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CTLA4Ig Alters the Course of Autoimmune Disease Development in Lyn^−/− Mice

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Lyn-deficient (Lyn^−/−) mice develop an age-dependent autoimmune disease similar to systemic lupus erythematosus, characterized by the production of IgG anti-nuclear Ab. To determine the extent to which this autoimmune phenotype is driven by T cell costimulation, we generated Lyn^−/− mice expressing a soluble form of the T cell inhibitory molecule, CTLA4 (CTLA4Ig). Surprisingly, although CTLA4Ig prevented myeloid hyperplasia, splenomegaly and IgG anti-nuclear Ab production in Lyn^−/− mice, it did not inhibit immune complex deposition and tissue destruction in the kidney. In fact, regardless of CTLA4Ig expression, Lyn^−/− serum contained elevated titers of IgA anti-nuclear Ab, although generally IgA deposition in the kidney was only revealed in the absence of self-reactive IgG. This demonstrated that activation of autoreactive B cell clones in Lyn^−/− mice can still occur despite impaired costimulation. Indeed, CTLA4Ig did not alter perturbed Lyn^−/− B cell development and behavior, and plasma cell frequencies were predominantly unaffected. These results suggest that when self-reactive B cell clones are unimpeded in acquiring T cell help, they secrete pathogenic IgG autoantibodies that trigger the fulminant autoimmunity normally observed in Lyn^−/− mice. The absence of these IgG immune complexes reveals an IgA-mediated axis of autoimmunity that is not sufficient to cause splenomegaly or extramedullary myelopoesis, but which mediates destructive glomerulonephritis. These findings have implications for the understanding of the basis of Ab-mediated autoimmune diseases and for their treatment with CTLA4Ig. The Journal of Immunology, 2010, 184: 757–763.
cell costimulation and therefore impairing T cell-dependent B cell activation in transgenic mice (16). In keeping with this, transgene-derived CTLA4Ig has been reported to ablate B cell responses following immunization with a T cell-dependent Ag and to prevent humoral responses to allogeneic islet grafts (17, 18). In addition, CTLA4Ig is under trial for use as a therapeutic in human SLE patients (19). Constitutive exposure to CTLA4Ig, although not mimicking a short-term, therapeutic regimen, provides a means for determining its potential to ameliorate chronic immunological abnormalities, such as those that develop in Lyn−/− mice. Many abnormalities that occur in Lyn−/− mice were obviated by CTLA4Ig expression, although surprisingly, kidney pathology was not ameliorated. CTLA4Ig did not alter intrinsic Lyn−/− B cell and plasma cell defects, but revealed their capacity to generate IgA-mediated autoimmunity in the absence of IgG immune complexes.

Materials and Methods

Mice

Transgenic mice expressing CTLA4Ig under the control of rat insulin promoter were generated on the B6.C-H-2bm1 (C57BL/6 with the bml mutation of H-2Kb) background and subsequently backcrossed to C57BL/6 (16). Lyn-deficient C57BL/6 mice (4) were bred with CTLA4Ig-expressing transgenic mice to generate Lyn-deficient offspring that carry one allele of CTLA4Ig. Lyn−/− littersmates not carrying the CTLA4Ig transgene were used as age- and sex-matched controls. Mice were maintained in a conventional animal facility. All procedures were approved by the Walter and Eliza Hall Institute animal ethics committee.

Abs for flow cytometry

Conjugates used in flow cytometry experiments included CD19-FITC (clone IA3), IgM-biotin (331.12), CD21-allophycocyanin (7G6), and CD23-FITC (RA3-6B2), and IgD-FITC (1126c), and purchased from BD Pharmingen (San Diego, CA). Avidin-Cy5 was obtained from Southern Biotechnology Associates (Birmingham, AL). Flow cytometry was performed on an LSR II or FACSCalibur (BD Biosciences, San Jose, CA). Cell death was measured using the BINDAZYME ANA Screen Kit (The Binding Site, Birmingham, U.K.) according to manufacturer’s instructions and detected using a pool of the biotinylated goat anti-mouse IgG, or IgA, isotype-specific secondary Ab used for ELISAs, followed by streptavidin-conjugated HRP.

