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The Lyn Tyrosine Kinase Differentially Regulates Dendritic Cell Generation and Maturation

Ching-Liang Chu and Clifford A. Lowell

The Src family kinase Lyn plays both stimulatory and inhibitory roles in hemopoietic cells. In this report we provide evidence that Lyn is involved in dendritic cell (DC) generation and maturation. Loss of Lyn promoted DC expansion in vitro from bone marrow precursors due to enhanced generation and accelerated differentiation of Lyn-deficient DC progenitors. Differentiated Lyn-deficient DCs also had a higher survival rate. Similarly, the CD11c-positive cell number was increased in aged Lyn-deficient mice in vivo. In contrast to their enhanced generation, lyn−/− DCs failed to mature appropriately in response to innate stimuli, resulting in DCs with lower levels of MHC class II and costimulatory molecules. In addition, IL-12 production and Ag-specific T cell activation were reduced in lyn−/− DCs after maturation, resulting in impaired Th1 responses. This is the first study to characterize Lyn-deficient DCs. Our results suggest that Lyn kinase plays uniquely negative and positive regulatory roles in DC generation and maturation, respectively. The Journal of Immunology, 2005, 175: 2880–2889.

Dendritic cells (DCs) are specialized hemopoietic cells that capture Ags in peripheral tissues, process the Ags to generate peptide-MHC complexes, then migrate to secondary lymphoid organs where they activate T cells to initiate adaptive immune responses (1, 2). Depending on the type of pathogen or the Ag dose, DCs can promote the specific polarization of Th cells, which, in turn, guides either cellular or humoral immune responses (3–5). In addition to the regulation of adaptive immunity, DCs are involved in innate immunity. For example, plasmacytoid DCs produce massive amounts of type I IFN in response to virus infection (6). Exposure of DCs to pathogen products or proinflammatory stimuli influences the way that cells mature during their migration from peripheral tissues to regional lymphoid organs. Many stimuli can induce DC maturation, such as microbial products (LPS and CpG), inflammatory cytokines (TNF-α and IL-1), and CD40L (7). These stimuli trigger a series of intracellular signaling pathways, consisting of kinases, phosphatases, adaptors, and transcription factors. Among these activation signals, the TLR signaling pathway has been demonstrated to activate NF-κB and MAPK pathways in MyD88-dependent and independent manners, resulting in the up-regulation of MHC class II (MHC-II)/costimulatory molecule expression and cytokine production (8–10). Several tyrosine kinases have been implicated in these signaling events. However, the contribution of Src family kinases to DC generation and maturation remains poorly studied.

Src family kinases play a critical role in many intracellular signaling events in hemopoietic cells, including involvement in innate immune signaling, responses to cytokines and growth factors, and stimulation of cells by Ags and immune complexes (11, 12). Numerous studies have implicated these kinases in LPS signaling, primarily in macrophages (13–20). In addition, Src family kinases are involved in signaling events in DCs. Upon TNF-related activation-induced cytokine-TNF-related activation-induced cytokine receptor ligation, Src is activated and, in turn, contributes to the activation of Akt/protein kinase B (21). Src family kinases may also participate in BDCA-2 signaling in plasmacytoid DCs (22). In monocyte-derived DCs, Src family kinases have been implicated in LPS signaling for cytokine production, either through controlling AP-1 formation (23) or regulating cAMP-protein kinase A-dependent pathway (24). Collectively, these studies provide evidence to suggest the importance of Src family kinases in innate immune signaling. However, the majority of these studies have relied on the use of tyrosine kinase inhibitors, which may have broader specificity than just Src family kinases, thus complicating the interpretation of these results.

Lyn is one of the major Src family kinases in B lymphocytes and myeloid cells, including DCs. Lyn is a positive regulator in BCR, CD40, IL-5R, and LPS signaling pathways (25–31). However, Lyn also plays a negative role in the regulation of signaling, based on the studies using Lyn-deficient mice (32–34). Lyn−/− B cells display a hyperactive response after BCR triggering, which may, in turn, contribute to the lupus-like autoimmune diseases observed in lyn−/− mice (35, 36). Macrophages and neutrophils are dramatically expanded in aged lyn−/− mice, suggesting that Lyn negatively regulates differentiation of these cell types (37). Other studies have shown that Lyn negatively regulates M-CSF signaling in macrophages (38) and integrin signaling in neutrophils and platelets (39, 40). Lyn−/− mast cells show a hyperpolarization in response to growth factors and enhanced IgE-dependent allergic responses (41, 42). Furthermore, the regulation of Lyn kinase activity is critical in mast cell degranulation (43). Despite many studies in myeloid cells, the effect of Lyn deficiency in DCs has not yet been explored.

