

2.3. ELISAs

Serum IgG ANAs were detected using a 1/250 dilution of serum [18]. Anti-dsDNA auto-Abs were detected by ELISA using plates

coated with purified dsDNA (50 ng/well)(Sigma). A 1/500 or 1/50 dilution of serum was used to assay for IgG and IgA anti-dsDNA auto-Abs while a 1/5 dilution of serum was used to assay for IgE anti-dsDNA auto-Abs. Serum Ig titers were determined as described [7].

2.4. Assessment of MHC-II on cultured B Cells

Purified splenic B cells at 500,000 cells/ml in proliferation media [19] were cultured overnight with no stimulus, 10 μg/ml IL-4 (Peprotech) or 5 μg/ml LPS (Sigma–Aldrich, USA), then analysed by flow cytometry to detect levels of MHC-II.

2.5. Histopathology and immunofluorescence

Tissues for histopathology or immunofluorescence were prepared as described [19].

2.6. Kidney function test

Kidney function (μg albumin/mg of creatinine), was determined by measuring albumin and creatinine levels in urine using a COBAS Integra 800 (Roche). A higher albumin to creatinine ratio indicates deterioration in kidney function.

2.7. Serum cytokine measurements

Serum cytokine levels were determined using a Bioplex Pro Multiplex Suspension Array system (Bio-Rad Laboratories) according to company instructions.

2.8. Statistical analysis

All statistical analyses were carried out using the non-parametric unpaired *t* test (Mann–Whitney) in GraphPad Prism software (version 4.03). Statistically significant differences between groups of data are indicated by asterisks.

3. Results

3.1. Deletion of STAT6 significantly reduces Th2 traits in young *Lyn*^{-/-} mice

To evaluate the influence of exaggerated Th2 immunity on the development of autoimmune disease in *Lyn*^{-/-} mice, we skewed the immune system away from Th2 immunity by genetic deletion of STAT6. We used BALB/c background mice for these studies, which are Th2-prone [20]. To determine whether loss of STAT6 reduced Th2 traits, we examined peritoneal eosinophilia and measured levels of IgE in serum, both characteristics of *Lyn*^{-/-} mice [10,12,13]. While young BALB/c *Lyn*^{-/-} mice had elevated numbers of peritoneal eosinophils, this spontaneous eosinophilia was lost in *Lyn*^{-/-} STAT6^{-/-} (DKO) mice (Fig. 1A). In addition, the elevated levels of IgE in the serum of 10-wk-old *Lyn*^{-/-} mice were markedly reduced in matched DKO mice (Fig. 1B). However, 2 out of 9 DKO mice displayed detectable IgE levels, suggesting that a pathway other than STAT6 can contribute to IgE class switching in *Lyn*^{-/-} mice. Since IL-4 directs class switching to IgG1 and IgE [21], we also measured levels of serum IgG1 and found that DKO mice had very low levels, similar to STAT6^{-/-} mice (Fig. 1C). Collectively, these results show that deletion of STAT6 in *Lyn*^{-/-} mice leads to a reduction in Th2 traits.

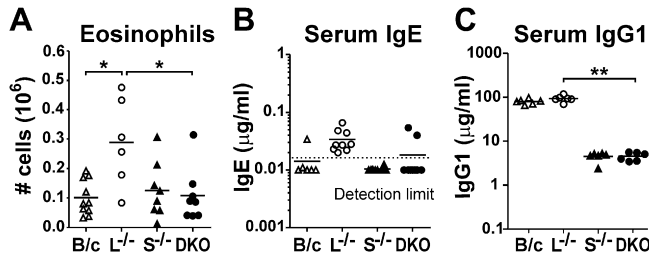


Fig. 1. Deletion of STAT6 reduces Th2 traits in *Lyn*^{-/-} mice. (A) Eosinophils in the peritoneal cavity of BALB/c (B/c), *Lyn*^{-/-} (L^{-/-}), *STAT6*^{-/-} (S^{-/-}) and DKO mice by flow cytometry and cell counting. Data are from three experiments and $n \geq 6$ mice. Circulating levels of (B) IgE and (C) IgG1 ($n = 6$ –10 10-wk-old mice/genotype). For data in (A) and (C), * $p < 0.05$, ** $p < 0.01$; in (B), statistical analyses could not be performed as many readings fell below the limit of detection of the assay (dashed line = 15.6 ng/ml).

3.2. DKO and *Lyn*^{-/-} mice share similar B cell characteristics but DKO B cells are hypo-responsive to IL-4

The immune cell subsets of unchallenged *STAT6*^{-/-} mice are indistinguishable from those of control mice [17,22,23], whereas *Lyn*^{-/-} mice show a major alteration in their B cell compartment [5,6], exhibit an expanded myeloid compartment [7] and with age show an expansion of regulatory T cells [18] and activated T cells [7,8,18]. To determine whether DKO mice were phenotypically different from *Lyn*^{-/-} or *STAT6*^{-/-} mice, flow cytometry studies were conducted. The splenic B cell compartment of young DKO mice looked similar to that of *Lyn*^{-/-} mice, showing decreased numbers of follicular B cells (Fig. 2A and B), loss of both marginal zone (MZ) and transitional 2 (T2) B cells (not shown) [24] and elevated numbers of plasma cells (Fig. 2A and B). Furthermore, DKO mice, like *Lyn*^{-/-} mice [5,6], showed a ten-fold elevation in serum IgM (Fig. 2C). The only significant difference observed was in B cell MHC-II expression (Fig. 2D). As reported [7,25], *Lyn*^{-/-} B cells have a higher mean fluorescence intensity (MFI) of MHC-II, reflecting increased B cell activation, whereas DKO B cells had levels of MHC-II that resembled those of control and *STAT6*^{-/-} B cells. This is consistent with previous studies showing that *STAT6* is critical for induction of MHC-II expression [17,22,23]. Despite lower levels of MHC-II, DKO B cells showed evidence of B cell activation as indicated by increased CD86 (Fig. 2D) and down-regulation of surface IgM (not shown).

Since we observed lower levels of MHC-II on DKO B cells *ex vivo*, this was assessed further by culturing purified splenic B cells overnight in IL-4 and LPS (Fig. 2E). Control B cells showed significant upregulation of MHC-II in response to both agonists, whereas *STAT6*^{-/-} B cells failed to upregulate MHC-II following IL-4 stimulation, confirming the importance of *STAT6* in this response [17,22,23]. Conversely, *Lyn*^{-/-} B cells dramatically upregulated MHC-II post-IL-4 stimulation compared to control B cells, confirming that they are hyper-responsive to IL-4 [12]. DKO B cells, like *STAT6*^{-/-} B cells, failed to upregulate MHC-II in response to IL-4, however, augmentation of MHC-II expression was observed in response to LPS, showing that the impairment is IL-4-specific. In the presence of LPS, B cells from both *Lyn*^{-/-} and DKO mice appeared hyper-responsive, showing more dramatic upregulation of MHC-II expression compared to BALB/c or *STAT6*^{-/-} B cells (Fig. 2E).