Histology and immunofluorescence were performed as described previously (20). Complement deposition in glomeruli was detected in 7-μm-thick kidney sections using FITC-conjugated goat anti-mouse C3 Ab (Cappel, Durham, NC). IgG immune complexes were detected using sheep anti-mouse IgG-FITC conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA), and IgA was detected using biotinylated goat anti-mouse IgA (Southern Biotechnology Associates), followed by streptavidin-FITC (Caltag Laboratories, Burlingame, CA).

Statistical analyses

Unpaired, two-tailed t tests were performed using Microsoft Excel software. Differences were deemed significant where p < 0.05.

Results

CTLA4Ig prevents splenomegaly and myeloid hyperplasia in Lyn−/− mice

To determine the contribution of CD28 T cell costimulation to the development of autoimmunity in mice lacking Lyn, we generated Lyn−/− mice that express soluble CTLA4Ig (16) (termed CTLA4Ig/Lyn−/−). This inhibitory molecule impairs T cell priming (Supplemental Fig. 1), enabling the resolution of cell-intrinsic aspects of the Lyn−/− phenotype from those that are exacerbated by T cell involvement. It should be noted that T cell activation still occurs in the presence of CTLA4Ig, distinguishing this from models involving T cell deficiency.

Splenomegaly develops early in Lyn−/− mice (7); however, when these mice express CTLA4Ig, splenomegaly is inhibited over their lifespan (Fig. 1A). In Lyn−/− mice, splenomegaly is thought to be due to the increased and abnormal presence of myeloid cells and their precursors in the spleen (8, 22). To determine whether the expansion in myeloid precursors present in Lyn−/− mice persists when CTLA4Ig is expressed, we measured colonies of myeloid cells that formed after culture of bone marrow and spleen cell suspensions with a range of CSFs. Colony-forming cells in the bone marrow are increased in Lyn−/− mice compared with Lyn-sufficient controls and are not significantly reduced by CTLA4Ig (Fig. 1B). In the spleen and blood (data not shown), wild-type animals contained no myeloid progenitor cells, but Lyn deficiency resulted in mobilization of precursor cells to these peripheral compartments. Demonstrating that T cell-mediated processes are a major contributor to the dysregulated myelopoiesis in Lyn−/− mice, CTLA4Ig resolves this, although the modest increase in myeloid progenitor cells in CTLA4Ig/Lyn−/− mice relative to wild-type was...
presumably due to the absence of Lyn in these cells or their precursors rather than the result of T cell activity in Lyn−/− mice. These data imply that the perturbed myelopoiesis in the Lyn−/− spleen is predominantly a result of T cell-driven inflammatory processes, whereas in the bone marrow, the increase in myeloid progenitors is probably caused by defects intrinsic to the Lyn-deficient cells.

**Lyn−/− kidney pathology is not inhibited by CTLA4Ig**

Mice lacking Lyn develop severe autoimmune glomerular pathology (4, 7). To determine the extent to which the nephropathy observed in Lyn−/− mice requires costimulation, we examined kidney sections for pathology and complement deposition. Immunofluorescence staining showed that CTLA4Ig diminished the deposition of IgG and C3′ in Lyn−/− glomeruli. Despite this, CTLA4Ig expression did not improve glomerulonephritis in Lyn−/− kidneys (Fig. 2A), as lobularity, crescent formation, and hypercellularity (as indicated by overlapping mesangial cell nuclei; Fig. 2B) were equally evident within CTLA4Ig/Lyn−/− glomeruli. Because autoreactive IgA is also able to form immune complexes and induce nephropathy, we measured IgA deposition in kidney tissue sections and found that CTLA4Ig/Lyn−/− glomeruli were positive for IgA (Fig. 2A). Serum titers of ANA showed a similar pattern of isotype preference (Fig. 2D). CTLA4Ig mostly prevented the development of IgG ANA over the life of Lyn−/− animals, demonstrating that IgG ANA development is exacerbated by costimulation (Fig. 2C). In contrast, sera from both Lyn−/− and CTLA4Ig/Lyn−/− mice contained very high titers of IgA ANA from an early age (Fig. 2D). These findings demonstrate that despite its ability to ameliorate splenomegaly and myeloid hyperplasia, CTLA4Ig does not prevent renal pathology and unmasks a costimulation-independent IgA-mediated arm of humoral autoimmunity in Lyn−/− mice that is otherwise outcompeted by IgG.