In this study we examine the contributions of Lyn to DC generation and maturation. Lyn negatively regulated the generation of DCs derived from bone marrow (BM) precursors, as shown by the increased expansion of lyn−/− bone marrow-derived DC (BMDCs). However, most of these BMDCs showed a less mature phenotype and, hence, had relatively reduced capacity to stimulate

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Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived DC; 5-FU, 5-fluorouracil; LN, lymph node; MHC-II, MHC class II; PIR-B, paired Ig-like receptor B; poly(1C), polyinosinic-polycytidylic acid; WT, wild type.
Ag-specific T cells. These data suggest that Lyn differentially reg- 
ulates aspects of DC function.

Materials and Methods

Mice

All experimental mice used were at 10–14 wk of age unless otherwise 
noted. Lyn−/− mice were previously described (44, 45). In brief, BM cells 
were isolated from femurs and tibias and seeded on Falcon 24-well culture 
plates (BD Biosciences Labware) or petri dishes (Kord-Valmark) in 1 or 10 
ml of RPMI 1640 medium (University of California Cell Culture Facility) 
supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, nones-
sential amino acids, sodium pyruvate, HEPES (all from Invitrogen Life 
Technologies), 5 × 10^−3 M 2-ME (Sigma-Aldrich), and 10 ng/ml recom-
binant mouse GM-CSF (PeproTech). On day 3, 1 or 10 ml of medium 
containing 10 ng/ml GM-CSF was added to plates or dishes. On days 5 and 
7, half the cell-free supernatant was exchanged, and fresh medium con-
taining 10 ng/ml GM-CSF was added. The 8- to 12-day-cultured BMDCs 
(>80% CD11c+ cells) were used for all experiments. In some experiments, 
DCs were purified by EasySep Positive Selection Kit (StemCell Technol-
ogy) according to the manufacturer’s instruction. The purity of CD11c+ 
cells was >95% (data not shown). To determine the number of DCs in 
mice, spleen cells were harvested and incubated for 30 min at 37°C in 
RPMI 1640 with collagenase and DNase (Roche). Cells were collected, 
washed in PBS (containing 5 mM EDTA), then counted by hemocytometry 
and analyzed by flow cytometry.

Immunoblotting

Western blots were performed as previously described (46). All Abs for 
Src, Hck, Fgr, Lyn, Lck, Fyn, Yes, and Erk2 were purchased from 
Santa Cruz Biotechnology. We used HRP-conjugated secondary Abs and 
Src, Hck, Fgr, Lyn, and Lck, but not Fyn, Blk, or Yes 
expressed Hck, Fgr, Lyn, and Src, but not Lck, Fyn, Blk, or Yes
profile of Src family kinases has not been reported in DCs. Using 
Src family kinases are critical regulators of many intracellular sig-
als in 96-well, flat-bottom plates (Costar Corning) with various stimuli 
and OVA323–339 peptide (a gift from Dr. L. Fong, University of California, 
San Francisco, CA), and incubated for 3 h. CD4 T cells were isolated from 
OT II transgenic mice with Dynabeads (Dynal Biotech) according to 
the manufacturer’s instructions and added to culture at 2 × 10^5/well to a 
final volume of 200 ml. Cells were incubated for 72 h. [3H]Thymidine (1 μCi/ 
well) was added during the last 16 h of culture, and incorporation was 
measured by scintillation counting. For in vivo assays, cells isolated from 
spleens and lymph nodes (LN) of OT II transgenic mice were labeled with 
5 μM CFSE for 10 min at room temperature. After washing, CFSE-labeled 
cells, containing 5 × 10^7 OT II T cells/mouse, were injected i.v. into B6 WT 
mice. One day later, mice were then injected with WT or lyn−/− DCs 
(10^5/mouse) that had been cultured with 1 μg/ml OVA323–339 peptide 
and 1 ng/ml LPS for 16 h previously. OT II T cell proliferation was determined 
by flow cytometry, assessing for CFSE dilution in CD4+ T cells.