3.3. Enhanced development of serum IgG ANAs in DKO mice

Since DKO mice are missing a key regulator of Th2 immunity and a major mediator of IgG1 production, we next determined whether this would affect development of Ab-mediated

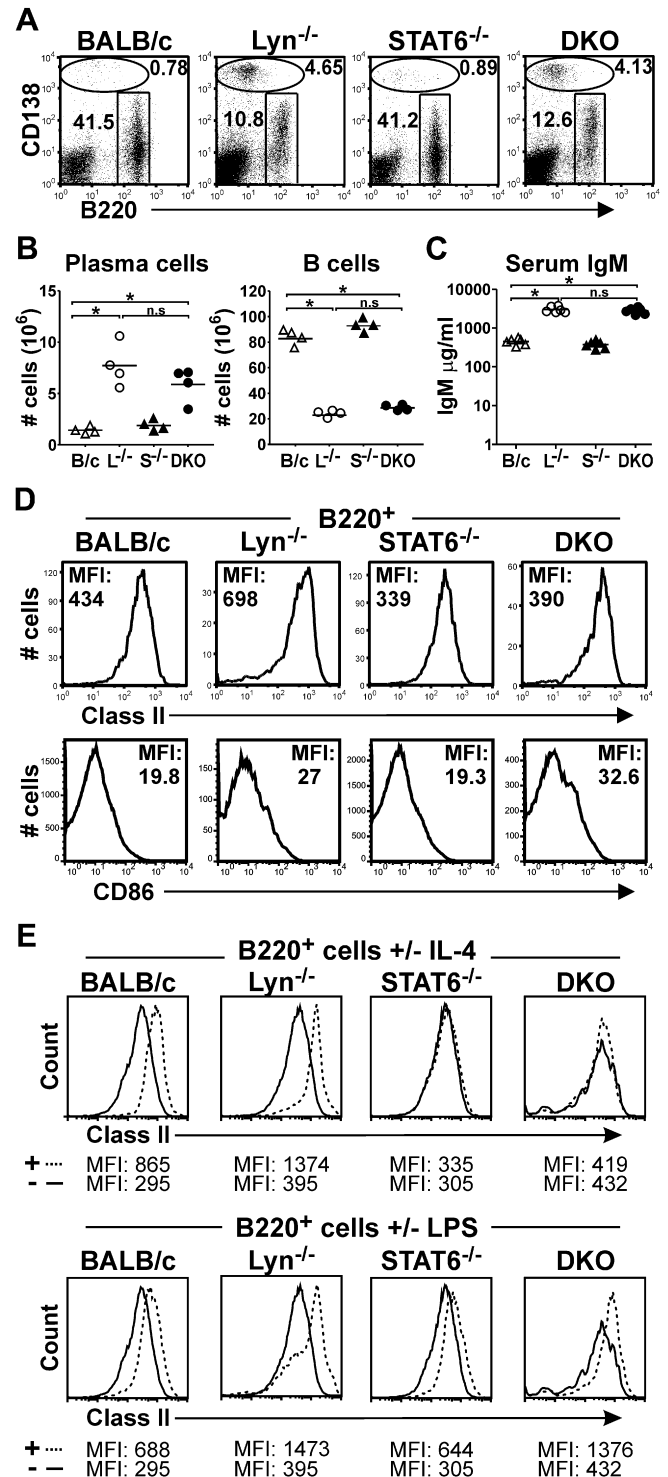


Fig. 2. The B cell compartment of DKO mice is similar to that of *Lyn*^{-/-} mice. (A) Representative flow cytometry of spleen cells from 8-wk-old BALB/c (B/c), *Lyn*^{-/-} (L^{-/-}), *STAT6*^{-/-} (S^{-/-}) and DKO mice. (B) Absolute numbers of plasma cells (B220⁺CD138^{hi}; ellipse in (A)) and B cells (B220⁺CD138^{lo}; rectangle in (A)); * $p < 0.05$. Data are from 4 mice/genotype in two experiments. (C) IgM in the serum of $n = 6$ mice/genotype; * $p < 0.05$. For data in (B) and (C) n.s. = non-significant. (D) Expression of MHC-II and CD86 on splenic B cells; MFI indicated. Data are representative of two experiments and $n = 4$ mice/genotype. (E) Purified splenic B cells were cultured in medium, IL-4 (10 μg/ml) or LPS (5 μg/ml) for 24 h. MHC-II expression on stimulated (dotted lines) vs. unstimulated (solid lines) B cells by flow cytometry is shown as histograms, MFI indicated. Data are representative of three experiments.

autoimmune disease. As early as 10 wks of age, DKO mice displayed IgG ANAs, by 14 wks all had detectable titres, and by 22 wks all DKO mice had maximal ANA titres as detected by the assay (Fig. 3A). In contrast, some $Lyn^{-/-}$ mice displayed ANAs at 14 wks of age, and by 26 wks not all mice had achieved saturating readings, findings consistent with slower ANA onset in BALB/c $Lyn^{-/-}$ mice compared to C57BL/6 $Lyn^{-/-}$ mice [18]. These results clearly demonstrate that loss of STAT6 leads to exacerbation of autoimmunity in $Lyn^{-/-}$ mice.

To determine if the more rapid acquisition of serum ANAs in DKO mice resulted in enhanced disease, kidneys from aged mice were analysed (Fig. 3B). Glomerular enlargement and hypercellularity were evident in both $Lyn^{-/-}$ and DKO kidneys, yet it was difficult to distinguish differences. However, kidney function tests demonstrated that aged DKO mice had a significantly elevated ratio of urine albumin to creatinine compared to $Lyn^{-/-}$ mice (Fig. 3C). Interestingly, there were no significant differences in kidney function between aged control and $Lyn^{-/-}$ mice, which may be due to the protective effects of the BALB/c background on the development of nephritis [19,26–29]. Indeed we have observed less severe glomerulonephritis in 9-mo-old BALB/c $Lyn^{-/-}$ mice

compared to matched C57BL/6 or mixed 129xB6 genetic background $Lyn^{-/-}$ mice [5,7,18]. Sections of kidneys were also assessed for immune complexes and complement (Fig. 3D). While there was no specific glomerular staining in BALB/c or STAT6 $^{-/-}$ mice, Abs to IgG2a and C3 revealed strong staining in the glomeruli of $Lyn^{-/-}$ and DKO mice.

