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BASOPHILS AND THE T HELPER 2 ENVIRONMENT CAN PROMOTE THE DEVELOPMENT OF LUPUS NEPHRITIS

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Summary

In systemic lupus erythematosus (SLE) self-reactive antibodies can target the kidney (lupus nephritis) leading to functional failure and possible mortality. We report that activation of basophils by autoreactive IgE, causes their homing to lymph nodes, promoting T_H2 cell differentiation, and enhancing the production of self-reactive antibodies that cause lupus-like nephritis in *Lyn*$^{-/-}$ mice. SLE patients also have elevated serum IgE, self-reactive IgE's, and activated basophils that express CD62L and the MHC Class II molecule, HLA-DR; parameters that were found to be associated with increased disease activity and active lupus nephritis. Basophils were also present in the lymph nodes and spleen of SLE patients. Thus, in *Lyn*$^{-/-}$ mice, basophils and IgE autoantibodies amplify autoantibody production that leads to lupus nephritis, and in SLE patients, the presence of IgE autoantibodies and activated basophils are factors associated with disease activity and nephritis.

Systemic lupus erythematosus (SLE) is a complex disease affecting various organs and may result in death when kidney damage (lupus nephritis) is severe1,2. Lupus nephritis is characterized by IgM-, IgG- and IgA-containing immune complexes deposited in the glomeruli. These immune complexes are formed by autoantibodies with specificity to nuclear components (ANA) or to nucleic acids (double stranded DNA (dsDNA)). However, little is known about how B cells are activated in SLE and thus, increased understanding of this process may uncover novel therapeutic strategies.

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While there is considerable evidence for the role of T_H1, T_H17, and regulatory T cells (Tregs) in SLE3–10, several studies suggest a possible T_H2 contribution11–13. As a disease with a strong humoral response14,15, it seems reasonable that SLE may have a T_H2 component and increases in immunoglobulin E (IgE) as well as the presence of autoreactive IgE in the sera of some SLE patients have been reported16, without associated increased atopy or allergy. Nonetheless, there is considerable uncertainty as to whether T_H2 cytokines (like IL-4) and IgE contribute in SLE and what cell type might be responsible for such contribution.

We and others17–19 have previously reported that mice deficient in the Src family protein tyrosine kinase Lyn (*Lyn*$^{-/-}$) developed a strong and constitutive T_H2 skewing in early life and show exacerbated responses to T_H2 challenges. In late life, *Lyn*$^{-/-}$ mice develop an autoimmune disease that mimics some of the features of human SLE20–22. *Lyn*$^{-/-}$ mice have circulating autoantibodies to double stranded DNA (dsDNA) and other nuclear antigens (ANA). A marked glomerular deposition of circulating immune complexes (CIC) is seen, which results in kidney damage and ultimately in death. Interestingly, a genetic association of Lyn with SLE, in a European-American population, was recently reported23. Additionally, B cells from some SLE patients have also been found to express reduced levels of Lyn kinase24. Thus, *Lyn*$^{-/-}$ mice provide a reasonable model to explore the influence of a T_H2 environment on the development of lupus-like nephritis.

Given the aforementioned uncertainty, we explored whether the T_H2 skewing of *Lyn*$^{-/-}$ mice plays a role in the development of late life lupus-like nephritis and whether similar characteristics might be seen in SLE patients. We found that the T_H2 phenotype is a contributory factor in the development of lupus-like nephritis in *Lyn*$^{-/-}$ mice and is also associated with lupus nephritis in human SLE. Importantly, the findings identify basophils and self-reactive IgE as key components that play a role in the development of autoantibody-mediated kidney disease.

Results

IL-4- and IgE-dependent lupus-like nephritis in *Lyn*$^{-/-}$ mice

Consistent with our prior results18, basophil-dependent T_H2-skewing was still present in aged *Lyn*$^{-/-}$ mice (Supplementary Figure 1) that develop an SLE-like disease. To study the importance of the T_H2 environment in the development of the SLE-like phenotype, mice deficient in both IgE and Lyn (*Igh7*$^{-/-}$*Lyn*$^{-/-}$), IL-4 and Lyn (*Il-4*$^{-/-}$*Lyn*$^{-/-}$), as well as mast cells and Lyn (*Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$) were used18. *Igh7*$^{-/-}$*Lyn*$^{-/-}$, *Il-4*$^{-/-}$*Lyn*$^{-/-}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice developed a peripheral B cell defect that was comparable to *Lyn*$^{-/-}$ mice and showed hyper IgM and IgA levels in the serum (Supplementary Fig. 2−6), demonstrating that IL-4 or IgE were not involved in these abnormalities. As shown in Supplementary Fig. 5, the level of IgE and IgG isotypes in *Igh7*$^{-/-}$*Lyn*$^{-/-}$ and *Il-4*$^{-/-}$*Lyn*$^{-/-}$ mice were associated with the phenotype reported for *Igh7* and *Il-4* single deficient mice25,26 and differed from *Lyn*$^{-/-}$ mice phenotype. IgE also contributed to the previously reported17 increase in mast cell numbers seen in *Lyn*$^{-/-}$ mice (Supplementary Fig. 7), consistent with a role for IgE in mast cell survival27,28. In contrast, the previously

described basophilia in *Lyn*$^{-/-}$ mice was independent of both IL-4 and IgE18 (Supplementary Fig. 7).

