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Lyn-Deficient Mice Develop Severe, Persistent Asthma: Lyn Is a Critical Negative Regulator of Th2 Immunity[1]

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The etiology of asthma, a chronic inflammatory disorder of the airways, remains obscure, although T cells appear to be central disease mediators. Lyn tyrosine kinase has been implicated as both a facilitator and inhibitor of signaling pathways that play a role in allergic inflammation, although its role in asthma is unclear because Lyn is not expressed in T cells. We show in the present study that *Lyn*$^{-/-}$ mice develop a severe, persistent inflammatory asthma-like syndrome with lung eosinophilia, mast cell hyperdegranulation, intensified bronchospasm, hyper IgE, and Th2-polarizing dendritic cells. Dendritic cells from *Lyn*$^{-/-}$ mice have a more immature phenotype, exhibit defective inhibitory signaling pathways, produce less IL-12, and can transfer disease when adoptively transferred into wild-type recipients. Our results show that Lyn regulates the intensity and duration of multiple asthmatic traits and indicate that Lyn is an important negative regulator of Th2 immune responses. *The Journal of Immunology,* **2005, 175: 1867–1875.**

Asthma is an incurable chronic inflammatory disease of the airways characterized by persistent eosinophilic mucosal inflammation, elevated IgE titers, mast cell hyperdegranulation, increased mucus production, and associated functional changes to the lung (1). The prevalence and severity of asthma are rising, and asthma is now a global epidemic (2). Considerable evidence suggests that molecular defects in Th lymphocyte immune regulation contributes to the pathogenesis of asthma (3, 4). In mouse models of asthma, Th2-biased T cells secrete cytokines, including IL-4, IL-5, IL-13, and IL-9. Transgenic mouse studies and analyses of mice deficient in these cytokines, as well as neutralizing Ab studies, have confirmed the likely role of Th2 cells in the induction of IgE, eosinophilia, mast cell growth and deregulation, and mucus (goblet) cell metaplasia. Additional research has identified key transcriptional regulatory mechanisms, including STAT-6, GATA-3, c-*maf*, and NFATc, as being important in Th2 induction, whereas STAT-1, STAT-4, and T-bet are important for Th1 populations that produce reciprocally cross-regulating IFN-γ, IL-2, and lymphotoxin (3). The paradigm, established >20 years ago, that Th1 and Th2 immunity counterregulate each other is now being questioned (5). Nonetheless, deregulated cytokine production remains a central element in asthma susceptibility (4, 6–8).

Lyn is a member of the Src family of nonreceptor protein tyrosine kinases that, with the exception of T lymphocytes, is expressed in the hemopoietic cells that infiltrate the inflamed asthmatic airway. Lyn has been directly implicated in a number of signaling pathways that play a role in asthma, including those for Ags (9), Igs (10), cytokines (11–13), and growth factors (14, 15). However, the role of Lyn in asthma and allergic inflammation is controversial. An in vivo study using a Lyn-binding peptide inhibitor has suggested that suppression of Lyn kinase activity might be a viable therapeutic strategy for treating allergic airway disease (16). However, we have shown previously that Lyn regulates eosinophil apoptosis (17) and that *Lyn*$^{-/-}$ mice are atopic (13) and have increased circulating histamine and increased numbers of mast cells in skin and peritoneum (18), suggesting that Lyn deficiency may promote asthma. In addition, a recent study has demonstrated that *Lyn*$^{-/-}$ mice show increased severity of experimental allergic encephalomyelitis (19), a Th1-mediated autoimmune disease, although paradoxically, in this model, *Lyn*$^{-/-}$ mice show decreased production of the Th1-related cytokines IL-12, IFN-γ, and TNF-α. These conflicting data leave open the question of the in vivo role of Lyn in modulating inflammatory diseases and Th2 immune responses.

To study the role of Lyn in Th2 immune responses, we have used a well characterized mouse model of asthma (7). We report that, rather than being protected from asthma, Ag-challenged *Lyn*$^{-/-}$ mice develop a severe, persistent multitrait asthma syndrome involving intense eosinophil-rich inflammation, hyper IgE, increased mucus production, overproduction of Th2 cytokines, mast cell and eosinophil hyperdegranulation, and deranged lung function. Myeloid dendritic cells (DCs)[4] derived from *Lyn*$^{-/-}$

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[4] Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff; AB, Alcian blue; DAB, diaminobenzidine; LN, lymph node; SHP-1, Src homology region 2 domain-containing phosphatase-1; cfeg, clusters of free eosinophil granule; PY, phosphotyrosine; me, mother-eaten; PIR-B, paired Ig-like receptor B.

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bone marrow (BM) mature inefficiently, exhibit defective inhibitory signaling pathways, and show a diminished ability to produce IL-12. Moreover, when adoptively transferred into naive recipient mice, Ag-pulsed $Lyn^{-/-}$ DCs intensify mucosal inflammation. Our data support the notion that Lyn is a negative regulator of Th2 mucosal inflammation and suggest that Lyn, or components of its signaling cascade, may be asthma severity and progression determinants.

Materials and Methods

Animals

For most studies, $Lyn^{-/-}$ mice or $Lyn^{+/+}$ controls were on a mixed 129Ola × C57BL/6 genetic background (20). Inbred $Lyn^{-/-}$ mice on C57BL/6 or 129Ola backgrounds were used for studies in Fig. 3, and $Lyn^{-/-}$ mice on a BALB/c background were used for experiments in Fig. 6. Experiments were approved by the Animal Ethics Committees of the Ludwig Institute for Cancer Research and Department of Surgery and the University of Melbourne.

Immunization and challenge protocol

$Lyn^{+/+}$ and $Lyn^{-/-}$ mice were immunized by i.p. injection with 20 μg of OVA in aluminum hydroxide on days 0 and 14 and challenged with aerosolized OVA in PBS for 20 min on days 21–23 using a DeVilbiss ultrasonic nebulizer (7, 21). In all cases, except for the dose response studies depicted in Fig. 2*A*, 50 mg/ml OVA were used. Sham mice were immunized with aluminum hydroxide alone. Twenty-four hours postallergen challenge, mice were euthanized with an overdose of ketamine/xylazine, and bronchoalveolar lavage fluid (BAL) was obtained with saline (4 × 0.3 ml). Total cell counts and viability were determined using ethidium bromide acridine orange staining in a Neubauer hemocytometer. Differentials (at least 500 cells/slide) were performed on cytospins (7, 21).

Microscopy and imaging

Images were obtained using a Nikon Elipse Microscope (E600) equipped with a Nikon DXM 1200F digital camera using Nikon Act-1 (version 2.51) software. The objectives/numerical apertures used were as follows: ×10/0.25; ×20/0.5; ×40/0.6; and ×100/1.3 oil immersion, as specified. With the exception of white balance obtained at the start of photomicrography, image manipulation procedures were not used.

Histology, cell staining, and morphometry

H&E and periodic acid-Schiff/Alcian blue (PAS/AB) stains were performed on paraffin-embedded, inflation-fixed 15-μm sections mounted on glass slides using Depex mounting medium, using standard histological methods. These sections were used to grade inflammation and to measure goblet cell parameters.