Proliferation assays

For in vitro assays, purified DCs were seeded at the indicated concentra-
tions in 96-well, flat-bottom plates (Costar Corning) with various stimuli 
and polyinosinic-polyribocytidyl acid (poly(I:C); Sigma-Aldrich), and 
Escherichia coli; Salmonella thyphimurium; CpG 
was included for 4 h before harvest. Staining was performed using the Fix 
and Perm Cell Permeabilization Kit (Caltag Laboratories). For ELISA, 
supernatants were collected from purified DC cultures after 24 h or from T 
cell cultures after 3 days. The production of IL-2, IL-6, IL-10, IL-12, 
TNF-α, and IFN-γ in DC cultures and that of IL-4 and IFN-γ in T cell 
cultures were measured by ELISA using cytokine-specific paired Abs ac-
cording to the manufacturer’s protocol (BD Pharmingen).

Results

Expression of Src family kinases in WT and lyn−/− DCs

Src family kinases are critical regulators of many intracellular sig-
aling pathways in immune cells (12). However, the expression profile of Src family kinases has not been reported in DCs. Using 
immunoblotting with specific Abs, we found that WT BMDCs 
expressed Hck, Fgr, Lyn, and Src, but not Lck, Fyn, Blk, or Yes (Fig. 1). Lyn-deficient DCs expressed an equivalent level of these 
kine, except for Lyn itself, indicating that the loss of Lyn did not 
leave hanging threads. Lyn is required for the expression of other Src family kinases in a compensa-
tory manner.

Immune response assays

In vitro. OT II T cells (10^6) were activated with 1 ng/ml LPS-treated WT 
or lyn−/− BMDCs (10^6) and 1 μg/ml OVA323–339 peptide in 12-well plates 
for 4 days, and supernatants were collected as the first stimulation. The OT II 
cells were then transferred to new wells and cultured for 24 h in the 
presence of 5 ng/ml IL-2 (PeproTech). The activated T cells were restim-
ulated with LPS-treated WT or lyn−/− BMDCs and OVA323–339 peptide 
for an additional 3 days, after which supernatants were collected, and IFN-γ 
and IL-4 were determined by ELISA.

In vivo. WT or lyn−/− BMDCs were incubated with 1 ng/ml LPS and 1 
μg/ml OVA323–339 peptide for 16 h, then transferred into OT II transgenic 
mice via i.v. injection (10^6 DC/mouse). Splenocytes were isolated from 
BMDC-immunized mice after 4 days and cultured in 96-well plates at 5 × 
10^5 cells/well with 1 μg/ml OVA323–339 peptide. Supernatants were 
collected from 3-day cultures, and IFN-γ and IL-4 were determined by 
ELISA.

WT or lyn−/− mice were immunized with 100 μg/mouse OVA (Sigma-
Aldrich) mixed with CFA (Sigma-Aldrich) via s.c. injection or alum 
(Pierce) via i.p. injection. Draining LN cells and splenocytes were isolated 
from immunized mice after 10 days and cultured in 96-well plates at 5 × 
10^5 cells/well with 100 μg/ml OVA for 3 days. Alternatively, LN or splenic 
CD4 T cells were isolated and cocultured with WT or lyn−/− BMDCs (T 
cell:DC ratio of 1:1) preincubated with 100 μg/ml OVA for 16 h for 3 days. 
Supernatants were collected from 3-day cultures, and IFN-γ and IL-4 were 
determined by ELISA. To measure Ag uptake by BMDCs, cells were incu-
bated with 100 μg/ml FITC-labeled albumin (Sigma-Aldrich) at 0 or 
37°C for 2 h. The incorporation of FITC-albumin was monitored by flow 
cytometry gating on CD11c+ cells.

WT or lyn−/− BMDCs were incubated with 100 μg/ml OVA for 3 h and 
then transferred into WT mice via i.v. injection (10^6 DCs/mouse). Spleno-
cytes were isolated from BMDC-immunized mice after 7 days and cultured 
in 96-well plates at 5 × 10^5 cells/well with 100 μg/ml OVA. Supernatants 
were collected from 3-day culture, and IFN-γ and IL-4 were determined by 
ELISA.
Enhanced expansion of DCs from lyn-/- BM precursors in vitro

A deficiency of Lyn results in exaggerated proliferative responses of macrophage precursors (37). Therefore, we first analyzed whether lyn-/- BM precursors can normally generate DCs. Freshly isolated BM cells from WT and lyn-/- mice were cultured in RPMI 1640 medium with various concentrations of GM-CSF. The unattached cells from both BM sources showed a characteristic DC morphology after 6 days of culture by microscopy and stained appropriately; CD11c+ DCs expressed CD11b, but not Gr-1 or F4/80, by flow cytometry (data not shown). However, as shown in Fig. 2A (left panel), lyn-/- BM generated significantly more DCs than WT BM at all concentrations of GM-CSF. The DC growth curves also indicated that more lyn-/- DCs were present in the BM culture on each day of incubation (Fig. 2A, right panel).