3.4. Auto-Ab isotypes in $Lyn^{-/-}$ and DKO mice

We next dissected auto-Ab isotypes by examining serum from $Lyn^{-/-}$ and DKO mice at two different time-points during disease progression (Fig. 4A). In 22-wk-old mice, DKO mice had close to maximal titers of IgG2a, IgG2b and IgG3 ANAs, whereas $Lyn^{-/-}$ mice had detectable but significantly lower titers. In 34-wk-old mice, IgG3 ANAs were similarly high, whereas titres of IgG2a and IgG2b ANAs were significantly lower in $Lyn^{-/-}$ mice. Interestingly, IgG1 did not appear to be a major component of the autoimmune response as the levels in both 22- and 34-wk-old $Lyn^{-/-}$ mice were much lower than those of the other IgG isotypes. As expected, DKO mice had negligible IgG1 ANAs.

Since a recent study has suggested that the Th2 environment and IgE ANAs can promote autoimmune disease in $Lyn^{-/-}$ mice [16], we next determined levels of IgE ANAs. Due to the low abundance of IgE in serum, we examined this using an anti-dsDNA ELISA, which shows greater specificity at high serum concentrations. Using a 1/5 dilution of serum, negligible levels of IgE anti-dsDNA Abs were found in 30-wk-old $Lyn^{-/-}$ mice but most unexpectedly, the levels in matched DKO mice were slightly but significantly elevated (Fig. 4B). Although elevated, the levels of IgE anti-dsDNA in DKO mice were at least 100-fold lower than the levels of IgG and IgA anti-dsDNA (Fig. 4B). When we analysed 40+ wk-old mice, both $Lyn^{-/-}$ and DKO mice had low but significantly elevated levels of IgE anti-dsDNA Abs (Fig. 4B).

To explore the curious ability of aged DKO mice to produce low level IgE auto-Abs, we next determined total serum IgE levels in aged mice. Control mice of all ages showed undetectable or low levels of IgE in serum (Figs. 1B and 4C). Unsurprisingly, STAT6 $^{-/-}$ mice did not have measurable levels of serum IgE throughout life, while $Lyn^{-/-}$ mice had detectable levels at all ages, which accumulated with age (Figs. 1B and 4C). Interestingly, although IgE levels were low or undetectable in the serum of DKO mice at 10 and 20 weeks of age, older DKO mice displayed levels of IgE akin to $Lyn^{-/-}$ mice (Figs. 1B and 4C). To establish whether aged DKO mice could also produce IgG1, we examined mice of 40+ weeks of age. IgG1 levels remained low and were even lower than the levels in STAT6 $^{-/-}$ mice, indicating that IgE was being produced in DKO mice by a mechanism independent of IgG1 (Fig. 4C).

3.5. Augmented serum levels of IgG2a, IgG2b and IgG3 isotypes in mutant mice

We next investigated whether mutant mice exhibited alterations in other IgG isotypes. Analysis of 22- and 34-wk-old STAT6 $^{-/-}$ mice showed modest yet significant increases in IgG2a, IgG2b and IgG3 compared with BALB/c mice (Fig. 4D). By comparison, DKO mice showed striking increases in all three isotypes, with the most dramatic increase seen in IgG2a. $Lyn^{-/-}$ mice showed a significant increase in IgG2a and IgG3, while IgG1 and IgG2b isotypes remained similar to controls.

3.6. Young DKO mice show myeloid compartment changes

We next examined the myeloid compartment of DKO mice to determine whether their enhanced development of autoimmune disease correlated with an early expansion of myeloid cells.

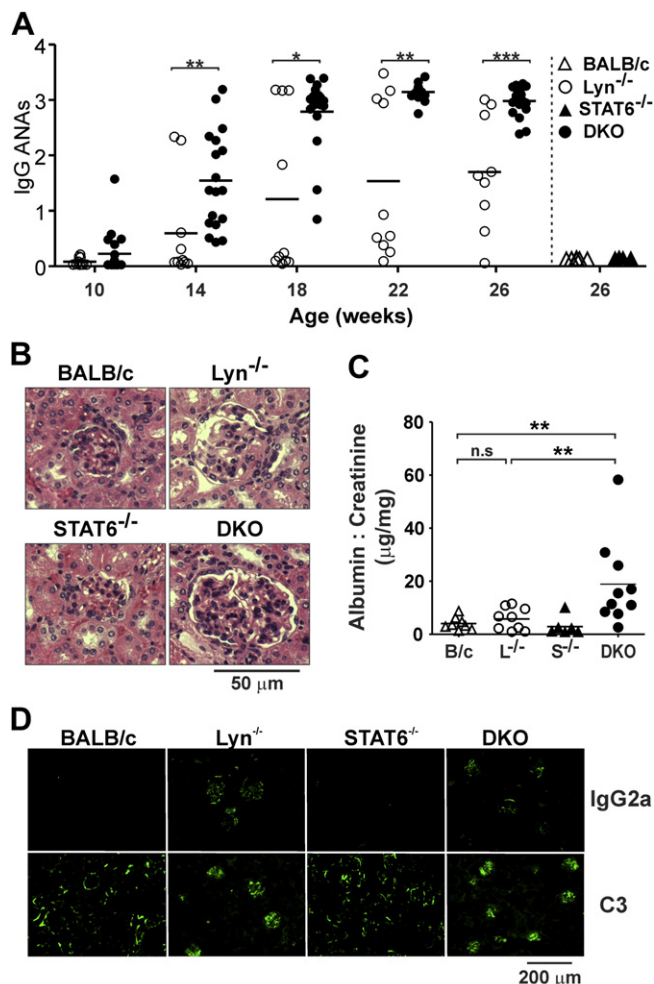


Fig. 3. Autoimmune disease is exacerbated in DKO mice. (A) Serum IgG ANAs in BALB/c, $Lyn^{-/-}$, STAT6 $^{-/-}$ and DKO mice ($n = 10–17$) at the indicated ages. (B) Representative images of H&E stained sections through the cortex of kidneys from 30-wk-old mice. (C) Kidney function determined by analysis of urine albumin and creatinine of 30-wk-old BALB/c (B/c), $Lyn^{-/-}$ ($L^{-/-}$), STAT6 $^{-/-}$ ($S^{-/-}$) and DKO mice; $n = 6–10$ mice, n.s = non-significant. (D) Immunofluorescent staining of IgG2a and C3 deposition in kidneys of 30-wk-old mice. Images are representative of staining in $n = 8$ mice. For all data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