Trachea-bronchial whole mounts were prepared as for rats (22). These preparations were used to measure eosinophil and mast cell indices. Eosinophils were stained for endogenous peroxidase using diaminobenzidine (DAB), and mast cells were stained with 0.05% w/v toluidine blue. These cells were enumerated manually, counting multiple fields in each intercartilaginous zone along the entire length of the trachea as previously described (22) using a calibrated eyepiece grid. Length measurements were performed manually using a calibrated eyepiece rule.

Airway inflammation of H&E-stained paraffin sections was graded on a 0–5 point scale on randomized blinded histological sections. Tissues (n = 6–8) were scored for intensity of peribronchial inflammation (around three to five small airways per section), and intensity of parenchymal inflammation was graded in multiple random parenchymal fields per section. Grading was assessed by the Wilcoxon rank sum test.

Goblet cells in PAS/AB-stained paraffin airway sections have a characteristic shape and a purple-magenta decoration. They were enumerated manually by measuring the number of goblet cells along a measured length of not <400 μm of epithelial basement membrane and are reported as cells/10 μm length of membrane. The fraction of epithelium positive for PAS/AB purple-magenta glycoconjugate staining was determined manually by area-counting using a calibrated morphometric grid and is reported as the percentage of total epithelial cell area including nuclei. Data were analyzed by two-tailed t test for n = 6–8 animals with at least 10 measurements/condition.

Eosinophilic inflammation was quantitated in DAB-stained tracheal whole mounts. Data were analyzed for n = 8–12 mice/group by ANOVA and Dunnet's multiple comparison test.

Mast cell granule proteoglycans (heparans and heparins) stain with toluidine blue and appear as elliptical blue-magenta structures with a central nucleus in intact mast cells. As mast cells degranulate, these granules are released into the surrounding tissue causing an apparent increase in cell size. The extent of degranulation was measured morphometrically by random manual counting of 500 mast cells/trachea, where they appeared as individual cells (overlapping clusters of degranulated mast cells were excluded). The maximal length and midline perpendicular width of each mast cell was measured in micrometers at ×100 magnification, and their product was defined as the size index. Data were analyzed for n = 8–12 mice/group by ANOVA and Dunnet's multiple comparison test.

Flow cytometry

BAL composition was determined by three-color flow cytometry as previously described (20) using the following Abs purchased from BD Pharmingen: RA3-6B2 (B220), 2C11 (CD3), GK1.5 (CD4), 53-6.7 (CD8a), PC61 (CD25), H1.2F3 (CD69), M1/70 (CD11b), 6B2-8C5 (Gr-1), and F4/80.

Eosinophil progenitor assay

BM from sham-immunized, immunized, and immunized/Ag-challenged mice was resuspended at 25,000 cells/ml in nutrient agar in 20 ng/ml IL-5 for 7 days at 37°C in 5% CO_2 in O_2. Plates were scored by light microscopy for eosinophil-containing colonies. Data is expressed as mean ± SEM for eight mice per group.

BrdU pulse-chase experiments

BM eosinophils were pulse labeled with a single i.p. injection of BrdU (50 mg/kg in saline) administered 48 h after secondary immunization, a period of peak BM eosinophilopoiesis (23). Labeled cells mature in the marginated marrow reserve and are released on subsequent aeroallergen challenge with OVA, where they infiltrate the lung. Two weeks postallergen inhalation, BAL was collected, and cytospins were prepared. $BrdU^+$ eosinophils show staining of the nucleus but not cytoplasmic granules; they were enumerated in Normaski interference microscopy and reported as the fraction of $BrdU^+$ cells in 200 eosinophils.

Anti-Fas-induced apoptosis

Apoptosis was induced by intranasal instillation of 35 μl of 1 mg/ml anti-Fas mAb (Jo2; BD Pharmingen), or an isotype-matched mAb, and assessed in BAL or morphometrically in DAB-stained whole mounts (17, 24).

Lung function and airway physiology

Lung function was measured in anesthetized, tracheostomized mice using a modification of the low-frequency forced oscillation technique (flexiVent; Scireg) as described previously (25). Following measurement of baseline lung function, mice sensitized to OVA were challenged with an aerosol of 1% OVA w/v in saline. Peak bronchospasm measured as Zrs was recorded over the following 10 min.

The in vitro sensitivity of airway smooth muscle was determined by Mulvaney-Halpern myography (26). The Mulvaney-Halpern myograph is a highly sensitive force transducer originally developed to measure isometric contractions in small blood vessels. Extrapulmonary bronchial 2-mm long tube segments were dissected and placed on the apposed wires of the myograph, and resting tension was increased to 0.1 mN. After stabilization, cumulative concentration response curves were constructed, and sensitivity was defined as the pEC_{50} (i.e., the $-\log_{10}$ of the molar concentration for half maximal effect). Tissue more sensitive to contraction is reflected by a higher pEC_{50} value.

T cell purification and cytokines

Thoracic-draining lymph node (LN) $CD4^+$ T cells were purified using flow cytometry or with MACS magnetic beads (Miltenyi Biotec) and anti-CD4 (BD Pharmingen). Purified $CD4^+$ T cells or BAL cells (2×10^5/well) were incubated overnight with plate-bound anti-CD3 (10 μg/ml; BD Pharmingen). Alternatively, LNs were depleted of $B220^+$ B cells and then incubated overnight with 0, 1, 10, or 100 μg/ml OVA. Cytokine production was quantitated by OptEIA ELISA sets (BD Pharmingen). Data represents pooled triplicate assays, limit of detection = 10 pg/ml, coefficient of variation (≥5%).

Biochemistry

B cells were sorted from the spleen and LN using mAbs against B220 (RA3-6B2). Splenic T cells ($CD3^+NK1.1^-$) were sorted from naive mice using mAbs to CD3 (2C11) and NK1.1 (PK136) (BD Pharmingen) and left

untreated or incubated for 4 days in 1000 U/ml IL-2. $CD4^+$ T cells were also sorted from the LN and spleen of Ag-challenged mice. Lyn expression was determined by Western blot analysis of total cell lysates using anti-Lyn antisera (Santa Cruz Biotechnology) (27). Blots were stripped and reprobed with anti-IgM (Jackson ImmunoResearch Laboratories), anti-CD22 (P. Crocker, Dundee University, Dundee, U.K.), anti-DokR (P. Lock, University of Melbourne, Melbourne, Australia), and anti-Src homology region 2 domain-containing phosphatase-1 (SHP-1) (H.-C. Cheng, University of Melbourne, Melbourne, Australia). For DC studies, total cell lysates were immunoprecipitated with rabbit anti-paired Ig-like receptor B (PIR-B) (BD Pharmingen) or rabbit anti-SHIP-1 (BD Pharmingen) and blotted with mouse anti-phosphotyrosine (PY, 4G10; Upstate Biotechnology). Blots were developed with HRP-conjugated goat anti-rabbit or sheep anti-mouse antiserum and the ECL system (Amersham Biosciences).

DC culture and adoptive transfer experiments

DCs were derived from the BM by culture for 10 days in GM-CSF (28). Purity was tested by flow cytometry using mAbs to Mac-1 and CD11c (BD Pharmingen). To induce maturation, cells were incubated for 24 h with 100 μg/ml OVA, stimulated for 1 h with 100 ng/ml LPS, then stained for MHC class II, CD40, CD80, and CD86. To assess cytokine production in vitro, DCs were plated at a density of 10^6 cells/well of a 24-well culture dish and then stimulated for 16 h in 0, 1, or 100 ng/ml LPS.