We conclude that lyn-/- DC precursors both proliferate more rapidly and have a lower GM-CSF dose response compared with WT cells. Interpretation of the increased expansion of lyn-/- BMDCs may be complicated by the fact that the mutant mice have higher numbers of progenitors than WT animals. Therefore, we analyzed the generation of DCs using a CFSE dilution method, followed by flow cytometry, to examine the proliferation of individual cells. This method allows examination of proliferative responses independent of the precursor frequency. BM cells isolated from mice treated with 150 mg/kg 5-fluorouracil (5-FU) for 6 days were labeled with 5 M CFSE for 10 min at room temperature and cultured in 10 ng/ml GM-CSF. 5-FU treatment is used to achieve a more uniform population of precursors that divide in a more synchronous fashion.

We observed that the generation of DCs from lyn-/- BM precursors was faster than that of WT BM precursors, as indicated by the greater loss of CFSE stain (Fig. 2B). Because the DC precursors do not divide synchronously in total BM cells, CFSE

FIGURE 2. Enhanced expansion of DCs in Lyn-deficient BM culture. BM cells isolated from WT and lyn-/- mice were cultured in 24-well plates as described in Materials and Methods. CD11c+ DC numbers were calculated by hemocytometry and flow cytometry. A: Left panel, Day 6 cultures in the presence of various concentrations of GM-CSF ranging from 0 to 40 ng/ml. Right panel, Growth kinetics were determined by culturing cells in 10 ng/ml GM-CSF, then counting daily for 6 days. B, BM cells were isolated from 5-FU-treated mice, labeled with CFSE, and then cultured in 10 ng/ml GM-CSF. The dilution of CFSE was detected by flow cytometry, gating only on the CD11c+ cells. The mean fluorescence intensity (MFI) of the entire CD11c+ population is indicated. C, Differentiation kinetics were determined daily during 6 days of culture with 2–50 ng/ml GM-CSF by flow cytometry, measuring the percentages of CD11c+ cells. D, DCs collected on day 8 were washed thoroughly and then cultured in RPMI 1640 without or with 10 ng/ml GM-CSF. Surviving cells were determined by trypan blue exclusion. All assays were performed in triplicate, and the results were displayed as the average ± SD. Data shown are representative of at least three independent experiments. *, p < 0.05; **, p < 0.01 (WT vs lyn-/- DCs).
CD11c+ splenic DCs in 9-mo-old lyn−/− mice was confirmed by immunohistochemistry (Fig. 3B). We concluded that the expansion of DCs was enhanced in lyn−/− mice, consistent with the results in vitro.

**Impaired maturation in lyn−/− DCs after innate immune stimulation**

Development of appropriate DC function is dependent on cell maturation after exposure to Ags and innate immune stimuli. To determine whether the lack of Lyn kinase affects DC maturation, we examined the maturation status of DCs under various culture conditions. IL-4 and TNF-α have been reported to promote DC maturation (45, 48). When we analyzed DCs in culture in the presence of 10 ng/ml IL-4 or 20 ng/ml TNF-α, a lower percentage of lyn−/− DCs expressed high levels of MHC-II (Fig. 4A). DC maturation can also be induced by incubation with LPS. After 24 h of 5 ng/ml LPS treatment, lyn−/− DCs showed a less mature phenotype, with fewer cells coexpressing MHC-IIhigh or costimulatory molecules, CD80, CD86, and CD40 (Fig. 4B). The lower sensitivity of lyn−/− DCs to LPS stimulation was not due to reduced expression of LPS receptors TLR4 or CD14, because we found equivalent cell surface levels of these proteins in WT vs lyn−/− cells (Fig. 4C). Furthermore, we tested the ability of lyn−/− DCs to mature to various other innate stimuli. Lyn−/− DCs did not mature appropriately after 50 ng/ml CpG and 50 ng/ml flagellin treatment, but did respond normally to 1 μg/ml poly(I:C) (Fig. 5). These results suggest that lyn−/− DCs have an intrinsic defect in maturation after innate immune stimulation.