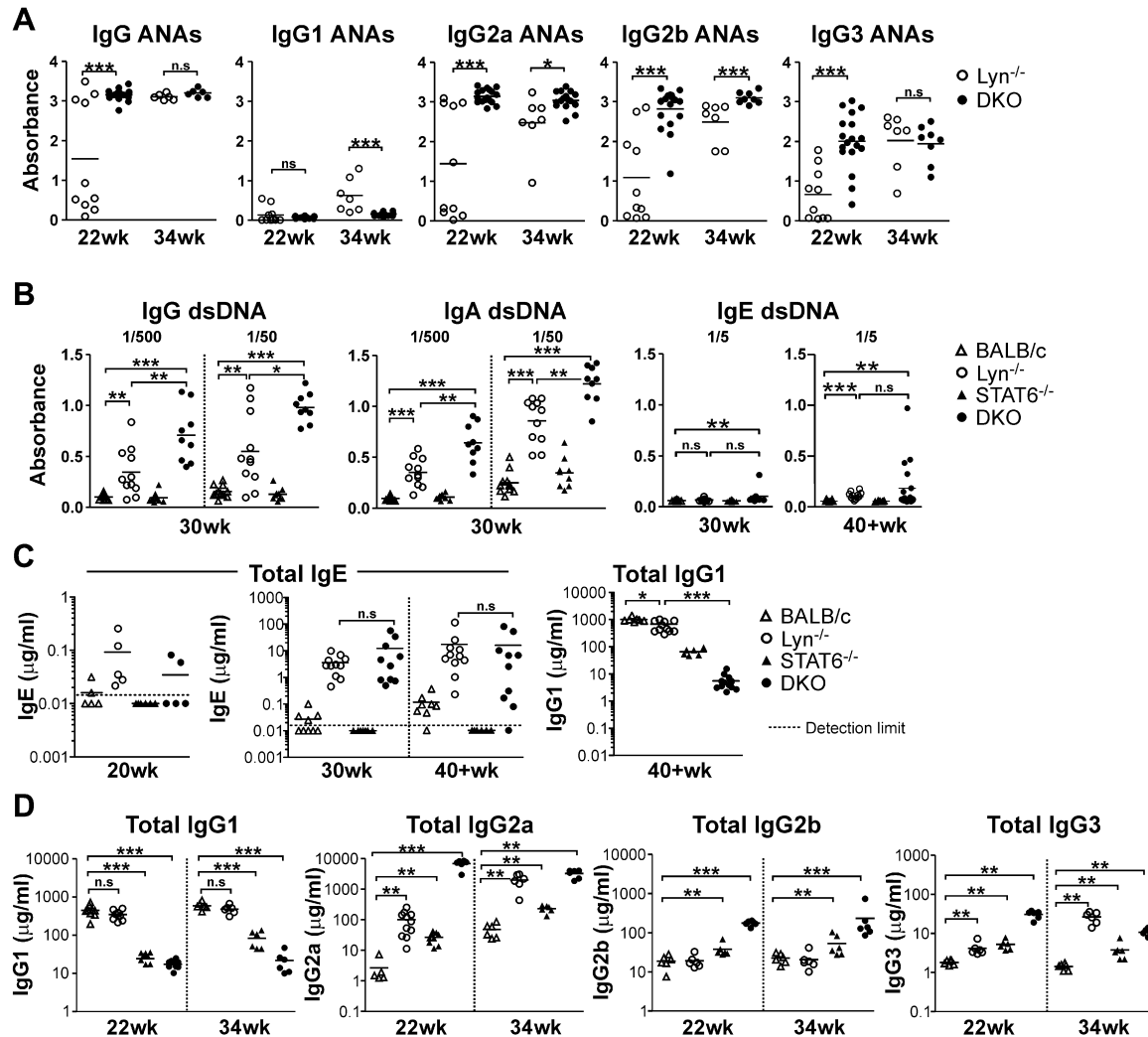


Fig. 4. Altered serum ANA, dsDNA and Ig isotypes in DKO mice. (A) Total IgG ANAs, and individual IgG1, IgG2a, IgG2b and IgG3 ANA isotypes in the serum of 22- and 34-wk-old Lyn^{-/-} and DKO mice; $n = 7$ –18 mice. Serum samples from BALB/c and STAT6^{-/-} controls were tested and were negative, so have been omitted for clarity. (B) Levels of IgG, IgA and IgE anti-dsDNA auto-Abs in serum of mice of the indicated ages; $n = 9$ –11 mice. Serum was diluted 1/500 and 1/50 for IgG and IgA anti-dsDNA and 1/5 for IgE anti-dsDNA. (C) Serum IgE in 20, 30 and 40+ wk-old mice and IgG1 in 40+ wk-old mice; $n = 5$ –11 mice. (D) Levels of total IgG1, IgG2a, IgG2b and IgG3 isotypes in the serum of the indicated mice; $n = 6$ –11 mice. For all data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; in (C), statistical analyses could not be performed on IgE levels in 20-wk-old and 30-wk-old mice as many fell below the limit of detection of the assay (dashed line = 15.6 ng/ml). In all data n.s. = non-significant.

Increased numbers of neutrophils and macrophages were found in 8-wk-old DKO mice and were further increased by 20 wks of age (Fig. 5A). While no changes were found in matched Lyn^{-/-} mice, by 30 wks of age, myeloid cell expansion was evident in both Lyn^{-/-} and DKO mice (Fig. 5A). This is consistent with earlier onset autoimmunity in DKO mice compared to Lyn^{-/-} mice.

3.7. Aged DKO mice show exacerbated T cell activation, increased numbers of Tregs and enhanced MHC-II on B cells

Since the T cell compartment of aged Lyn^{-/-} mice becomes activated with development of autoimmune disease [7,8,18], we next compared the temporal development of T cell changes between Lyn^{-/-} and DKO mice. CD4 T cells of 22-wk-old DKO mice were highly activated, showing substantial upregulation of CD69 and CD44 and significant down-regulation of CD62L (Fig. 5B). By comparison, changes were less substantial in 22-wk-old Lyn^{-/-} mice; when absolute numbers of activated CD4 T cells were determined they were elevated but significantly lower than those in matched DKO mice (Fig. 5C). This suggests that disease-related changes are occurring more rapidly in DKO mice.