For in vivo experiments, DCs were derived from control or $Lyn^{-/-}$ mice on a BALB/c background, loaded with 3 μg/ml of the immunodominant epitope $OVA_{323–339}$ peptide, and 10^6 DCs were administered intranasally into lightly anesthetized recipient BALB/c mice ($n = 7$ recipients/donor genotype). Recipient mice were allowed to recover for 7 days, challenged on 3 consecutive days with aerosolized OVA (50 mg/ml in PBS for 15 min), and analyzed the following day. BAL cells were recovered and analyzed for cellularity and cytokine production as above. Data was analyzed by one-tailed *t* test.

Results

Enhanced asthma-like inflammation in Ag-challenged $Lyn^{-/-}$ mice

Eosinophilic inflammation is a hallmark trait of human asthma, and its intensity correlates with disease severity and progression (29). Therefore, we measured eosinophil numbers in BAL from control and $Lyn^{-/-}$ mice immunized and challenged with OVA, which reflects eosinophils in the airspace. Basal BAL cell profiles, which comprised >99% macrophages, were indistinguishable (data not shown). In inhaled Ag dose-response studies, threshold concentrations of OVA that barely elicited a response in $Lyn^{+/+}$ BAL produced eosinophil numbers that were significantly higher in $Lyn^{-/-}$ BAL 24 h after challenge (Figs. 1, *A* and *B*, 2, *A* and *B*, and 3, *A–C*). This intense inflammatory infiltrate also included lymphocytes and macrophages (Fig. 2*B*), with the latter being enlarged and vacuolated, which is indicative of activation (Fig. 1, *A* and *B*). Differential analysis of cytospins coupled with BAL cellularity indicated that while the inflammation was predominantly eosinophilic, lymphocyte numbers were also significantly higher in $Lyn^{-/-}$ BAL (Fig. 2*B*). Additional analysis of BAL lymphocyte subpopulations by flow cytometry revealed similar percentages of B and T cells (10% $B220^+$ B cells; 90% $CD3^+$ T cells) but an increase in both the percentage and absolute number of $CD4^+$ T cells in $Lyn^{-/-}$ mice. Furthermore, these cells had an activated phenotype (Fig. 2, *C–F*), with >50% of $CD25^+$ cells being $CD69^+$ (data not shown). Thus, following Ag challenge, Lyn-deficient mice mount an exacerbated eosinophilic inflammatory response in BAL with concomitant increases in numbers of activated $CD4^+$ T cells.

The inflammation in Lyn-deficient mice extended beyond the BAL, with histological examinations revealing marked peribronchial and parenchymal inflammation (Fig. 2*G*) and extensive goblet (mucus) cell metaplasia in conducting airways in $Lyn^{-/-}$ mice compared with controls (Figs. 1, *C* and *D*, and 2*H*). Furthermore, eosinophil peroxidase-stained tracheal whole mounts revealed extensive eosinophilic inflammation and clusters of free eosinophil granules (cfegs) only in $Lyn^{-/-}$ mice (Fig. 1, *E* and *F*).

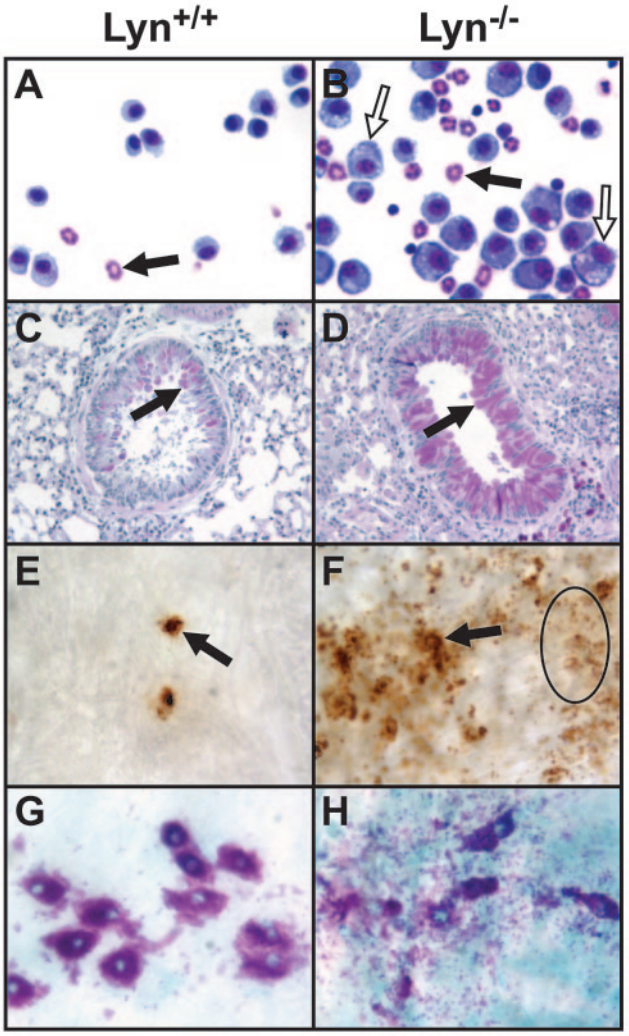

FIGURE 1. Severity and persistence of Ag-induced lung inflammation in $Lyn^{-/-}$ mice. BAL cytospins 24 h post-OVA challenge from $Lyn^{+/+}$ (*A*) and $Lyn^{-/-}$ (*B*) mice showing numerous eosinophils (→) and activated/vacuolated macrophages (⇒) in $Lyn^{-/-}$ mice (×40; Diff-Quick). Lung parenchyma 72 h post-OVA challenge from $Lyn^{+/+}$ (*C*) and $Lyn^{-/-}$ (*D*) mice showing enhanced Ag-induced goblet cell metaplasia (→) in $Lyn^{-/-}$ small bronchus and extensive parabronchial leukocyte infiltration (×10, PAS/AB). Tracheal whole mounts 4 wk after Ag challenge from $Lyn^{+/+}$ (*E*) and $Lyn^{-/-}$ (*F*) mice. $Lyn^{-/-}$ mice fail to resolve inflammation and show extensive infiltration of eosinophils (→) and cfegs (*circled*), an index of activated eosinophil degranulation (×40, DAB). Mast cells in tracheal whole mounts 24 h post-Ag challenge from $Lyn^{+/+}$ (*G*) and $Lyn^{-/-}$ (*H*) mice. Note annular, irregular contour of partially degranulated $Lyn^{+/+}$ mast cells compared with extensive degranulation of $Lyn^{-/-}$ mast cells (×40, toluidine blue).

Impaired resolution of inflammation in $Lyn^{-/-}$ mice

To determine the basis of the amplified eosinophilia in $Lyn^{-/-}$ mice, we assessed whether this was due to a combination of accelerated eosinophil production, enhanced survival, and/or defective apoptosis.