Unlike *Lyn*$^{-/-}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice, *Igh7*$^{-/-}$*Lyn*$^{-/-}$ and *Il-4*$^{-/-}$*Lyn*$^{-/-}$ mice did not develop glomerulonephritis (Fig. 1a,b **and** Supplementary Fig. 8). Glomerular deposits of circulating immune complexes (CIC) containing IgG (Fig. 1c), IgM, IgA and complement factor 3 (C3) (Supplementary Fig. 9a–c) were markedly reduced in the kidney's of *Igh7*$^{-/-}$*Lyn*$^{-/-}$ and *Il-4*$^{-/-}$*Lyn*$^{-/-}$ mice, but were still present in the kidney's of *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ at comparable levels to *Lyn*$^{-/-}$ mice (Fig. 1c **and** Supplementary Fig. 9a–c). Kidney function (as measured by the albumin/creatinine ratio (ACR) in the urine) was rescued in *Igh7*$^{-/-}$*Lyn*$^{-/-}$ and *Il-4*$^{-/-}$*Lyn*$^{-/-}$ mice, whereas the ACR was similarly elevated in both *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ and *Lyn*$^{-/-}$ mice (Fig. 1d). These findings show that the lupus-like nephritis observed in *Lyn*$^{-/-}$ mice is dependent on IgE and IL-4, but is independent of mast cells.

Basophils support autoreactive plasma cells in *Lyn*$^{-/-}$ mice

Aged *Lyn*$^{-/-}$ mice produce large amounts of autoantibodies against dsDNA and nuclear antigens (Fig. 2a, b), which are at the origin of the damage seen in the kidney29,30. We explored if the recovery of kidney function in *Igh7*$^{-/-}$*Lyn*$^{-/-}$ and *Il-4*$^{-/-}$*Lyn*$^{-/-}$ mice was associated with a concomitant decrease in autoantibody production and found a two fold decrease in anti-dsDNA and ANA when compared to *Lyn*$^{-/-}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice (Fig. 2a, b). Depletion of basophils in aged *Lyn*$^{-/-}$ mice (>32 weeks) or in younger *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice (~20 weeks) showed a marked reduction in ANA autoantibodies (Fig. 2c, d). Loss of basophils also decreased the proportion of plasma cells in the spleen (Fig. 2e) and reduced the pro-inflammatory environment in the kidney (Fig. 2f **and** Supplementary Fig. 10). Collectively, the findings show that basophils support plasma cells in the spleen and amplify the production of autoantibodies in an IL-4 and IgE-dependent manner, leading to a pro-inflammatory environment and kidney disease in *Lyn*$^{-/-}$ mice.

Lyn$^{-/-}$ mice produce basophil-activating self-reactive IgE

Our findings showed the IgE dependence of the SLE-like phenotype, thus we investigated if self-reactive IgE that might activate FcεRI-bearing basophils could be found in the circulation of these mice. Sera from *Lyn*$^{-/-}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice had high levels of IgE anti-dsDNA (Fig. 3a) and IgE anti-ANA (data not shown) as compared to their WT counterparts. The presence of self-reactive IgEs was reduced in *Il-4*$^{-/-}$*Lyn*$^{-/-}$ mice and as expected were not detected in *Igh7*$^{-/-}$*Lyn*$^{-/-}$ mice (Fig. 3a). CIC were purified as previously described31 and IgE-containing CIC (IgE-CIC) were found in varying amounts in all the sera from *Lyn*$^{-/-}$ and *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice (Fig. 3b **and** Supplementary Fig. 11a,d), whereas the sera of *Il-4*$^{-/-}$*Lyn*$^{-/-}$ and *Igh7*$^{-/-}$*Lyn*$^{-/-}$ mice was essentially void of IgE-CIC (Fig. 3b **and** Supplementary Fig. 11d). IgG-containing (IgG-CIC)- as well as IgM and IgA containing- CIC were observed in all the mutant strains of mice, but a marked reduction of these CICs was also observed in *Il-4*$^{-/-}$*Lyn*$^{-/-}$ and *Igh7*$^{-/-}$*Lyn*$^{-/-}$ mice, correlating with the reduced amount of autoantibodies found in these mice (Fig. 3c **and** Supplementary Fig. 11b, c, e). We next examined if IgE or IgG ICs could stimulate basophil IL-4 production. While IgG-ICs failed to stimulate basophil IL-4 production, IgE-ICs were able to induce IL-4

production by basophils (Fig. 3d **and** Supplementary Fig. 12a, b). Moreover, basophils from *Lyn*$^{-/-}$ mice showed increased sensitivity to IgE-ICs as compared to their WT counterparts (Fig. 3d **and** Supplementary Fig. 12a). Importantly, all of the stimuli tested (PMA/Ionomycin, IgE-antigen (Ag), IgE-ICs and IgG-ICs) failed to induce IL-12p40 or IFN-γ production by basophils from WT or *Lyn*$^{-/-}$ mice (Supplementary Fig. 12c, d). The findings demonstrate that the presence of IgE-ICs (which are present as circulating IgE CICs in *Lyn*$^{-/-}$ mice) can lead to basophil activation and selective T_H2 cytokine expression.