In previous studies we have assessed the Treg population in Lyn^{-/-} mice, finding that aged Lyn-deficient mice had a considerably expanded Treg compartment [18]. When function was assessed in an *in vivo* colitis model, Lyn^{-/-} Tregs were able to efficiently suppress disease, suggesting that Tregs are expanding to control disease but are overwhelmed by the disease process. Given the association between IL-4, STAT6 and FoxP3 [30], we examined the Treg compartment of DKO mice and found that it is also expanded, with the expansion occurring more rapidly to what is observed in Lyn^{-/-} mice (Fig. 5D). This is consistent with the disease process occurring more quickly in DKO mice, and demonstrates that a deficit of Tregs does not underlie the acceleration of autoimmune disease in DKO mice.

We also assessed levels of MHC-II on B cells of aged mice to determine if this phenotype had altered with disease state. Consistent with young mice, B cells from aged Lyn^{-/-} mice expressed higher levels of MHC-II than control B cells, while B cells from aged STAT6^{-/-} mice had similar or lower levels (Figs. 5E and 2D). In contrast, B cells from aged DKO mice had MHC-II levels that were higher than on young DKO mice, resembling the levels on Lyn^{-/-} B cells (Fig. 5E). This suggests that pathways independent of

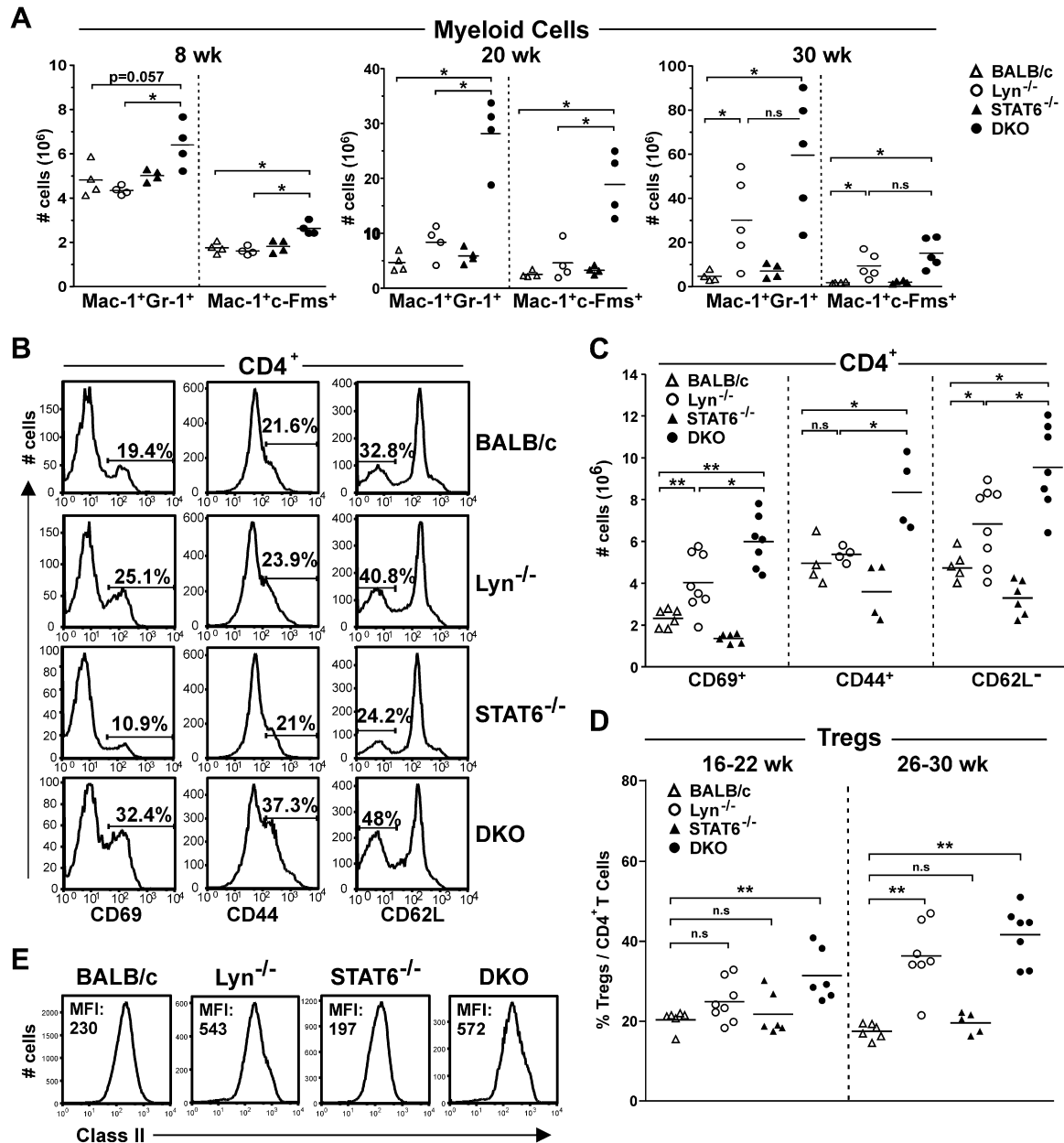


Fig. 5. Myeloid cell expansion and T cell activation occurs more rapidly in DKO mice. (A) Absolute numbers of splenic neutrophils (Mac-1⁺Gr-1⁺) and macrophages (Mac-1⁺c-Fms⁺) in mice of the indicated ages based on total cell counts and flow cytometry; data from $n \geq 4$ mice/genotype in 2 experiments. (B) CD4⁺ spleen cells from the indicated 22-wk-old mice were gated to assess expression of CD69, CD44 and CD62L. (C) Absolute numbers of CD69⁺, CD44⁺ and CD62L⁻ CD4⁺ T cells based on total cell counts and flow cytometry; data from $n = 4-7$ mice in at least 2 experiments. (D) Tregs as a proportion of the total CD4⁺ T cell population in the spleen of the indicated 16–22-wk-old mice and 26–30-wk-old mice based on flow cytometry. (E) MHC-II expression on B220⁺ spleen cells from the indicated 30-wk-old mice, MFI indicated. Data are representative of 3 experiments. In (A), (C) and (D), * $p < 0.05$, ** $p < 0.01$, ns = not significant.

STAT6 may be induced in aged DKO mice to promote up-regulation of MHC-II on B cells.

3.8. Increased numbers of immature basophils in aged *Lyn*^{-/-} and DKO mice

Previously it has been reported that basophil proportions were increased in aged *Lyn*^{-/-} mice and there was an increase in basophil expression of MHC-II and CD62L [16]. On examination of the basophil compartment, we also observed increased numbers of basophils in the spleen of aged *Lyn*^{-/-} mice, however we found that they expressed reduced levels of MHC-II and CD62L compared to control mice (Fig. 6A). Interestingly, the same phenotype was

observed in DKO mice (Fig. 6A). Evaluation of absolute numbers revealed that the number of Class II⁺ basophils was unaltered in *Lyn*^{-/-} and DKO mice compared to controls; however, the number of CD62L⁺ basophils was significantly reduced (Fig. 6B). These data, which show less mature basophils in the secondary lymphoid tissue of *Lyn*^{-/-} and DKO mice, suggests that basophil activation is not associated with the onset of Th2 responses or exacerbated autoimmune responses in these mice.