Previous studies have shown that Lyn contributes to IL-5 signal transduction and terminal differentiation of eosinophils (30), and we have shown that myelopoiesis is markedly enhanced in $Lyn^{-/-}$ mice (27, 31). Therefore, we assessed eosinophil generation in BM

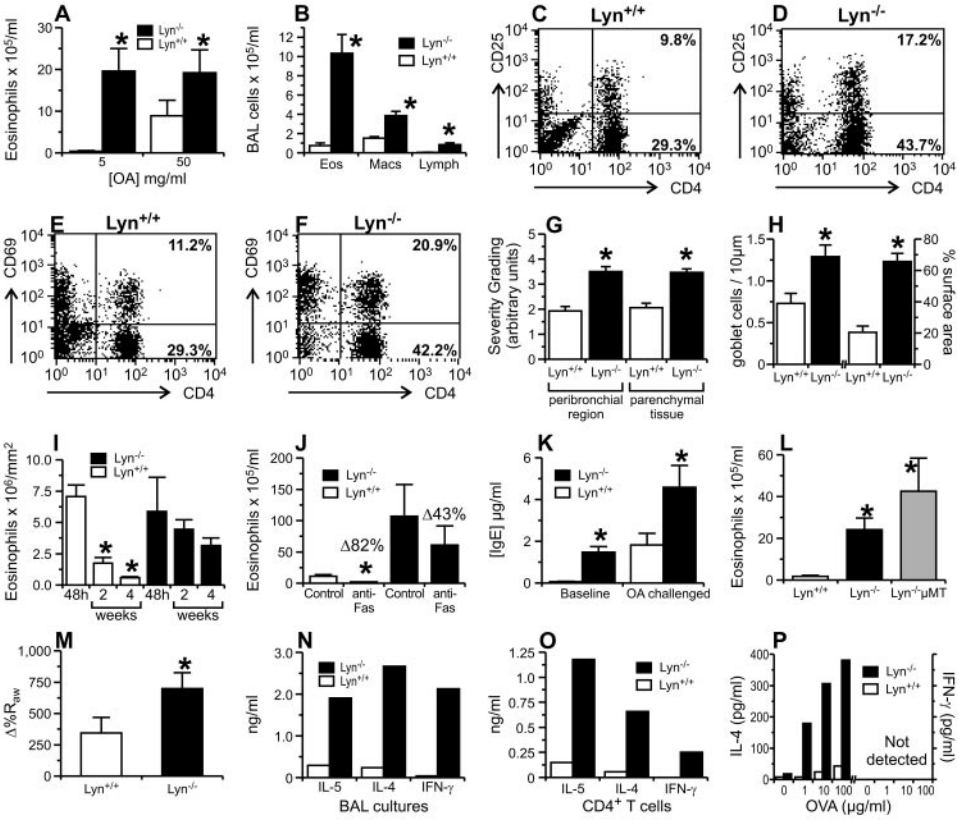

FIGURE 2. Severe multitrait asthma in OVA-challenged $Lyn^{-/-}$ mice. $Lyn^{+/+}$ mice, □; $Lyn^{-/-}$ mice, ■. *A*, BAL eosinophilia following aerosol challenge with the indicated doses of OVA. *B*, Differential analysis of BAL recovered from Ag-challenged mice indicating numbers of eosinophils (Eos), macrophages (Macs), and lymphocytes (Lymph). FACS analysis of BAL derived from Ag-challenged (*C* and *E*) $Lyn^{+/+}$ and (*D* and *F*) $Lyn^{-/-}$ mice gated on lymphocytes using forward and side scatter criteria and stained with Abs to CD4 and CD25 (*C* and *D*) or CD4 and CD69 (*E* and *F*). *G*, Quantitation of inflammation in the peribronchial and parenchymal regions of lung. *H*, Quantitation of numbers of goblet cells lining the bronchus (*left side of graph*) and determination of the PAS/AB (mucus)-stained surface area within the bronchus (*right side of graph*). *I*, Kinetics of eosinophil resolution. Tracheal whole mounts were quantitated for tissue eosinophils by morphometric analysis under conditions where the initial intensity of response was comparable. *J*, Sensitivity of BAL eosinophils to Fas-induced apoptosis. Anti-Fas Ab (Jo-2) was used to trigger apoptosis; an isotype-matched Ab was used as a control. *K*, Serum IgE levels in unsensitized (baseline) and OVA-challenged mice. *L*, BAL eosinophilia in $Lyn^{+/+}$ mice (□), $Lyn^{-/-}$ mice (■), and $Lyn^{-/-}$ *µMT* mice (B cell-deficient $Lyn^{-/-}$ mice, ▨) 48 h post-OVA challenge. *M*, Enhanced bronchospasm induced by inhalation of OVA was measured as percent increase in basal airway resistance (R_{aw}). *N*, Quantitation of IL-4, IL-5, and IFN-γ cytokines from unfractionated BAL cells, and (*O*) purified $CD4^+$ thoracic LN T cells after restimulation on CD3ϵ-coated plates. *P*, Quantitation of IL-4 and IFN-γ levels produced by B220-depleted thoracic LN cells following overnight culture in the indicated concentrations of OVA. In all panels, ∗ indicates $p < 0.05$, unpaired *t* test or ANOVA/Dunnets. Except where pooling of data occurred, all data are mean ± SEM for $n = 8–12$ observations and are typical of three replicate experiments.

colony assays (CFU-eo), pre- and post-Ag challenge. $Lyn^{-/-}$ mice had lower numbers of BM eosinophil progenitors (control sham immunized, $Lyn^{+/+}$ 10.50 ± 0.88 vs $Lyn^{-/-}$ 7.74 ± 0.47*; OVA sensitized, $Lyn^{+/+}$ 8.03 ± 0.90 vs $Lyn^{-/-}$ 5.23 ± 0.63*; sensitized and challenged, $Lyn^{+/+}$ 10.57 ± 0.97 vs $Lyn^{-/-}$ 5.17 ± 0.67* ($p < 0.05$) CFU-eo/25,000 BM cells, $p < 0.05$), indicating that accelerated eosinophilopoiesis does not contribute to the enhanced eosinophilia in Ag-challenged $Lyn^{-/-}$ mice.

To assess the ability of $Lyn^{-/-}$ mice to spontaneously resolve airway inflammation, we measured eosinophilic inflammation in airway tissues up to 1 mo after Ag challenge using the whole-mount technique. Unlike in BAL, there was no significant difference in the degree of eosinophilic inflammation in the tracheas of $Lyn^{+/+}$ or $Lyn^{-/-}$ mice (Fig. 2*I*), presumably because the regulation of eosinophilic inflammation in the alveolar space is different from that in tracheal tissue. However, in $Lyn^{+/+}$ mice, tissue eosinophilic inflammation resolved rapidly and, after 2 wk, had almost completely dissipated (Fig. 2*I*). In contrast, $Lyn^{-/-}$ mice showed persistent, intense eosinophilia in tracheal tissue lasting at least 1 mo (Figs. 1*F* and 2*I*). In BrdU pulse-chase survival studies, more eosinophils in $Lyn^{-/-}$ BAL measured 2 wk postallergen inhalation were BrdU positive (95 vs 73% in wild type, $n = 5$), indicating extended survival.