Lyn$^{-/-}$ basophils express immunoregulatory molecules

We next explored if basophils can home to the secondary lymphoid tissues of *Lyn*$^{-/-}$ mice where they might influence B and T cell responses. Circulating basophils from *Lyn*$^{-/-}$ mice showed increased expression of CD62L (L-Selectin) (Fig. 4a), which allows for the homing of leukocytes to secondary lymphoid tissues. In the context of Lyn-deficiency, the absence of IL-4 or IgE (*Il-4*$^{-/-}$*Lyn*$^{-/-}$ and *Igh7*$^{-/-}$*Lyn*$^{-/-}$ mice), but not of mast cells (*Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice), inhibited the expression of CD62L on circulating basophils (Fig. 4b). As shown in Fig. 4c and d, *Lyn*$^{-/-}$ mice had high levels of basophils in both the lymph nodes (cervical and inguinal) and spleen. In the lymph nodes, the constitutive presence of basophils was markedly reduced when IL-4 or IgE were also absent (*Il-4*$^{-/-}$*Lyn*$^{-/-}$ and *Igh7*$^{-/-}$*Lyn*$^{-/-}$ mice) but not when mast cells where absent (*Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$ mice) (Fig. 4c). Some reduction was also seen in the spleen but it was not as marked as in the lymph nodes (Fig. 4d). As expected, due to the basophilia seen in the absence of Lyn, no change in the proportion of circulating basophils was observed for any of the strains studied (Fig. 4e). We also found that lymph node resident basophils expressed membrane associated BAFF (Fig. 4f), which was not accounted for by the low levels of BAFF receptor expressed on these cells (data not shown), demonstrating the potential of lymph node resident basophils to influence B cell survival and differentiation. Moreover, both lymph node- (Fig. 4g) and spleen- (Supplementary Fig. 13) localized basophils from *Lyn*$^{-/-}$ mice showed increased MHC II expression. These findings demonstrate that *Lyn*$^{-/-}$ basophils upregulate CD62L expression and home to the lymph nodes and spleen, where increased expression of MHC II32–34 and/or BAFF may allow communication with T and B cells.

Self-reactive IgE is associated with SLE and lupus nephritis

The cohort of SLE patients analyzed had large amounts of C1q-reactive CIC that can fix complement (Fig. 5a), as previously described2,30. When analyzed relative to disease activity (based on SLEDAI score)35, CIC (C1q) is strongly elevated in mild (SLEDAI of 1.0–4.0) and active disease (SLEDAI of > 4.0). SLE patients also had self-reactive IgEs recognizing dsDNA and their levels were associated with increased disease activity (Fig. 5b). IgG directed towards IgE (IgG anti-IgE) was also present in the sera of SLE patients (Fig. 5c) with significantly elevated levels in patients with active disease. IgE anti-dsDNA levels were found to be highly associated with active lupus nephritis (Fig. 5d). Moreover, total IgE levels were increased in patients and were associated with disease activity, and patients showed a modest to strong IgG1, IgG3 and IgE autoantibody response (Supplementary Fig. 14a–c). Thus, the findings show that SLE patients have both T_H1 and T_H2 autoantibodies and self-reactive IgE's and IgG anti-IgE antibodies that are associated with increased disease activity and active nephritis.

SLE basophils express HLA-DR and home to lymphoid tissues

To investigate the activation state of basophils in SLE patients the marker CD203c was used as its expression is upregulated in activated basophils36. All SLE patients showed increased CD203c expression, relative to healthy controls, indicating that their basophils are active (Fig. 6a). CD62L expression was also increased on SLE basophils, and this was associated with increased disease activity (Fig. 6b). Expression of HLA-DR$^+$ was also enhanced on SLE basophils (Fig. 6c, **inset**). This suggested increased homing of SLE basophils to the secondary lymphoid tissues. As shown in Fig. 6d, the absolute numbers of basophils in the circulation decreased in SLE patients. While this decrease was associated with immunosuppressive treatment (IST) (Supplementary Fig. 15), IST had no effect on the activation of basophils (as indicated by HLA-DR$^+$). Importantly, basophils were found in the lymph nodes and spleen of the two SLE patients tested, but not of normals (Fig. 6e, f). The findings show that basophils in SLE patients are activated, and home to secondary lymphoid organs and express the appropriate molecules to present antigen. This is associated with the presence of self-reactive IgE in SLE patients.

Discussion

While SLE has long been considered a B cell disease, self-reactive T cells that promote B cell class switching37 and other cell types like dendritic cells, macrophages, etc.38,39 have also been implicated, for example, through secretion of factors influencing B cell survival and differentiation, such as BAFF and APRIL40. Here we demonstrate that the basophil is a key contributor to the production of self-reactive antibodies in SLE. Our findings in *Lyn*$^{-/-}$ mice demonstrate that depletion of basophils or the absence of IL-4 or IgE caused a marked reduction in autoantibody production and preserved kidney function. This suggests that without basophils the levels of autoantibodies are insufficient to cause kidney disease. Thus, basophils function to amplify the pre-existing loss of B cell tolerance.

Basophils have long been associated with allergy41,42. However, the role of the basophil in immunity has long been unclear. The recent discovery that basophils can induce T_H2 cell differentiation *in vivo*18,43, amplify humoral memory responses44, and present antigen via MHC II32,34,43, provides evidence of a role for this cell type in regulating T_H2 immunity. In the *Lyn*$^{-/-}$ mouse model, T_H2-skewing is driven by the absence of Lyn kinase in the basophil, upregulating GATA-3 expression in these cells leading to copius production of IL-4 *in vivo*18. In humans, our preliminary analysis of the Lyn content in the basophils of SLE patients did not reveal significant differences relative to healthy controls (data not shown). However, there is increasing evidence of a role for Lyn kinase in SLE, particularly in populations of European descent23,24. Thus, further studies are required to determine the role of Lyn in human SLE.