**Perturbed Lyn−/− B cell development and behavior are independent of costimulation**

B cell selection and maturation are influenced by local lymphoid microenvironments that are in turn influenced by autoimmune inflammatory processes. The persistence of ANA in CTLA4Ig/Lyn−/− mice reveals a failure to delete or anergize autoreactive B cell clones and suggests that Lyn−/− B cell differentiation is intrinsically defective regardless of the environment in which the cells develop. To evaluate whether impairing T cell costimulation alters Lyn−/− B cell development, we used flow cytometry to delineate B cell populations in lymphoid tissues of the mice. The proportions of pre-/pro-B, immature and transitional B cells in the bone marrow of both Lyn−/− and CTLA4Ig/Lyn−/− mice did not differ substantially from wild-type controls (Fig. 3A). However, mature recirculating B cells were severely diminished in Lyn−/− bone marrow regardless of CTLA4Ig expression. The elevated proportion of plasma cells, as revealed by Syndecan-1 expression and measured by flow cytometry, in Lyn−/− bone marrow and spleen was also independent of T cell costimulation as it occurred identically in Lyn−/− and CTLA4Ig/Lyn−/− mice (Fig. 3C, 3D).

Despite being enlarged, spleens of Lyn-deficient mice contain a diminished proportion of T2, marginal zone, and follicular B cells and an increase in plasma cell frequency relative to C57BL/6 controls. These changes also occur in the spleens of Lyn−/− mice that express CTLA4Ig (Fig. 3B, 3D), which are normal in size, demonstrating that the changes in B cell populations are intrinsic defects in cell survival or differentiation in the absence of Lyn. Among Lyn−/− splenic CD23+ B cells, however, is a population expressing low amounts of CD21 but high for surface IgM (Fig. 3B). This population does not appear in spleens of either C57BL/6 or CTLA4Ig/Lyn−/− mice, suggesting its appearance may depend on disease status in what is currently an unknown way.

Lower BCR surface expression is assumed to be a general feature of B cells that lack negative regulators of BCR signaling and consequently downregulate surface Ig to compensate for elevated signaling (23). Indeed, Lyn−/− B cells have lower levels of BCR surface expression than C57BL/6 B cells (23). To estimate the activation status of Lyn−/− B cells in the presence of CTLA4Ig, we used flow cytometry to measure surface IgM levels and intracellular calcium flux in response to BCR ligation (Fig. 4). IgM surface expression is equally reduced on Lyn−/− and CTLA4Ig/Lyn−/− follicular B cells relative to Lyn-sufficient cells, indicating that B cells lacking Lyn experience elevated BCR signaling.
regardless of CTLA4Ig expression (Fig. 4A). In support of this, we found that ligation of the BCR induced delayed but elevated calcium flux in CTLA4Ig/Lyn−/− follicular B cells relative to C57BL/6 B cells, identical to that typical for Lyn−/− B cells (Fig. 4B). The defects in BCR signaling and the changes in B cell populations in Lyn−/− mice are therefore due to the absence of Lyn in those cells and are not affected by an altered immunological environment.