Apoptotic lung eosinophils are rapidly cleared by macrophages (24), making it difficult to assess rates of apoptosis in vivo. Thus, susceptibility to apoptosis was measured indirectly using FasR cross-linking Abs, which directly induce apoptosis (24). We found that compared with controls, $Lyn^{-/-}$ eosinophils were markedly resistant to Fas-induced apoptosis, particularly in the BAL airway compartment (Fig. 2*J*). Collectively, these data suggest that enhanced survival and defects in apoptosis, but not eosinophil production, contribute to the intensity of inflammation in $Lyn^{-/-}$ mice.

Contribution of mast cells, B cells, and IgE to asthma pathology in $Lyn^{-/-}$ mice

Atopy is an extremely common trait in human asthma where cross-linking of Ag-specific IgE bound to FcϵRI on mast cells results in the release of inflammatory mediators. As Lyn participates in signaling from FcϵRI (10) and B cell IgE class switching by regulating IL-4 sensitivity (13), we assessed IgE and IgE-related functional responses. $Lyn^{-/-}$ mice showed elevated titers of IgE at

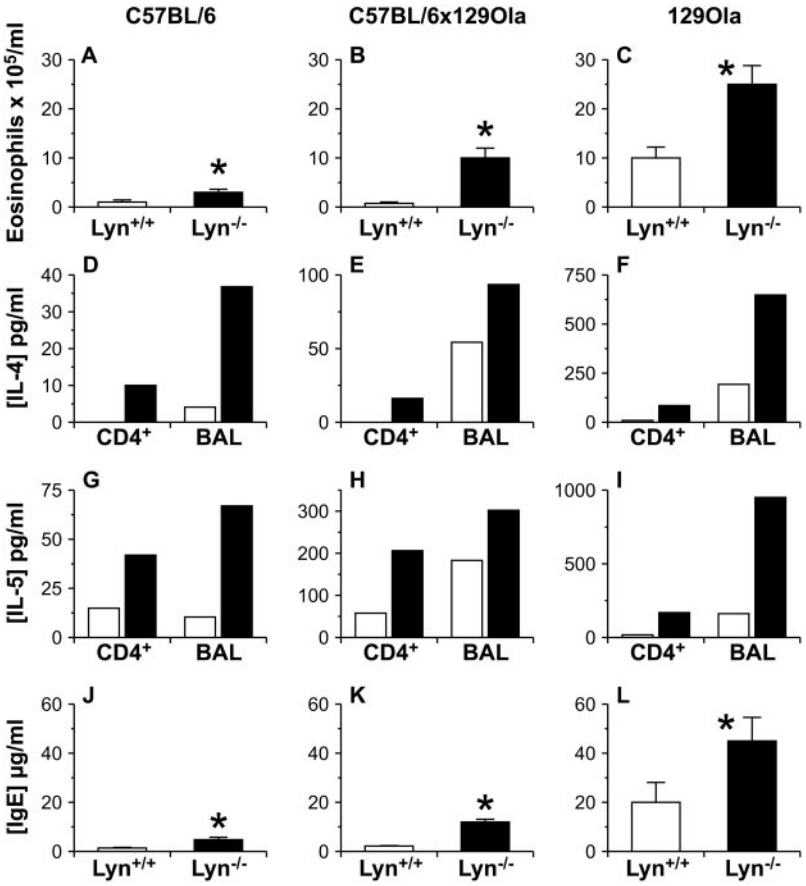

FIGURE 3. Effect of genetic background on eosinophilia, cytokine production, and serum IgE levels in Ag-challenged $Lyn^{-/-}$ mice. Eosinophilia (*A–C*); IL-4 levels in $CD4^+$ LN T cells or BAL cells (*D–F*); IL-5 levels in $CD4^+$ LN T cells or BAL cells (*G–I*); and IgE serum concentrations in C57BL/6 background mice, 129Ola × C57BL/6 mixed genetic background mice, or 129Ola background mice as indicated (*J–L*). Results are mean ± SEM ($n = 8–12$ mice/group) ∗, $p < 0.05$ unpaired *t* test. ELISA samples were pooled and assayed in triplicate (coefficient of variation ≤ 5%).

baseline that were further enhanced after Ag challenge (Fig. 2*K*) (13). Furthermore, we observed hyperdegranulation of mast cells in tracheal whole mounts in OVA-challenged $Lyn^{-/-}$ mice, although mast cell numbers were not significantly different (Table I; Fig. 1, *G* and *H*). These results are consistent with studies showing that FcεRI-dependent degranulation is enhanced in mast cells from $Lyn^{-/-}$ mice (32). However, it is unlikely that elevated titers of IgE or IgE-dependent mediators make a significant contribution to the intensity of mast cell degranulation in $Lyn^{-/-}$ mice because Ag-challenged compound $Lyn^{-/-}\mu MT$ mice (B cell-deficient $Lyn^{-/-}$ mice) lacking IgE also showed mast cell hyperdegranulation (Table I). Interestingly, compound $Lyn^{-/-}\mu MT$ mice also mounted profound eosinophilia following OVA challenge (Fig. 2*L*), suggesting that neither the elevated titers of IgE nor IgE-dependent pathways mediate the intensity or persistence of eosinophilic inflammation in $Lyn^{-/-}$ mice.

Enhanced bronchospasm in $Lyn^{-/-}$ mice

Because mast cell mediators are potent bronchoconstrictors, we assessed lung function in $Lyn^{-/-}$ mice (25). $Lyn^{-/-}$ mice showed significantly enhanced bronchoconstriction following inhalation of OVA compared with similarly treated control animals (Fig. 2*M*), although in vitro airway smooth muscle responses to methacholine (26) were not different between genotypes (pEC_{50}: $Lyn^{+/+}$ 5.8 ± 0.08 vs $Lyn^{-/-}$ 5.7 ± 0.14, NS).

Enhanced mucosal immunity in Ag-challenged $Lyn^{-/-}$ mice

Deregulated T cell cytokine production is a very prominent trait in human asthma (33) and, supported by extensive evidence in mouse models, is widely held to be an essential process in asthma pathogenesis (6–8). Therefore, we measured the concentration of cytokines associated with mucosal inflammation (IL-4, IL-5, and IFN-γ) in purified thoracic-draining LN $CD4^+$ T cells and unfractionated BAL cells, following culture on CD3-coated plates. Compared with cultures of $Lyn^{+/+}$ BAL and $CD4^+$ LN T cells, we found that $Lyn^{-/-}$ BAL and $CD4^+$ LN T cells produced significantly higher levels of IL-4, IL-5, and IFN-γ (Fig. 2, *N* and *O*). As the high concentration on IFN-γ was unexpected, we determined whether this could be a bystander effect by assessing the ability of Ag-specific T cells to produce IL-4 and IFN-γ when cultured with

Table I. *Allergen-induced mast cell activation*[a]

	Wild Type Baseline[b]	Wild Type Postallergen[b]	$Lyn^{-/-}$ Postallergen[b]	$Lyn^{-/-}$ μMT Postallergen[b]
No. of cells	n.d.[c]	356 ± 62	480 ± 79	291 ± 27
Degranulation index	121 ± 15	246 ± 29[d]	521 ± 82[d,e]	766 ± 98[d,e]

[a] Mast cell number and degranulation index were assessed in toluidine blue-stained tracheal whole mounts as described in *Materials and Methods.*
[b] $n = 6–8$
[c] n.d., not done.
[d] $p < 0.05$ compared with wild-type baseline.
[e] $p < 0.05$ compared with wild-type postallergen.