Of particular interest is the finding that basophils contribute to the production of autoantibodies that cause lupus-like nephritis in the *Lyn*$^{-/-}$ mice. Activation of these cells caused enhancement of CD62L expression and their accumulation in the lymph nodes of *Lyn*$^{-/-}$ mice and SLE patients. MHC II expression on mouse and human basophils was increased, and in the mouse, expression of membrane-bound BAFF was observed, similar to what has been described in human basophils after engagement of IgD on their cell

surface45. Depletion of these cells in *Lyn*$^{-/-}$ mice decreased splenic plasma cells and suppressed autoantibody production, which drives lupus nephritis29,30. Depletion of basophils also reduced the production of IL-1β, IL-4, IL-6, IL-13, and IFN-γ in the kidney of *Lyn*$^{-/-}$ mice. Thus, a reduction in the pro-inflammatory environment in the kidney suggests a possible therapeutic benefit from basophil inactivation or depletion.

Our findings show that IgE-ICs can activate basophils, and removal of self-reactive IgEs that form functional CICs (by deletion of the *Igh7* locus or by eliminating IL-4 production) ablated kidney disease. These IgE-CICs were also associated with lupus nephritis in both *Lyn*$^{-/-}$ mice as well as in SLE patients. Given that circulating IgE levels can be reduced by an existing anti-allergy drug, omalizumab (Xolair®, Genentech), an anti-IgE antibody that functions to reduce circulating IgE levels and can cause decreased FcεRI expression on basophils46, a treatment with potential therapeutic benefit may already exist. While in SLE patients the association of increased levels of IgE anti-dsDNA antibodies with increased disease activity and active lupus nephritis strongly argues for a link between increased T_H2 responses and development of nephritis in these patients, it is clear that T_H1-mediated responses are also found in this patient population. The presence of increased circulating IgG1 and IgG3 autoantibodies demonstrates a strong T_H1 component. This suggests that direct modulation of the T_H2 response, such as by the use of IL-4 and IL-13 receptor antagonists47, as a therapeutic strategy could have the unwanted effect of exacerbating disease by shifting towards a T_H1 (or possibly T_H17) phenotype. Nonetheless, as we did not find the presence of IgE-CIC in the kidney's of *Lyn*$^{-/-}$ mice (data not shown), it appears that these CICs do not contribute to the kidney pathology per se but instead are important in basophil activation. Thus, perhaps, the strategy of IgE or basophil depletion may avoid the complications of altering the T_H1/T_H2 balance.

The view of SLE as a disease with a T_H2 component has been controversial. There is considerable evidence for involvement of T_H1 and possibly T_H17 cells in SLE11,12,48–50 as well as for the alteration or loss of regulatory T cell (Treg) activity9,51. Some mouse models of spontaneous SLE, like BXSB and MRL-*Fas*^{lpr} show increases in the T_H1 cytokine IFN-γ as linked to the expression of the T_H1 mediated isotypes, IgG2a and IgG3, and deletion of the IFN-γ gene in the context of these backgrounds was shown to eliminate disease4,49. Nonetheless, it is less well known that many of the spontaneous mouse models (*NZB*, *NZW*, *BXSB*, and *MRL*/*Fas*^{lpr}) of lupus-like disease have high circulating levels of IgE52, suggesting the possibility of a contributory role for a T_H2 component in these models. SLE patients showed both T_H1 and T_H2 responses and both IgG-CIC's and IgE-CIC's were associated with increased disease activity. Several studies have suggested that the balance of T_H1 and T_H2 cell responses may determine the phenotype of lupus nephritis3,11–13,48. A strong T_H1 response was shown to be associated with diffuse proliferative lupus nephritis whereas a dominant T_H2 response was associated with a membranous lupus nephritis. These observations argue that both T_H1 and T_H2 responses can contribute to lupus nephritis but the disease may manifest differently depending on the dominance of one or the other response.

Our findings show that basophils and the T_H2 environment influences the production of autoantibodies and that the depletion of basophils or deletion of the *Igh7* or *Il-4* gene, in the

context of Lyn-deficiency, caused a reduction in the circulating levels of these self-reactive antibodies. In SLE patients, self-reactive IgE is associated with active disease and active lupus, and basophils are active and were found in the secondary lymphoid tissues, of two tested individuals, where they can influence T and B cell function. Thus, our findings suggest the possibility that reduction of the circulating levels of self-reactive IgE or the dampening of basophil activity could have therapeutic benefit in lupus nephritis.

Online Methods

Mice

All animals used in the present study were described previously18. Unless otherwise noted, mice were aged for 32–40 weeks and were aged matched for group comparisons. Mice were maintained in specific pathogen-free conditions and used in accordance with NIH guidelines and NIAMS–approved animal study proposal A007-03-01.

Patients

Patient samples were collected from adult patients enrolled in a long term natural history study of systemic lupus erythematosus (SLE). The study was approved by the Institutional Review Board of NIAMS. All patients provided written informed consent. All patients fulfilled the American College of Rheumatology classification criteria for SLE53,54. Patient characteristics and lupus activity scoring system are shown in Supplementary Table 1 and Supplementary Methods. Control samples were obtained from random healthy blood donors.

Antibodies and flow cytometry

DNP-specific mouse IgE was produced as previously described55. All other antibodies were from commercial sources and are described in Supplementary Table 2. Flow cytometry acquisition was done with a FACSCalibur (BD Biosciences) as previously described18. Data analysis was with Flowjo software (Treestar Inc.).

In vivo basophil depletion and *ex vivo* analysis of splenic T cells

In vivo basophil depletion and *ex vivo* analysis of splenic T cells ($CD4^+$) were previously described18.