3.9. Elevated serum cytokines in aged *Lyn*^{-/-} and DKO mice

As *Lyn*^{-/-} mice age they have an increase in inflammatory cytokines in their serum [7–9,18,31]. To determine whether there

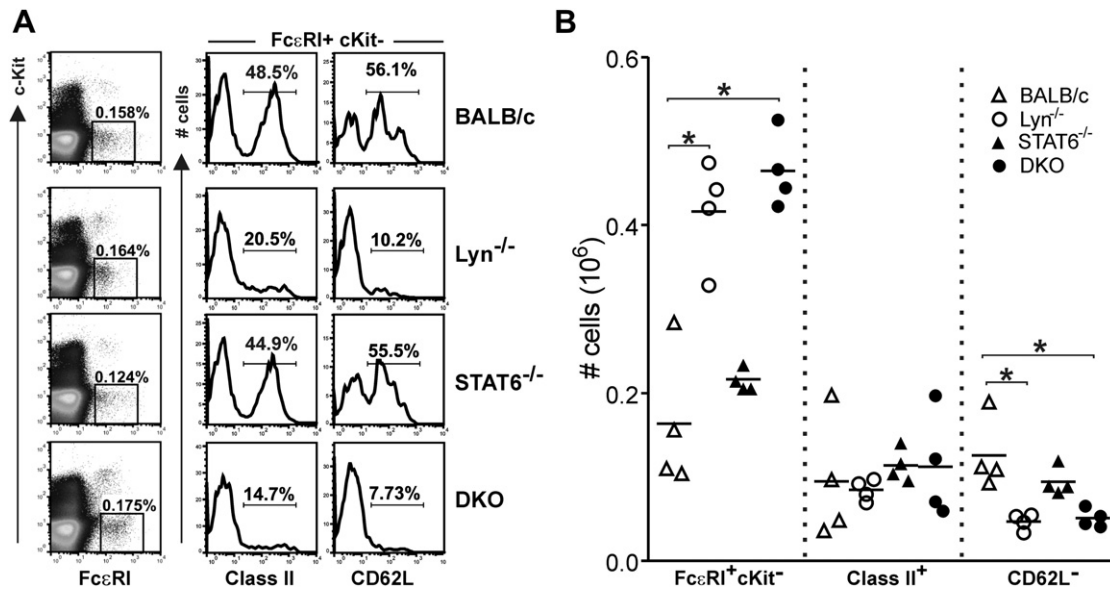


Fig. 6. Increased numbers of immature basophils in Lyn^{-/-} and DKO mice. (A) Representative flow cytometry of spleen cells from 30-wk-old BALB/c, Lyn^{-/-}, STAT6^{-/-} and DKO mice staining with antibodies to c-kit and FcεRI (left) and gating on FcεRI⁺ c-kit⁻ basophils to examine expression of MHC-II and CD62L (right). (B) Absolute numbers of splenic basophils and MHC-II⁺ and CD62L⁺ basophils in the mice shown in (A). Data from $n = 4$ mice/genotype in 2 experiments.

was differential expression of Th1 vs. Th2 cytokines, we assayed serum of 30-wk-old mice finding that both Lyn^{-/-} and DKO mice had elevated levels of IL-2, IL-4, IL-5, IL-6, IL-10, G-CSF, GM-CSF and IFN- γ (Fig. 7). These data show that there is an age-dependent increase in both Th1 and Th2 cytokines and indicate that defective Th2 responses do not affect the concomitant upregulation of both Th1 and Th2 cytokines that is intrinsic to Lyn deficiency.

4. Discussion

Recent epidemiological studies suggest that Th1 inflammation may suppress the development of atopy and in turn, atopy may impact the severity of autoimmunity [1]. Indeed, many studies have shown that enhanced Th2 immunity can protect against the development of autoimmunity [32–36] and the incidence of autoimmune diseases is low in regions where helminth infections are endemic [37]. Other studies show that the severity of rheumatoid arthritis is lower in patients with allergic hay fever and that the prevalence of hay fever in rheumatoid arthritis patients is reduced [38]. Furthermore, asthma incidence is correlated inversely with type 1 diabetes mellitus [39], while helminth infections may contribute to a decreased incidence of type 1 diabetes [40]. These findings suggest that inducing a Th2 response or supplementing the Th2 pathway may suppress the onset of Th1 responses and restrain autoimmune disease development. However, recent new but seemingly contradictory evidence suggests that self-reactive IgE and a Th2 environment are associated with SLE and lupus nephritis [16].

Here we show that genetic deletion of STAT6, the key transcription factor for development of Th2 immunity, in lupus-prone Lyn-deficient mice results in a profound skewing towards autoimmunity. This is despite using mice on the Th2-prone BALB/c genetic background, which has previously been shown to be protective against autoimmune disease severity when compared to the C57BL/6 genetic background [19,26–29]. This is also true for mice harboring null mutations in Lyn; C57BL/6 Lyn^{-/-} mice develop earlier onset pathogenic auto-Abs and more severe autoimmune-driven nephritis than BALB/c Lyn^{-/-} mice [7,18]. Here, we have clearly shown that autoimmune disease develops more quickly in

DKO mice compared with Lyn^{-/-} mice as evidenced by (1) more rapid development of pathogenic auto-Abs; (2) earlier expansion of the myeloid compartment; (3) faster onset of activated T cells; and (4) enhanced development of kidney disease as indicated by impaired kidney function. The altered kinetics of autoimmune disease development in DKO mice is consistent with deletion of a key Th2 transcription factor and the creation of an intensely Th1-polarizing environment (Table 1). What is highly surprising is that Th2 traits can eventually develop in aged DKO mice even though they are missing a prototypic Th2 transcription factor and are phenotypically Th1-biased (Table 1). While young DKO mice show a disruption of Th2 traits that are characteristic of Lyn^{-/-} mice, with the exception of IgG1 production, there is restoration of most Th2 traits in older mice including IgE, IL-4 and IL-5 production, and basophil expansion (Table 1). These data show that there is intense selection pressure to create a Th2 environment in the absence of Lyn and demonstrate the importance of Lyn in maintaining a brake on Th2 cell differentiation and function. What is also clear from our data is that, under certain conditions, there is an alternate STAT6-independent pathway for development of Th2 immune responses.