OVA. Under these conditions, B220-depleted LN cells from Ag-challenged $Lyn^{-/-}$ mice produced much higher amounts of IL-4 than controls (Fig. 2*P*); however, IFN-γ was not detectable in either genotype (Fig. 2*P*). Thus, collectively these data indicate that when stimulated with Ags, $Lyn^{-/-}$ lymphocytes secrete predominantly Th2 cytokines and that IFN-γ is most likely produced by bystander cells as a result of nonspecific T cell stimulation. Therefore, Lyn might also contribute to the severity of asthma via an influence on bystander T cell IFN-γ release.

Mucosal immunity and airway inflammation is independent of genetic background

The exaggerated cytokine profile we observed suggests that Lyn might affect the penetrance of genes regulating allergic inflammation in mixed genetic background 129Ola × C57BL/6 mice. Therefore, we re-examined the asthma response of $Lyn^{-/-}$ mice backcrossed onto C57BL/6 (Th1-biased) or 129Ola (Th2-biased) backgrounds. Although considered Th1-biased, C57BL/6 $Lyn^{-/-}$ mice still mounted enhanced eosinophilia (Fig. 3*A*) with matching elevated IL-4 and IL-5 cytokines in both BAL and $CD4^+$ LN T cell cultures (Fig. 3, *D* and *G*). This mirrored a near identical, but stronger, pattern on the 129Ola background (Fig. 3, *C*, *F*, and *I*). Similarly, IgE levels were elevated in $Lyn^{-/-}$ mice, regardless of genetic background (Fig. 3, *J–L*). These data indicate that Lyn exerts an influence on the intensity of asthma-like inflammation independent of background genetic determinants.

Role of T cells and DCs in deregulated mucosal immunity

It is generally accepted that Lyn is not expressed in primary T lymphocytes; however, the observed lung inflammation in Ag-challenged $Lyn^{-/-}$ mice suggests that Lyn might play an important role in the T cell response. Because Lyn has been observed in T cells producing human T cell leukemia virus-I (34), we examined whether Lyn expression can be induced in primary T cells following immunization. We sorted T cells from the spleen and LN of naive mice and mice that had been immunized with OVA and assessed Lyn expression by Western blotting. Although Lyn was expressed in LN and spleen B cells, it was not detected in $CD4^+$ T cells purified from Ag-sensitized mice (Fig. 4*A*) or in $CD3^+$ T cells purified from naive mice, even following culture with IL-2 (Fig. 4*B*).

Because the exaggerated T cell cytokine production we observed was unlikely to be a direct T cell effect, we explored whether Lyn was expressed in DCs, which are critically involved in the initiation of T cell-dependent immune responses. DCs were derived from the BM of $Lyn^{+/+}$ and $Lyn^{-/-}$ mice in GM-CSF, and Lyn levels were examined by Western blotting. Fig. 4*C* shows that in addition to PIR-B, SHP-1, and SHIP-1 Lyn is also expressed in DCs. Although $Lyn^{+/+}$ and $Lyn^{-/-}$ DCs were $CD11c^+MHCII^+Mac1^+$ (data not shown), $Lyn^{-/-}$ DCs had a more immature phenotype in that they consistently expressed lower levels of MHC class II, CD86 (B7-2), CD40, and CD80 (B7-1) before and following OVA uptake and LPS stimulation (Fig. 5, *A* and *B*, and data not shown). This phenotype closely resembles that of DCs from Th2-prone $PIR\text{-}B^{-/-}$ mice (35), and because we and others (27, 36, 37) have shown previously that Lyn regulates the phosphorylation of PIR-B in myeloid cells and B lymphocytes, we assessed the tyrosine phosphorylation status of PIR-B in $Lyn^{-/-}$ DCs. Ag-pulsed DCs from $Lyn^{-/-}$ mice showed substantially less phosphorylation of PIR-B and diminished SHP-1 association, indicating that Lyn regulates the PIR-B pathway in

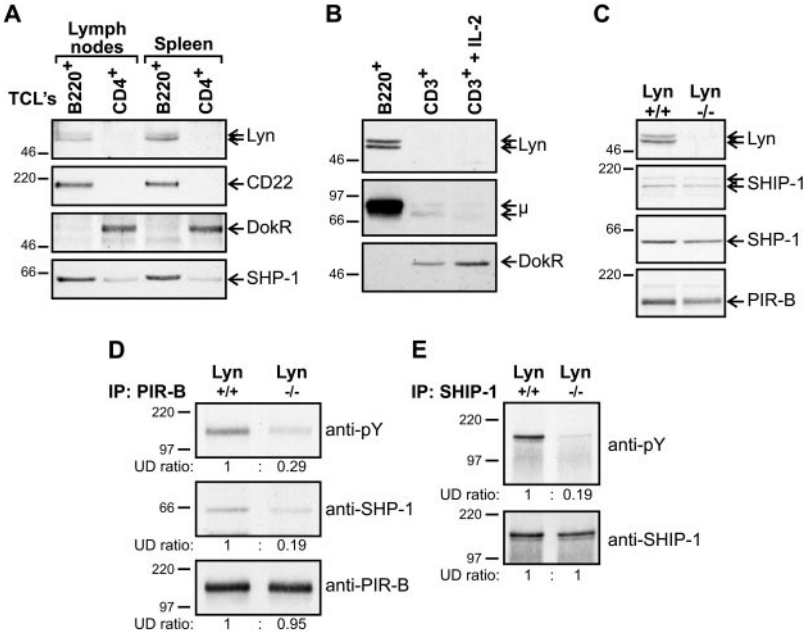

FIGURE 4. Lyn expression pattern in T cells and DCs and Lyn-dependent signaling in DCs. *A*, Western blot analysis of Lyn expression in $B220^+$ B cells and $CD4^+$ T cells sorted from the LN or spleen of OVA-immunized and -challenged mice. The blot was stripped and reprobed with anti-CD22 as a marker of B cells, anti-DokR as a marker of T cells, and anti-SHP-1 for protein loading. *B*, Western blot analysis of Lyn expression in sorted splenic B cells ($B220^+$), T cells ($CD3^+$), and IL-2-stimulated T cells ($CD3^+$ + IL-2). The blot was stripped and reprobed with anti-IgM (μ) and anti-DokR to indicate purity. *C*, Western blot analysis of Lyn expression in BM-derived DCs from $Lyn^{+/+}$ and $Lyn^{-/-}$ mice. The blot was stripped and reprobed with anti-SHIP, anti-SHP-1, and anti-PIR-B. In *A–C*, the two isoforms of Lyn are indicated by arrows. *D*, PIR-B was immunoprecipitated from $Lyn^{+/+}$ and $Lyn^{-/-}$ DC lysate and blotted with anti-PY. The blot was stripped and reprobed with anti-SHP-1 to demonstrate coassociation of SHP-1 and PIR-B, as well as anti-PIR-B to demonstrate equal protein loading. *E*, SHIP-1 was immunoprecipitated from $Lyn^{+/+}$ and $Lyn^{-/-}$ DC lysate and blotted with anti-PY. The blot was stripped and reprobed with anti-SHIP-1 for protein loading. (UD = units of density, as measured by densitometry. For *D* and *E*, the density of bands was calculated and expressed as a ratio of the $Lyn^{+/+}$ band)