Glomerulonephritis, analysis of glomerular deposition of CIC, and kidney function

Aged (~40 week old) mice were euthanized, kidneys were removed. One kidney was fixed with 10% buffered formalin (Sigma), embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) (American Histolabs). The other kidney was placed in a vinyl mold in OCT medium and the sample was frozen in liquid nitrogen. Four-micrometer-thick frozen sections were fixed in cold acetone, blocked in PBS containing 1% bovine serum albumin (BSA) and stained in the same buffer with the specific fluorescein-conjugated antibodies or isotype controls (see Supplementary Table 2 for antibodies used).

For assessment of kidney function the albumin/creatinine ratio (ACR) was determined. Urine was collected from at least ten aged mice per genotype and the albumin concentration was measured with a mouse albumin ELISA (Bethyl laboratories). A creatinine assay (R&D

systems) was used to determine urine creatinine concentrations. Results are expressed as ACR in μg of albumin per mg of creatinine.

Methods for assessment of glomerulonephritis and cytokine content in the kidney are described in Supplementary Methods.

Measurement of autoantibodies, CIC, and precipitation of CIC

Mouse anti-dsDNA IgG, mouse ANA IgG and mouse circulating immune complexes (CIC (C1q) IgG+A+M) ELISA kits were from ADI. ELISA for human circulating immune complexes (C1q coated plates) was from ALPCO and for human IgE was from Mabbiotech. All commercial ELISA's were performed according to the manufacturer's instructions. To measure both human and mouse, anti-dsDNA IgE and anti-dsDNA IgG subclasses, dsDNA coated plates (Calbiotech) were incubated with serial dilutions of serum in PBS containing 10% FCS (Invitrogen). The corresponding HRP-conjugated secondary antibodies were used (see Supplementary Table 2). Optical density at 450 nm was measured after TMB substrate incubation (Invitrogen). Data shown are from 1:200 dilution plates (where the best signal to noise ratio was obtained). The same approach was used to measure the levels of circulating anti-IgE IgGs in patient and healthy controls, using plates coated with human IgE (Abbiotec) at 2 μg ml^{-1} in PBS.

Circulating immune complexes (CIC) were precipitated from sera of aged mice as described previously31. Samples were analyzed by SDS-PAGE followed by Western blot using the indicated antibodies (see Supplementary Table 2). The LiCor Odyssey System was used to detect signal.

Basophil cultures, basophil detection, and measurement of IL-4 production

Bone marrow derived cultured basophils were previously described18. At day nine of culture, cells were washed, resuspended at one million cells per ml in medium containing only IL-3 and incubated overnight at 37°C. Cells were then resuspended in the same medium at five million cells per ml and stimulated as indicated. For IgE and antigen (IgE +Ag) stimulation, cells were sensitized with 1 μg ml^{-1} of IgE anti-DNP for 30 min, washed and then stimulated with 20 ng ml^{-1} of DNP-HSA (Sigma). For IgE-IC and IgG-IC stimulations, IgE- or IgG-containing immune complexes were prepared by incubating either IgE and anti-mouse IgE or IgG1 and anti-mouse IgG1 at 1:2 ratio for 30 min at 37°C (see Supplementary Table 2). The indicated concentration was then added to the cells for 4 hrs at 37 °C. Two hours prior to the end of this incubation, 10 μM monensin were added to the cells. Intracellular staining was previously described18.

Immunohistochemistry for basophil detection was performed as previously described56,57.

Statistical Analysis

For comparisons between two populations an unpaired two-tailed student *t* test was performed, unless otherwise specified. When three or more populations were compared, a one way ANOVA test was first performed and if significance was reached ($p<0.05$) an

unpaired two-tailed student *t* tests was performed between each compared population, unless otherwise indicated. Statistical analysis was performed using GraphPad Prism 5.01 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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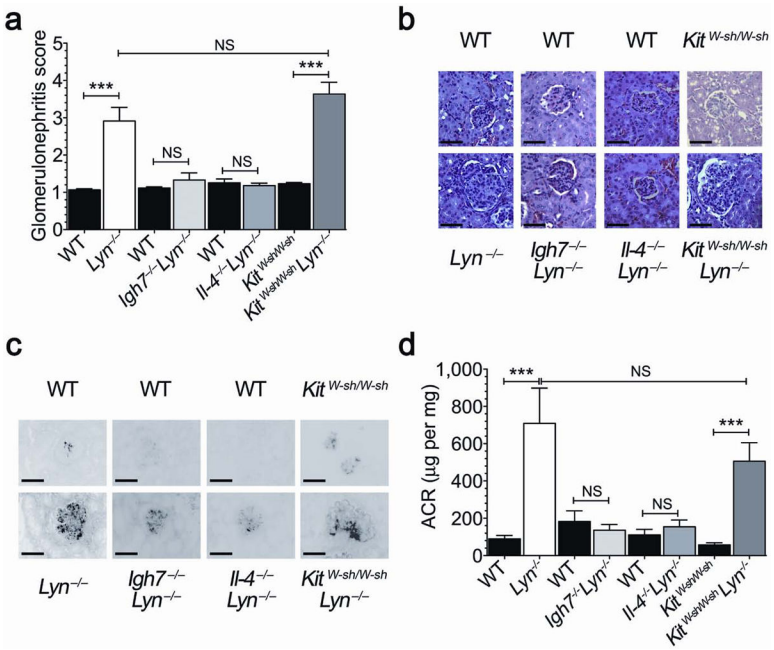

Figure 1.