An analysis of the spectrum of auto-Ab isotypes in the serum of aged DKO mice has revealed high levels of IgG2a, IgG2b, IgG3 and IgA. Not surprisingly, IgG1 auto-Abs are not detectable in DKO mice and interestingly are present at comparatively low levels in Lyn^{-/-} mice compared to other auto-Ab isotypes, even though IgG1 is the predominant isotype in the serum of BALB/c Lyn^{-/-} mice. Remarkably, IgE auto-Abs are found in DKO mice before they are detectable in Lyn^{-/-} mice, albeit in very low amounts. However, the onset of IgE autoantibodies is markedly delayed compared to IgG; when coupled with their low abundance, this suggests that they are not central to disease induction. Curiously, aged DKO mice, even though they lack STAT6, have high levels of IgE in serum, similar to the levels found in matched Lyn^{-/-} mice and 100-fold higher than in control mice. Since young DKO mice have low or undetectable levels of IgE, these data suggest that STAT6-independent factors become operative over time in the Lyn-deficient environment to promote IgE class-switching. There is now mounting evidence that class-switching to IgE can occur in the absence of an IgG1 intermediate [41,42], and very recently it has been shown that immature

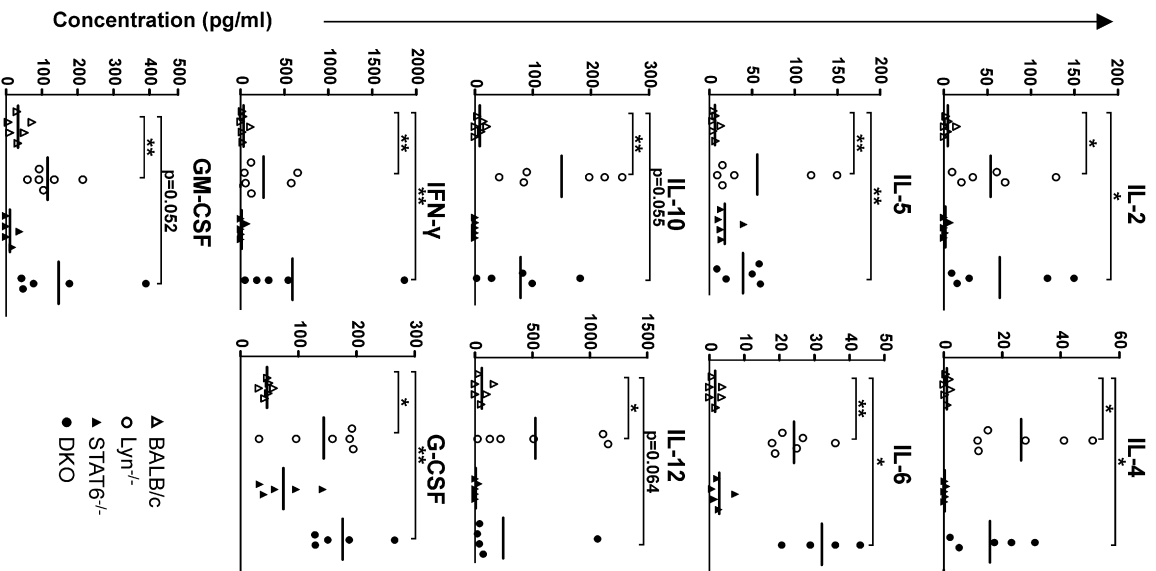


Fig. 7. Altered serum cytokine profiles in $Lyn^{-/-}$ mutants. Amounts of indicated cytokines in the serum of 30-wk-old BALB/c, $Lyn^{-/-}$, $STAT6^{-/-}$ and DKO mice; $n = 5-6$ mice; * $p < 0.05$, ** $p < 0.01$.

B cells have a greater propensity to switch directly to IgE and in a milieu of reduced STAT6 activation [43]. However, this cannot explain the presence of IgE in DKO mice as they, like $Lyn^{-/-}$ mice [24], have a reduced immature B cell compartment.

Although IgE has long been considered a key mediator of allergy, recent studies have suggested it may play a role in the development of autoimmunity. Recently it has been proposed that the presence of autoreactive IgE immune complexes in $Lyn^{-/-}$ mice, through interaction with the high affinity IgE receptor on basophils, triggers the production of IL-4 that acts on B cells to amplify auto-Ab production and nephritis [16]. These previous studies, which suggest that the Th2 environment is promoting autoimmune disease, were done in mixed genetic background $Lyn^{-/-}$ mice [16]. Here, we are also able to detect autoreactive IgE in both $Lyn^{-/-}$ and DKO mice, however the amounts in the BALB/c genetic background animals are particularly low compared to other autoreactive iso-types. Nonetheless, it is conceivable that these may be sufficient to trigger the FcεRI on basophils, which is a high affinity receptor [44]. However, basophils are rare cells and ordinarily represent <1% of

Table 1

Summary of immune response differences between $Lyn^{-/-}$ and DKO mice compared to control mice.

	Parameter	$Lyn^{-/-}$	DKO
Disease indices	Total IgG ANA	40% penetrant in 14-wk-old mice, 100% penetrant in aged mice	100% penetrant in 14-wk-old mice, 100% penetrant in aged mice
	Kidney function in aged mice	1.4-fold reduced	4.6-fold reduced
Th1 traits	Serum IgG2a	3-fold elevated in young mice, 41-fold elevated in aged mice	9-fold elevated in young mice, 67-fold elevated in aged mice
	IgG2a ANA	76-fold elevated in 22-wk-old mice, 233-fold elevated in 34-wk-old mice	166-fold elevated in 22-wk-old mice, 286-fold elevated in 34-wk-old mice
	Serum IFN-γ	8-fold elevated in aged mice	18-fold elevated in aged mice
	IgE ds-DNA	1.7-fold elevated in aged mice	3.5-fold elevated in aged mice
Th2 traits	Serum IgG1	Unaltered in young mice, 1.5-fold reduced in aged mice	18-fold reduced in young mice, 182-fold reduced in aged mice
	Serum IgE	Elevated in young mice, 130-fold elevated in aged mice	Undetected in young mice, 130-fold elevated in aged mice
	Serum IL-4	24.2-fold elevated in aged mice	14.4-fold elevated in aged mice
	Serum IL-5	8.5-fold elevated in aged mice	6-fold elevated in aged mice
	Basophil numbers	2-fold elevated in aged mice	2-fold elevated in aged mice
	CD62L ⁺ basophils	2.7-fold reduced in aged mice	2.5-fold reduced in aged mice
	Peritoneal eosinophils	3.1-fold elevated in young mice	Unaltered in young mice
Other disease indicators	Mac-1 ⁺ myeloid cell expansion	Unaltered in young mice, 6-fold elevated in aged mice	2-fold elevated in young mice, 12-fold elevated in aged mice
	CD69 ⁺ T cells	1.7-fold elevated in aged mice	2.6-fold elevated in aged mice
	CD62L ⁺ T cells	1.4-fold elevated in aged mice	2-fold elevated in aged mice
	Tregs	1.2-fold elevated in 16-wk-old mice, 2.1-fold elevated in 30-wk-old mice	1.5-fold elevated in 16-wk-old mice, 2.4-fold elevated in 30-wk-old mice
	MHC-II ⁺ B cells	1.6-fold elevated in young mice, 2.4-fold elevated in aged mice	Unaltered in young mice, 2.5-fold elevated in aged mice