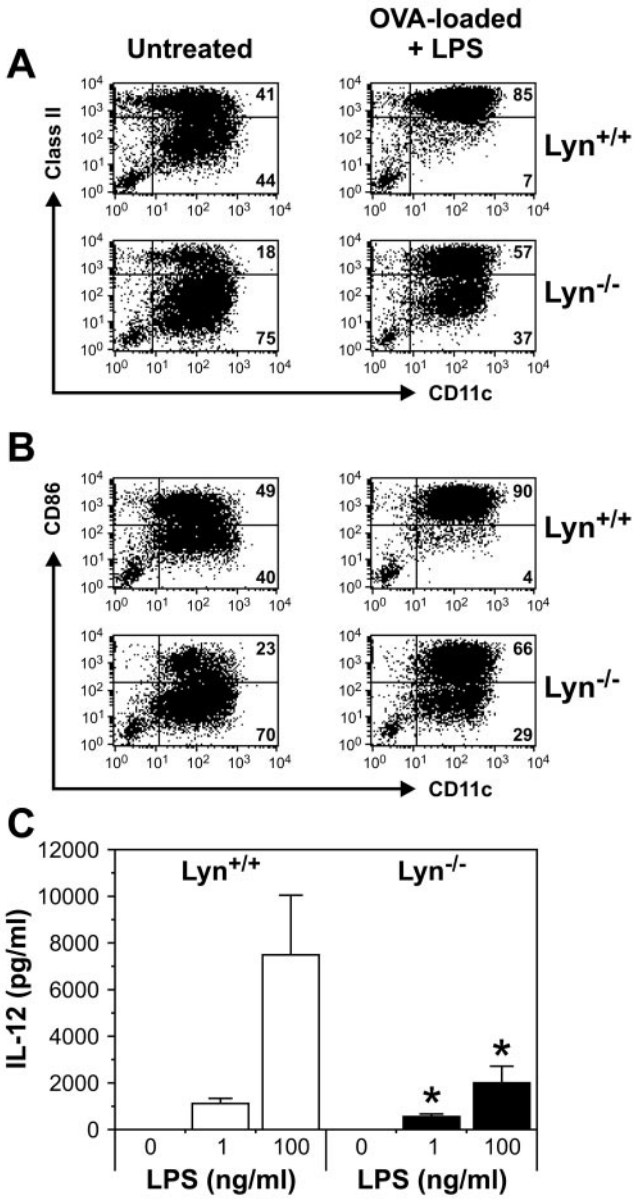

FIGURE 5. BM-derived DCs from $Lyn^{-/-}$ mice are less mature than wild-type DCs and show diminished IL-12 production. *A* and *B*, Representative two-color fluorescence analysis of BM DCs from $Lyn^{+/+}$ or $Lyn^{-/-}$ mice derived in GM-CSF for 10 days. Cells were either untreated or OVA-loaded (100 μg/ml) for 24 h and stimulated with 100 ng/ml LPS for 1 h. Staining was performed with Abs against (*A*) CD11c and class II or (*B*) CD11c and CD86 as a marker of maturation. *C*, Quantitation of IL-12 levels produced by $Lyn^{+/+}$ DCs (□) or $Lyn^{-/-}$ DCs (■) following stimulation with 0, 1, or 100 ng/ml LPS. Results are mean ± SD and are a representative of four experiments. *, $p < 0.02$, unpaired *t* test.

DCs (Fig. 4*D*). These results suggest that PIR-B function and Lyn activity are intimately linked and associated with DC maturation.

Cytokines produced by DCs are critical for shaping T cell differentiation and function. IL-12 is critical for Th1 cytokine responses and considered an inhibitory factor for allergic responses and Th2 cytokine production. Indeed, it has been shown recently that overexpression of IL-12 by DCs strongly polarized naive OVA-specific $CD4^+$ T cells toward a Th1 phenotype in vitro and in vivo, and these DCs failed to induce eosinophilic airway inflammation (38). Therefore, we measured IL-12 production by $Lyn^{-/-}$ DCs in response to LPS and found it to be impaired compared with control DCs (Fig. 5*C*). This difference was observed at all LPS doses tested. Because the PI3K pathway negatively regulates IL-12 synthesis by DCs (39) and the inhibitory phosphatase SHIP-1 is an antagonist of this pathway (40, 41), we investigated the phosphorylation status of SHIP-1. In contrast to resting $Lyn^{+/+}$ DCs where SHIP-1 was heavily tyrosine phosphorylated, SHIP-1 showed minimal tyrosine phosphorylation in $Lyn^{-/-}$ DCs (Fig. 4*E*), indicating that its phosphorylation is regulated by Lyn.

We next examined the ability of $Lyn^{-/-}$ DCs to induce mucosal inflammation. Ag-pulsed DCs derived from $Lyn^{+/+}$ or $Lyn^{-/-}$ mice were adoptively transferred into Ag-naive $Lyn^{+/+}$ recipients. Following challenge of the recipient mice with 50 mg/ml aerosolized OVA, animals adoptively transferred with $Lyn^{-/-}$ DCs displayed a more intense inflammation in BAL than mice reconstituted with $Lyn^{+/+}$ DCs (Fig. 6, *A* and *B*), consistent with responses in Ag-challenged $Lyn^{-/-}$ mice (Fig. 2*B*). Animals reconstituted with $Lyn^{-/-}$ DCs had enhanced numbers of macrophages, eosinophils, and lymphocytes in BAL compared with mice receiving $Lyn^{+/+}$ DCs (Fig. 6*B*). The relatively weak lung inflammatory response seen in recipient animals is presumably because their BM eosinophil reserves have not been boosted by prior immunization (42). Nonetheless, CD3-stimulated BAL cells from animals adoptively transferred with $Lyn^{-/-}$ DCs displayed exaggerated cytokine production compared with those from mice reconstituted with $Lyn^{+/+}$ DCs (Fig. 6, *C–F*), which is in keeping with the cytokine patterns seen in Ag-challenged mice (Fig. 2, *N* and *O*). Thus, these data strongly support the notion that Lyn negatively regulates