Isotype switching of Lyn−/− B cells is largely unaffected by CTLA4Ig

An additional defining feature of the Lyn−/− phenotype is the accumulation of plasma cells in the bone marrow and spleen, with Lyn-deficient mice having up to 10-fold more plasma cells than C57BL/6 mice (4). Flow cytometry revealed the proportion of Lyn−/− plasma cells to be significantly elevated regardless of CTLA4Ig expression (Fig. 3). To determine whether the Ab class restriction of plasma cells was also unaffected in the transgenic animals, plasma cell isotypes were examined by ELISPOT assay (Fig. 5). IgM-secreting plasma cell frequencies were not reduced in CTLA4Ig/Lyn−/− bone marrow, suggesting that the formation of these cells is independent of processes requiring costimulation and that their persistence does not require inflammation-induced survival niches (Fig. 5A). Although the Lyn−/− spleen contained a significantly lower proportion of IgM-secreting plasma cells when CTLA4Ig was expressed (Fig. 5B), it remained substantially elevated compared with C57BL/6. Hyperresponsiveness of autoreactive IgM-expressing B cells does not intrinsically result in switching to pathogenic IgG isotypes (24, 25). Indeed, the proportions of Lyn−/− plasma cells secreting IgG of various subtypes did not generally differ from C57BL/6 and nor were they remarkably altered by CTLA4Ig expression (Fig. 5). Changes in the concentrations of IgG subtypes in the serum were also minor (Fig. 6). A generalized but modest CTLA4Ig-induced reduction in plasma cell frequency was observed for IgG2c, IgG2b, and IgG3 isotypes, indicating either a direct or indirect requirement for CD28 T cell costimulation for their production (Figs. 5, 6). Consistent with a previous report on serum titers (7), IgA-secreting plasma cell frequencies in the spleen of Lyn−/− mice were elevated irrespective of the costimulation blockade (Fig. 5B), and serum titers of IgA were even higher in CTLA4Ig/Lyn−/− mice than in their nontransgenic Lyn−/− littermates (Fig. 6). Thus, plasmacytosis in Lyn−/− mice appears to be because of a cell-intrinsic capacity to accumulate and persist in the absence of Lyn. This particularly applies to plasma cells of autoreactive specificities that secrete IgA autoantibodies irrespective of the availability of T cell help.

Discussion

The study of Lyn’s role within specific cell types is complicated by the development of Ab-mediated autoimmunity in mice lacking this negative regulator of signaling. Lyn-deficient mice develop splenomegaly and myeloeXpansion, high-serum IgG ANA titers,
We generated Lyn through their interaction with self-reactive B cells (26, 27), which are due to cell-extrinsic factors, including the recruitment of other cell types into the process of disease development. As T cells which are unable to compensate for the removal of IgG immune complexes to drive myelopoiesis in the spleen. Previously, it has been reported that M-CSF signaling is enhanced in Lyn−/− precursor cells (28) and Lyn−/− bone marrow macrophage and dendritic cell precursors were hyperproliferative in response to GM-CSF (8, 29), suggesting that Lyn−/− myeloid precursor cells are intrinsically hyperproliferative to CSFs. However, myelopoiesis surges in inflammatory environments (30, 31), and therefore, the increased myelopoiesis in Lyn−/− mice may develop as a secondary result of fulminant autoimmunity. We found that the elevated frequency of myeloid precursors in the spleen of Lyn−/− mice was at least partially dependent on T cell costimulation. However, in the bone marrow of Lyn−/− and CTLA4Ig/Lyn−/− mice, the comparable increase in myeloid precursor frequencies indicate that here the amplified myelopoiesis is due to the primary deficiency of Lyn in precursor cells. In Lyn−/− mice, the autoimmune milieu of the spleen may better support the seeding and survival of precursor cells emigrating from the bone marrow. Therefore, both cell-intrinsic and -extrinsic effects contribute to the myeloid phenotype of Lyn−/− mice. Extrinsic factors may include IgG autoantibodies, which were resolved by impairing T cell costimulation, and inflammatory mediators. For example, IL-6 skews hematopoiesis toward myeloid differentiation, resulting in lymphopenia and myeloeexpansion in SHIP−/− mice (32). In this study, we demonstrate that regardless of the autoimmune microenvironment arising in CTLA4Ig/Lyn−/− mice, autoreactive IgA is unable to compensate for the removal of IgG immune complexes to drive myelopoiesis in the spleen.