The lupus-like nephritis of *Lyn*$^{-/-}$ mice is IL-4 and IgE dependent. (**a**) H&E stained histological kidney sections from aged mice (over 40 weeks) of the indicated genotype were scored for glomerulonephritis as indicated in methods. Data shown as means ± s.e.m (WT & *Lyn*$^{-/-}$: n= 8; WT & *Igh7*$^{-/-}$*Lyn*$^{-/-}$: n=6; WT & *Il-4*$^{-/-}$*Lyn*$^{-/-}$: n= 4 and 5; *Kit*$^{W\text{-}sh/W\text{-}sh}$ & *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$: n= 11). Statistical analysis was by a two tailed unpaired student *t* test; ***: *p<0.001*; NS: not significant. (**b**) Representative glomeruli in H&E stained histological kidney sections of aged mice (40 weeks old) with the indicated genotype. Scale bar, 50 µm. (**c**) Immunofluorescent detection of glomerular IgG deposits in aged mice (40 weeks) of indicated genotypes after staining with fluorescein-conjugated anti-mouse IgG. Scale bar, 50 µm. (**d**) Albumin/Creatinine ratio (ACR) measured in the urine of at least 15 aged mice (40 weeks) of the indicated genotype. Data are means ± s.e.m. Statistical analysis was by a two tailed unpaired student *t* test; ***: *p<0.001*; NS: not significant.

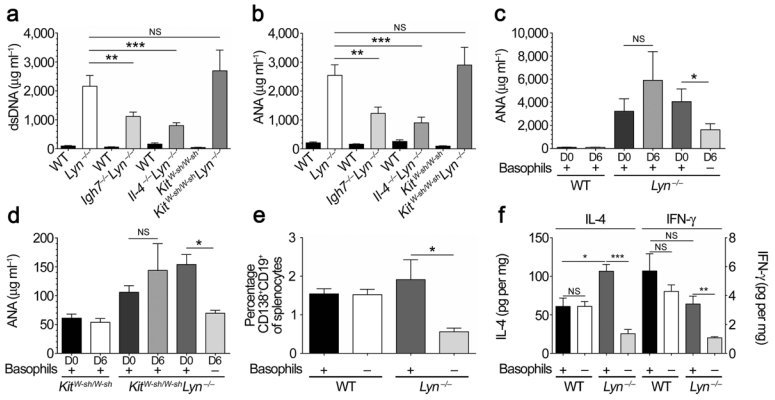

Figure 2.

IgE, basophils, and IL-4 regulate autoantibody production in *Lyn*$^{-/-}$ mice and basophils promote the kidney cytokine environment. (**a**) Quantitation of IgG anti-dsDNA in the serum of aged mice (40 weeks) of the indicated genotype. Data are means ± s.e.m (at least 15 mice per group). (**b**) Quantitation of IgG anti-nuclear antigen (ANA) in the above mice. (**c**) Quantitation of IgG ANA autoantibodies in the serum of aged mice (32 weeks) of the indicated genotype before (D0) and six days after (D6) injection of the basophil depleting antibody MAR-1 (−) or isotype control (+). Data are means ± s.e.m (WT: n= 3; *Lyn*$^{-/-}$ (+): n=4; *Lyn*$^{-/-}$ (−): n=5). (**d**) Same as (**c**) for the serum of mice (20 weeks-old) of the indicated genotype. Data are means ± s.e.m (for each group, n=3). (**e**) Proportion of splenic CD138^{+}CD19^{+} plasma cells determined by flow cytometry in mice six days after basophil-depletion (−) or isotype injection (+). (**f**) Quantitation of IL-4 (left) and IFNγ (right) in kidney homogenates from 40 weeks old WT and *Lyn*$^{-/-}$ mice six days after basophil depletion (−) or isotype injection (+). Cytokine amounts were normalized to the total protein content of the respective homogenates. (**e**,**f**) Data are means ± s.e.m (WT and *Lyn*$^{-/-}$, at least n=4 per group). Statistical analysis was by a two tailed unpaired (**a**,**b**,**e**,**f**) or paired (**c**,**d**) student *t* test; *: *p<0.05*; **: *p<0.01*; ***: *p<0.001*; NS: not significant.

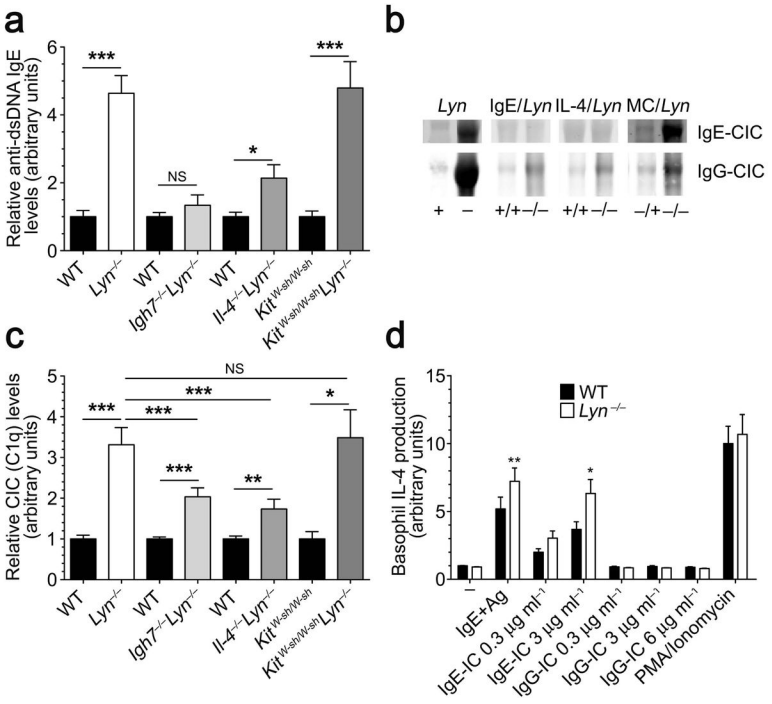

Figure 3.