the total leukocyte population [44]. $\text{Lyn}^{-/-}$ mice have elevated numbers of basophils in the blood and peritoneal cavity [9]. We have also found increased numbers in DKO mice, although like $\text{Lyn}^{-/-}$ mice, this expansion is only two-fold. In our hands, the basophils of $\text{Lyn}^{-/-}$ and DKO mice have an immature phenotype expressing low levels of MHC-II and CD62L despite the elevation in total and autoreactive IgE. While we do not know the reasons for the discrepancy between studies, other than possible genetic background differences, our data suggests that basophil activation does not underlie the onset of Th2 responses or autoimmune responses in $\text{Lyn}^{-/-}$ and DKO mice. The increased numbers of basophils may actually be providing a survival signal to plasma cells, since it has recently been reported that basophils can support mouse plasma cell survival via production of IL-4 and IL-6 [45], two cytokines that are elevated in both $\text{Lyn}^{-/-}$ and DKO mice. Our data shows that DKO mice have impaired kidney function and glomerulonephritis by 30 wks of age, yet at this stage they only have very low levels of IgE auto-Abs in a milieu of very high levels of other autoreactive Ab isotypes. Thus it is difficult to see how IgE auto-Abs are a major contributor to disease in these mice, with our study suggesting that IgG auto-Abs remain the main driver of pathology. Given that autoimmune disease development in $\text{Lyn}^{-/-}$ mice is complex and involves B cells, autoantibody, inflammation and the eventual recruitment of T cells into the disease, it appears that the best way to define the cellular compartment(s) responsible for disease would be through the generation of conditional mice, where *Lyn* could be deleted from specific immune cell subsets such as B cells, macrophages or basophils.

There is direct evidence that IL-4 can induce lupus-like autoimmune disease. For example, constitutive expression of IL-4 leads to B cell hyperactivity, enhanced production of IgG1 and IgE and glomerulonephritis [46], while IL-4 neutralization or deficiency decreases auto-Ab production and renal disease [47,48]. Interestingly, removal of IgE or IL-4 from $\text{Lyn}^{-/-}$ mice can have significant effects on IgG-driven pathology [16]. However, this may well be due to direct or indirect effects on B cells. IL-4 was initially discovered as a B cell growth factor, and it is well known that IL-4 can rescue B cells from apoptosis and promote their survival.

Autoimmune diseases such as experimental allergic encephalomyelitis (EAE) and lupus nephritis have recently been associated with Th17 responses [49,50] and there is a report suggesting that $\text{Lyn}^{-/-}$ mice may have altered Th17 responses showing increased severity of EAE [51]. While we have been unable to detect Th17 cells in secondary lymphoid tissue of unchallenged $\text{Lyn}^{-/-}$ or DKO mice (unpublished observations), it remains possible that occult Th17 cells may be a component of the disease process at some stage in its diathesis. Deficiencies in Treg numbers or Treg function can also be a feature of human SLE and mouse lupus (reviewed in [52]). The consequence of Treg deficiency is exemplified in IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) disease [53] and by the scurfy mouse that rapidly develops systemic inflammation and autoimmunity [54]. The scurfy mouse has a truncation in the *Foxp3* gene essential for Treg development [55] and is characterized by a prominent Th2 inflammation [56]. However, in a background of STAT6 deficiency, disease is unabated, with deficiency of IFN- γ also having little effect [57]. This highlights the complexity of autoimmune disease and challenges the theory of mutual exclusivity of Th1/Th2 responses in immune system disorders. While a reduction in Tregs may be a possible mechanism underlying the accelerated disease onset and severity in DKO mice compared with $\text{Lyn}^{-/-}$ mice, we have found that this compartment expands more quickly in DKO mice. While these data are compatible with the finding that STAT6 inhibits Treg generation via suppression of *Foxp3* [30], the more likely explanation for the enhanced amplification of the Treg compartment in aged DKO mice

is simply to control early-onset disease. Nevertheless, in the extreme inflammatory environment they are unable to keep pace with the activation of effector T cells. Very interestingly, while IL-4 can inhibit the generation of FoxP3⁺ Tregs in a STAT6-dependent manner [30], in the *Lyn*-deficient environment this is clearly not the case since there is simultaneous expansion of Tregs (Fig. 5D, [18]) and increase in levels of IL-4 (Fig. 7, [31]).

Our data indicates that STAT6 deficiency on a $\text{Lyn}^{-/-}$ background suppresses spontaneous Th2 immunity in young mice but leads to early onset autoimmune disease and atopy in older mice. It is of note that current therapies in the treatment of asthma are designed to target the Th2 pathway via inhibition of IL-4/13 or STAT6 signaling. These types of strategies should now be viewed with caution, as they could lead to severe unwanted autoimmune responses or atopy in intrinsically susceptible individuals. Our data may also prove to be very informative for understanding new mechanisms of asthma other than those classically linked to the STAT6-dependent Th2 pathway. Clinical studies with reagents that should inhibit Th2 immunity by blocking IL-4 and IL-13 have proven to be of limited usefulness [58]. The most promising of these, an antibody directed against IL-13 (Lebrikizumab), was found to significantly improve asthma but only in a small subset of patients with high blood levels of periostin, a biomarker induced by IL-13 [59]. Interestingly, a recent study has shown that clinical asthma can be divided into two distinct molecular phenotypes defined by degree of Th2 inflammation, and suggest that the Th2 pathway is only a relevant target for a subset of patients [60]. This weakens the hypothesis that inhibitors of STAT6 will prove to be useful anti-asthma drugs, thus opening a new era of research into alternative mechanisms of disease.

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Authorship contributions

ML, ET, GPA and MLH designed research; ML, ET and MJM performed research; ML, ET, MJM, DMT, GPA and MLH analysed data; ML, ET and MLH wrote the paper; and MJM, DMT and GPA provided editorial comment.

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