Despite its ability to ameliorate splenomegaly, myeloid hyperplasia, and IgG ANA, CTLA4Ig did not prevent glomerular tissue destruction in Lyn−/− mice. This renal pathology may result from defects in glomerular endothelial cells, which normally express Lyn (33). In addition, the persistence of autoreactive Lyn−/− B cell clones is not affected by CTLA4Ig and in the relative absence of pathogenic IgG, IgA autoantibodies seem to predominate as the major iso-type-precipitating humoral autoimmunity. This indicates an intrinsic failure to negatively select or anergize autoantibodies in Lyn−/− mice. This renal pathology may result from defects in glomerular endothelial cells, which normally express Lyn (33). In addition, the persistence of autoreactive Lyn−/− B cell clones is not affected by CTLA4Ig and in the relative absence of pathogenic IgG, IgA autoantibodies seem to predominate as the major iso-type-precipitating humoral autoimmunity. This indicates an intrinsic failure to negatively select or anergize autoantibodies in Lyn−/− mice. It was previously unclear which aspects of the Lyn−/− phenotype occur as a direct result of the loss of Lyn in particular cells and which are due to cell-extrinsic factors, including the recruitment of other cell types into the process of disease development. As T cells may be particularly important in compounding the autoimmunity through their interaction with self-reactive B cells (26, 27), we generated Lyn−/− mice that express CTLA4Ig to provide an in vivo mechanism of inhibiting T cell priming in this model. We reasoned that CTLA4Ig would reduce the contribution of costimulation-dependent processes to disease development and delineate Lyn-related defects from those that are compounded by T cell involvement. CTLA4Ig does not block absolutely T cell activation but rather moderates the degree or extent to which this occurs. This clearly distinguishes this model from those lacking T cells completely.

CTLA4Ig is of interest as a therapy for lupus nephritis (19), and contrary to our expectations, lifelong exposure to CTLA4Ig did not prevent autoimmune kidney destruction in Lyn−/− mice. Instead, the suppression of costimulation by CTLA4Ig revealed an IgA-mediated axis of disease in the Lyn−/− model of humoral autoimmunity that causes glomerular pathology of a different nature, although similar severity, to that arising in an environment of sufficient costimulation. CTLA4Ig blocked splenomegaly in Lyn−/− mice, which was presumably due to the inhibition of extramedullary myelopoiesis in the spleen. Previously, it has been reported that M-CSF signaling is enhanced in Lyn−/− precursor cells (28) and Lyn−/− bone marrow macrophage and dendritic cell precursors were hyperproliferative in response to GM-CSF (8, 29), suggesting that Lyn−/− myeloid precursor cells are intrinsically hyperproliferative to CSFs. However, myelopoiesis surges in inflammatory environments (30, 31), and therefore, the increased myelopoiesis in Lyn−/− mice may develop as a secondary result of fulminant autoimmunity. We found that the elevated frequency of myeloid precursors in the spleen of Lyn−/− mice was at least partially dependent on T cell costimulation. However, in the bone marrow of Lyn−/− and CTLA4Ig/Lyn−/− mice, the comparable increase in myeloid precursor frequencies indicate that here the amplified myelopoiesis is due to the primary deficiency of Lyn in precursor cells. In Lyn−/− mice, the autoimmune milieu of the spleen may better support the seeding and survival of precursor cells emigrating from the bone marrow. Therefore, both cell-intrinsic and -extrinsic effects contribute to the myeloid phenotype of Lyn−/− mice. Extrinsic factors may include IgG autoantibodies, which were resolved by impairing T cell costimulation, and inflammatory mediators. For example, IL-6 skews hematopoiesis toward myeloid differentiation, resulting in lymphopenia and myeloeexpansion in SHIP−/− mice (32). In this study, we demonstrate that regardless of the autoimmune microenvironment arising in CTLA4Ig/Lyn−/− mice, autoreactive IgA is unable to compensate for the removal of IgG immune complexes to drive myelopoiesis in the spleen.

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CTLA4Ig expression did not rescue defects in Lyn−/− B cell development and behavior, as Lyn−/− mice and their transgenic CTLA4Ig/Lyn−/− littermates both had a deficiency of T2, marginal zone, and follicular B cells in the spleen and an excess of IgM-secreting plasma cells in the spleen and bone marrow (1, 4). Polyclonal B cell hyperactivation is a hallmark of lupus (35), and
lower levels of surface IgM and an augmented calcium response to BCR ligation are both features of B cells that lack Lyn (4), although both of these parameters were unaffected by impairing T cell costimulation.