Autoreactive IgE and IgE-circulating immune complexes (IgE-CIC) are present in the sera of aged *Lyn*$^{-/-}$ mice. (**a**) Anti-dsDNA IgE in the sera of aged mice (40 weeks), of the indicated genotype, was determined by semi-quantitative ELISA. Data are means ± s.e.m (> ten mice per group) normalized to the respective WT and expressed as arbitrary units. Statistical analysis was by a two tailed unpaired student *t* test; *: *p<0.05*; ***: *p<0.001*; NS: not significant. (**b**) IgE- and IgG-CIC were PEG-precipitated from serum samples of aged animals (>30 weeks) of the indicated genotype. The precipitated CIC were submitted to SDS-PAGE, transferred to nitrocellulose, and probed with anti-mouse IgE or anti-mouse IgG. One representative of at least ten mice per genotype is shown. (**c**) Serum levels of CIC (IgA+IgM+IgG) were determined by semi-quantitative ELISA from at least ten aged mice per genotype on complement factor 1q (C1q) coated plates. Data are means ± s.e.m normalized to levels in WT mice and reported as arbitrary units. Statistical analysis was by a two tailed unpaired student *t* test; *: *p<0.05*; **: *p<0.01*; ***: *p<0.001*; NS: not significant. (**d**) Basophil (bone marrow-derived) IL-4 production induced by the indicated stimuli. IL-4 production is expressed as the relative mean fluorescence intensity (MFI) detected by intracellular staining. The MFI was normalized to the unstimulated (−) control response. Data are means ± s.e.m. (n=6 per condition for three independent experiments). Statistical analysis was by using a two tailed paired student *t* test; *: *p<0.05*; **: *p<0.01*.

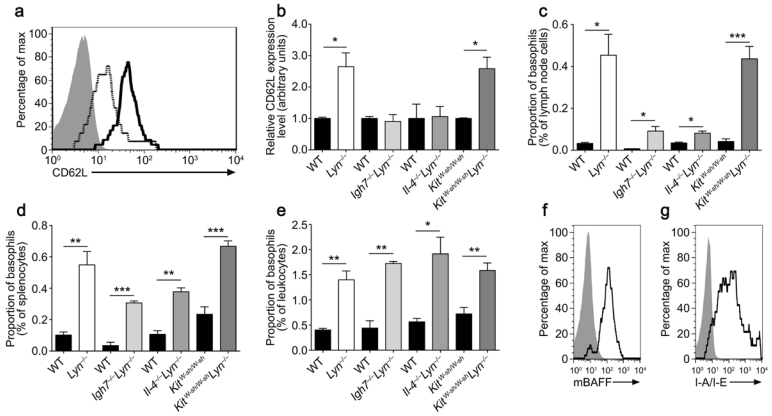

Figure 4.

Basophils from aged *Lyn*$^{-/-}$ mice upregulate CD62L expression, home to secondary lymphoid tissues, and express membrane BAFF and MHC II. (**a**) Representative flow cytometric analysis of blood basophil CD62L expression in aged (40 weeks) WT (grey dashed line) and *Lyn*$^{-/-}$ mice (black line) relative to isotype control (grey fill). (**b**) Compilation of all experiments as in (**a**) from aged mice of the indicated genotype. Data are the mean fluorescence intensity (MFI) of CD62L expression on blood basophils normalized to corresponding WT controls expressed as means ± s.e.m (WT & *Lyn*$^{-/-}$: n= 4 and 7; WT & *Igh7*$^{-/-}$*Lyn*$^{-/-}$: n=3; WT & *Il-4*$^{-/-}$*Lyn*$^{-/-}$: n= 3; *Kit*$^{W\text{-}sh/W\text{-}sh}$ & *Kit*$^{W\text{-}sh/W\text{-}sh}$*Lyn*$^{-/-}$: n= 4 & 7). Statistical analysis was by a two tailed unpaired student *t* test; *: $p<0.05$. (**c–e**) Flow cytometric analysis of basophils (defined as FcεRI^{+} CD11b^{+} CD49b^{+} cells) in lymph nodes (cervical and inguinal) relative to the total cell number (**c**), spleen (**d**), blood (**e**), of the indicated mice strains. (**f, g**) Representative flow cytometric analysis of basophil membrane BAFF (**f**) or MHC II (I-A/I-E) (**g**) expression in the lymph nodes of *Lyn*$^{-/-}$ mice (black line) relative to isotype control (grey fill).