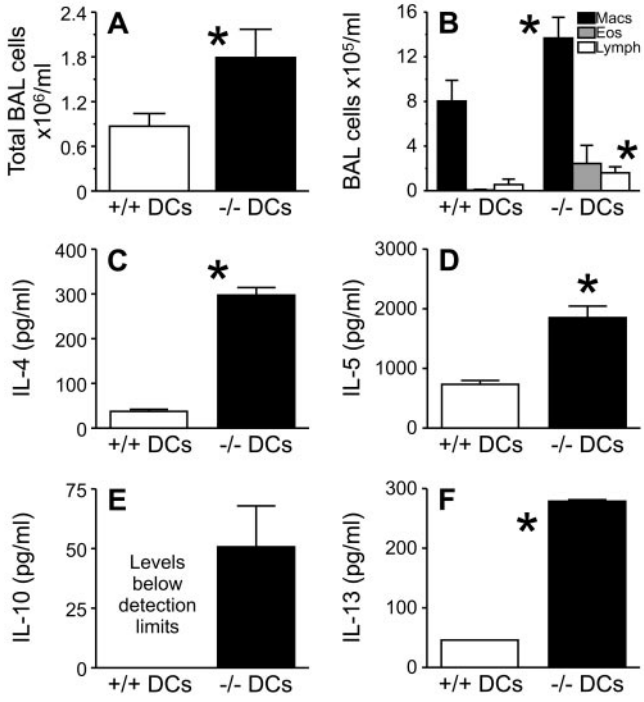

FIGURE 6. Adoptive transfer of OVA-loaded $Lyn^{-/-}$ DCs into wild-type mice induces an exaggerated mucosal immune response to aeroallergen. Total cellularity (*A*) and BAL cell profile (*B*) indicating numbers of macrophages (Macs), eosinophils (Eos), and lymphocytes (Lymph) recovered from animals adoptively transferred with $Lyn^{+/+}$ DCs or $Lyn^{-/-}$ DCs. Cytospins were stained with Diff-Quick, and differential cell counts were performed on a minimum of 300 cells ($n = 7$ mice/group). Quantitation of IL-4 (*C*), IL-5 (*D*), IL-10 (*E*), and IL-13 (*F*) levels produced by culture of BAL cells on CD3-coated plates following harvest from mice adoptively transferred with $Lyn^{+/+}$ DCs (□) or $Lyn^{-/-}$ DCs (■). Results are expressed as mean ± SEM and are representative of two experiments. *, $p < 0.05$, *t* test.

T cell cytokine production at the DC level and suggest that Lyn plays a critical role in negative regulation of mucosal inflammatory responses to aeroallergen.

Discussion

This study demonstrates that a single molecular defect in Lyn disturbs a multiplicity of cellular and molecular processes that culminate in a severe, persistent asthma-like syndrome. Our data indicate that Lyn regulates, either directly or indirectly, several stages of asthma diathesis that include at least 1) atopy, 2) intensity and 3) persistence of mucosal inflammation, 4) T cell cytokine profile, 5) DC polarization, and 6) the intensity of bronchospasm after allergen challenge. Although it is known that $CD4^+$ T cells are essential for the initiation of asthma (43), our data indicates that defects outside the T cell compartment can regulate the intensity and persistence of Th2-driven mucosal inflammation.

Although Lyn participates in positive signaling, Lyn is largely classified as a negative regulatory kinase. In B lymphocytes and myeloid cells, Lyn recruits phosphatases such as SHP-1, SHP-2, and SHIP-1 to Lyn-dependent phosphorylation sites in inhibitory receptors such as CD22, FcγRIIB1, PIR-B, and SIRP-α for signal down-regulation (27, 36, 37, 44–47). Altered signaling through several of these pathways has been associated with increased allergic airway inflammation and Th2 immunity (35, 48). SHP-1 appears to play a key role as a negative regulator of the asthma phenotype because heterozygous *moth-eaten* mice (*me*/+) that carry a mutation in one allele of *SHP-1* show enhanced Th2-dependent airway inflammation (48). This is attributed to the role of SHP-1 in down-regulating IL-4R signaling in T cells because naive *me*/+ CD4 T cells show enhanced and prolonged IL-4-induced STAT-6 phosphorylation, increased Th2 cell differentiation, and augmented Th2 cytokine production (14). PIR-B, as with Lyn, is not expressed in T cells; however, *PIR-B*$^{-/-}$ mice showed significantly augmented IgG1 and IgE responses (35). In *PIR-B*$^{-/-}$ mice, this enhanced Th2 immune response has been attributed to Th2-polarizing DCs because adoptive transfer of *PIR-B*$^{-/-}$ DCs into wild-type mice leads to enhanced IL-4 production (35). Intriguingly, *Lyn*$^{-/-}$ mice have a similar phenotype. *Lyn*$^{-/-}$ mice have increased numbers of IgG1 Ab-forming cells and 10-fold increases in serum IgE, and *Lyn*$^{-/-}$ B cells show an increased propensity to isotype switch in response to IL-4 (13). We have now demonstrated that *Lyn*$^{-/-}$ DCs show substantially reduced phosphorylation of PIR-B and, as with *PIR-B*$^{-/-}$ DCs, are less mature and defective in IL-12 production. Moreover, our studies, such as those of *PIR-B*$^{-/-}$ mice, indicate that DCs play a pivotal role in this phenotype because *Lyn*$^{-/-}$ DCs are capable of inducing an asthma-like phenotype when adoptively transferred. We suggest that in DCs, PIR-B is operating through a Lyn-dependent mechanism to induce T cell polarization. Although substantially impaired, PIR-B is still tyrosine phosphorylated to a small degree in *Lyn*$^{-/-}$ DCs, which may reflect the activity of other Src family kinases operating in DCs. Interestingly, a recent study has implicated the myeloid-specific Src family kinases Hck and Fgr in negative regulation of chemokine signaling in neutrophils and DCs that operate through PIR-B (49).

The PI3K pathway is critical for negatively regulating the synthesis of IL-12 by DCs (39), and we have shown that SHIP-1, an antagonist of this pathway (40, 41) that is constitutively phosphorylated in resting control DCs, has significantly reduced tyrosine phosphorylation in *Lyn*$^{-/-}$ DCs. Although Lyn has been implicated in the phosphorylation status of SHIP-1 in several different cell types (27, 50–53), two recent studies have also shown that SHIP-1 activity is impaired in the context of Lyn deficiency (53, 54). We propose that SHIP-1 may not be appropriately functional in modulating the PI3K pathway in *Lyn*$^{-/-}$ DCs, explaining the diminished IL-12 synthesis. Additionally, IL-12 production is dependent on the costimulatory activity of CD40 and MHCII because anti-CD40L Abs can block IL-12 production, and activated T cells from CD40L-deficient mice fail to elicit IL-12 secretion from splenic monocytes or to mount Th1 immune responses (55, 56). Therefore, the failure of Lyn-deficient DCs to appropriately upregulate costimulatory molecules may contribute to their inability to secrete IL-12, thus biasing immunity toward a Th2 phenotype. The defective expression of costimulatory molecules on *Lyn*$^{-/-}$ DCs may also extend to inhibitor family members such as programmed death ligand 1 and PD-L2, which are known to effect T cell tolerance and cytokine synthesis (57, 58) and would provide a mechanism by which the absence of Lyn, in cells other than T cells, could regulate both Th1 immunity such as the experimental allergic encephalomyelitis model (19), as well as the OVA model described in the present study.

The constellation of asthma traits in *Lyn*$^{-/-}$ mice closely resembles chronic, severe human asthma. In particular, we demonstrate that Ag-challenged *Lyn*$^{-/-}$ mice develop cfegs, a feature of human asthma that is seldom observed in mouse models (59, 60). Because Lyn is not expressed in T cells, our study offers new insights into the molecular pathogenesis of this often life-threatening disease. We propose that reduction in the net efficiency of Lyn-dependent signal transduction pathways in a multitude of immune and inflammatory cells may explain why some people develop severe, persistent asthma. Although our studies clearly do not lend support to the concept that inhibiting this enzyme would be a viable strategy for treating allergic airway disease, we nonetheless suggest that Lyn, or components of the Lyn-dependent signaling machinery, may prove to be valuable targets for therapeutic manipulation of DC-induced immune responses. It now remains to determine the role of Lyn-dependent signal transduction pathways in human disease.

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Disclosures

The authors have no financial conflict of interest.

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