In addition, plasmacytosis in Lyn−/− mice is driven by factors other than those causing myeloproliferation and splenomegaly, because CTLA4Ig expression did not lessen plasma cell frequencies in the bone marrow or spleen. Inflammation in peripheral tissues is known to influence the availability and location of plasma cell niches, particularly in an autoimmune environment (36–38). However, the majority of Ab-secreting cells in Lyn−/− mice are unswitched, IgM-secreting plasma cells (4), suggesting that dysregulated plasma cell differentiation and survival occurs regardless of the immune environment. Despite IgM plasma cells being present at the same frequency in bone marrow and spleen, CTLA4Ig reduced Lyn−/− IgM serum levels presumably by preventing splenomegaly. No significant differences were detected in the frequencies of plasma cells secreting IgG isotypes, although interestingly, Lyn−/− serum IgA levels and the frequency of IgA-secreting plasma cells were in fact elevated by the expression of CTLA4Ig.

The CTLA4Ig/Lyn−/− model has some interesting similarities to IgA nephropathy (IgAN), the most common primary glomerulonephritis leading to end-stage renal failure in humans, and the related condition Henoch-Schonlein purpura (39). IgAN is characterized by glomerular hypercellularity and inflammation associated with the glomerular deposition of IgA1 and, less prominently and consistently, IgG, IgM, C3, and C4. There is evidence for T cell involvement and active IgAN is associated with high titres of serum IgA (40, 41). It is intriguing that the CTLA4Ig transgene converts a lupus-like IgG-dominated renal disease to one closely resembling IgAN. It could be that such modulation of the T cell response biases in favor of IgA deposition, or that reduced IgG production biases the Ab response in favor of IgA (perhaps by increasing the number of extramucosal plasma cell niches available for IgA production). Another model of autoimmune disease mediated by IgG and IgA autoantibodies is mice transgenic for the B cell activating factor belonging to the TNF family, in which IgA but not IgG autoantibody production is T cell dependent (42). This finding highlights the variability in mechanisms that may underlie disease development in otherwise superficially similar models. CTLA4Ig has been shown to not prevent IgA switching in certain immune responses (43), whereas the requirement for T cells in IgA switching may be overridden by B cell production of IL-6 (44). Levels of IL-6 are strongly correlated with IgAN disease severity (45, 46) and we have found that deletion of IL-6 in Lyn−/− mice abolishes IgA ANA (data not shown), suggesting disease develops as a result of a failure of CTLA4Ig to impede IL-6–driven IgA production in CTLA4Ig/Lyn−/− mice. It will be important to determine the relevance of this model to IgAN, for which no good animal model is currently available, because this might have clinical implications. Our observations also raise the possibility that IgAN might complicate CTLA4Ig treatment of SLE or other autoimmune conditions, but CTLA4Ig may be considered for use in combination with therapies that block B cell differentiation factors. It is important to note that in the model system used here, CTLA4Ig is present from birth, whereas in a clinical setting CTLA4Ig or its equivalent would be delivered after disease onset. It will be interesting to determine the consequences of such therapeutic application of CTLA4Ig in the Lyn disease model.

Our results show that mutations in B cells that trigger autoimmunity, such as Lyn deficiency, are not adequate to drive fulminant autoimmunity in these models. Instead, chronic stimulation of autoreactive lymphocyte specificities in a situation where tolerance has been breached, coupled to an inability to resolve inflammation, potentially lead to the cyclical exacerbation and compounding of the phenotype into an advanced state of autoimmunity (11). Following from this, previous studies have assumed that the cycle of Ab-mediated autoimmunity may be interrupted by selectively ablating individual pathways involved. For example, disease development has been intercepted at the level of B cell activation, B cell maturation, cytokine signaling, and T cell activation. Despite the disparate nature of these facets of disease development, disruption of a single one superficially appears to attenuate disease (47–54). Additional mutations in Lyn−/− B cells that impair B cell responses, such as deletion of the signaling molecules Btk or CD19, also seem to block the development of autoimmunity (55, 56) as does removal of the TLR signaling molecule MyD88 (15). Our results call for caution in the interpretation of such studies, which may fail to identify more subtle indicators of a disease whose nature has been changed. This work also highlights the need for careful interpretation of results from preclinical studies aiming to treat Ab-mediated autoimmunity and provides insights into the effects of CTLA4Ig treatment on mice with SLE-like autoimmunity that may have clinical relevance.

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Disclosures

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