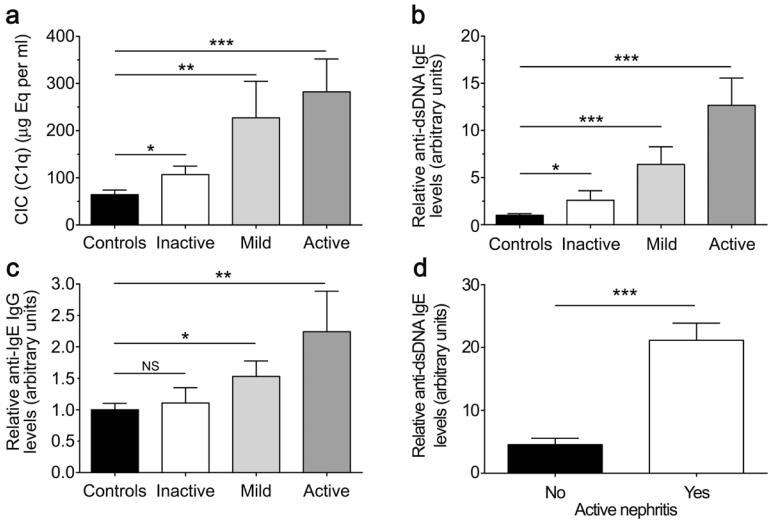

Figure 5.

IgE anti-dsDNA and IgG anti-IgE are associated with human SLE disease activity and lupus nephritis. (**a**) Total CIC's in serum from healthy controls (n=37), inactive SLE patients (SLEDAI=0) (n=13), patients with mild disease (SLEDAI 2.0 to ≤4.0) (n=15), and patients with active disease (SLEDAI >4) (n=15) were measured by ELISA. Data are means ± s.e.m. Statistical analysis was by a two tailed unpaired student *t* test; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. (**b**) IgE anti-dsDNA was determined by semi-quantitative ELISA. dsDNA-coated plates were incubated with sera from healthy controls and SLE patients (same populations as in (**a**)). Data are means ± s.e.m (same n as in (**a**)) normalized to healthy controls. Statistical analysis was by a two tailed unpaired student *t* test; *: $p<0.05$; ***: $p<0.001$; NS: not significant. (**c**) IgG anti-IgE levels were determined by incubating sera from healthy controls and SLE patients on human IgE-coated plates, and anti-IgE IgG was detected with anti-human IgG (Fcγ specific). Data are means ± s.e.m (same n as in (**a**)) normalized to healthy controls. Statistical analysis was by a two tailed unpaired student *t* test; **: $p<0.01$. (**d**) IgE anti-dsDNA in sera of SLE patients classified on the basis of active nephritis (Yes, n=8) or not (No, n=34) (see Supplemental Methods). Data are means ± s.e.m. Statistical analysis was by a two tailed unpaired student *t* test.

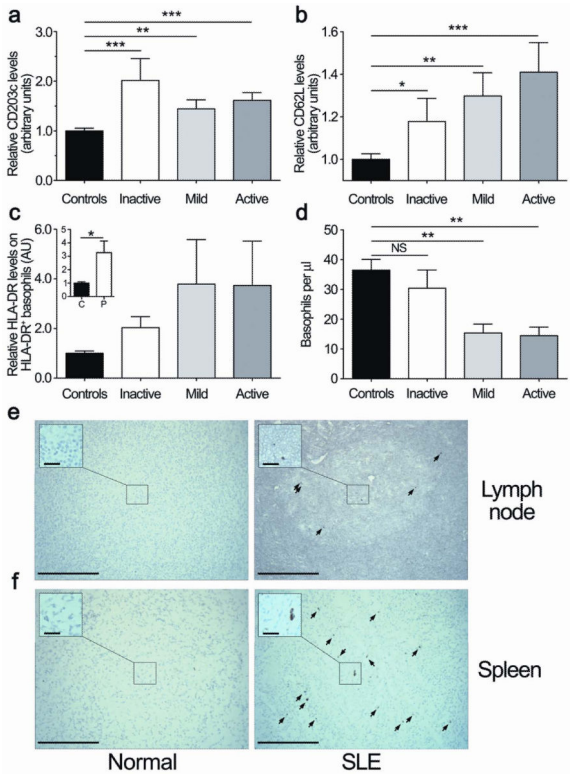

Figure 6.

Basophils in SLE patients are active, upregulate CD62L and HLA-DR, and home to secondary lymphoid organs. (**a**) Flow cytometric analysis of CD203c expression levels on blood basophils from healthy controls and inactive/mild/active SLE patients ((n=13/15/15) as described in Fig. 5a) relative to controls (n=41). Data are the ratio of CD203c mean fluorescence intensity (MFI) normalized to controls. (**b**) Same as in (**a**) showing expression of CD62L. Data are means ± s.e.m (healthy controls: n=14; SLE patients: inactive/moderate/active, n=4/6/6). (**c**) Flow cytometric analysis of relative HLA-DR levels on HLA-DR$^+$ blood basophils compared to healthy controls. Data are means ± s.e.m (healthy controls: n=13; SLE patients: inactive/mild/active n=4/6/6). (**d**) Absolute number of blood basophils in healthy controls (n=41) or inactive/mild/active SLE patients (n=13/15/15) as determined by flow cytometry. Data are means ± s.e.m. (**a–d**) Statistical analysis was by a two tailed unpaired student *t* test; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; NS: not significant. (**e, f**) Immunohistochemistry (with the 2D7 monoclonal antibody) of basophils in the lymph nodes (**e**) or spleen (**f**) of healthy (normal) controls or SLE patients (n=2). Basophils were found in the B cell zone of lymph node germinal centers for SLE patients only (**e**). A spleen biopsy from healthy (normal) controls or SLE patient shows the localization of basophils in the germinal centers of patients but not normals (**f**). Similar results were obtained with a second basophil specific antibody (BB1). Original magnification x20. Scale bar, 200 µm. (*inset*) Original magnification x40. Scale bar, 25 µm.