**Impaired IL-12 production in lyn−/− DCs after LPS and CpG stimulation**

The production of IL-12 by activated DCs plays an important role in regulation of the immune response. We studied the production of IL-12 from LPS- and CpG-treated DCs by intracellular staining and measuring cytokine release into the supernatant by ELISA. As shown in Fig. 6A, fewer IL-12+ cells were detected in lyn−/− DCs after LPS and CpG stimulation for 6 h. Over a wide dose range of LPS or CpG stimulation, lyn−/− DCs showed a lower level of IL-12 release into the supernatant compared with WT cells, especially in the lower LPS dose range (Fig. 6B). Reduced IL-12 production by lyn−/− DCs is consistent with impaired maturation after LPS or CpG stimulation. In contrast, the production of IL-6 was not significantly different between WT and lyn−/− DCs after LPS or CpG treatment. Lyn−/− DCs tended to produce slightly less TNF-α after CpG stimulation, but slightly more TNF-α after LPS stimulation. The production of IL-2, IL-10, and IFN-γ was not detected in these cultures. We conclude that the reduced maturation of lyn−/− DCs correlates with a significant impairment in IL-12 production by these cells.

**Reduction of Ag-specific T cell activation by lyn−/− DCs in vitro and in vivo**

The major function of mature DCs is to induce specific T cell activation and proliferation. We set up an Ag-specific T cell proliferation assay in vitro to assess the functions of WT and lyn−/− DCs. CD4 T cells isolated from OT II transgenic mice were cocultured with OVA323–339 peptide-pulsed and LPS-treated DCs for 72 h, and T cell proliferation was determined by [3H]thymidine incorporation. As shown in Fig. 7, A and B, lyn−/− DCs induced a lower level of OT II T cell proliferation at various T cell:DC ratios and concentrations of OVA peptide. Furthermore, induction of T cell proliferation by CpG- and flagellin-treated, but not poly(I:C)-treated, lyn−/− DCs was also reduced (Fig. 7C). These results demonstrate that the impaired maturation of lyn−/− DCs after LPS,
FIGURE 4. Lyn-deficient DCs fail to mature appropriately. DC cultures were treated with cytokines and LPS, and maturation was determined by flow cytometry for the expression of MHC-II, CD80, CD86, and CD40. CD11c⁺ cells were gated for analysis. Control Ig. A: Left panel, BM cells were cultured in GM-CSF without (gray lines) or with (black lines) IL-4, and cells were collected on day 6 for flow cytometry. Right panel, PBS (gray lines) or TNF-α (black lines) was added on day 6, and cells were collected on day 8 for assay. The percentages of MHC-IIbright cells are shown by the regional markers, and the increase in percentage from control to treated cells is indicated. The increases in mean fluorescence intensity (MFI) values were: for IL-4: WT, 295 to 492; lyn⁻/⁻, 223 to 274; and for TNF-α: WT, 438 to 614; Lyn⁻/⁻, 259 to 383. B, Six-day-cultured cells were stimulated with PBS (gray lines) or LPS (black lines) for an additional 24 h, then analyzed for maturation. The percentages of highly positive cells are shown by the regional markers, and the increase in percent stimulation from the control is indicated. The increases in MFI were: for MHC-II: WT, 350 to 425; lyn⁻/⁻, 303 to 219; for CD80: WT, 101 to 256; lyn⁻/⁻, 108 to 162; for CD86: WT, 53 to 222; lyn⁻/⁻, 59 to 81; and for CD40: WT, 20 to 145; lyn⁻/⁻, 17 to 68. C, The expression of TLR4 and CD14 was determined using cells in day 6 culture (black lines). The MFI of the CD11c⁺ population is indicated. Data shown are representative of at least three independent experiments.

CpG, or flagellin stimulation leads to reduced ability to activate Ag-specific T cell proliferation in culture.

To determine whether lyn⁻/⁻ DCs showed impaired activation of Ag-specific T cells in vivo, we conducted a series of adoptive transfer experiments. CFSE-labeled OT II cells (splenic and LN) were transferred into WT mice, then 24 h later the same mice were injected i.v. with OVA323–339 peptide-pulsed and LPS-treated WT and lyn⁻/⁻ DCs. The OT II T cell proliferation was determined by CFSE dilution after 3 days. As shown in Fig. 7D, OT II T cells activated by lyn⁻/⁻ DCs had delayed proliferation in vivo. The reduced ability of lyn⁻/⁻ DCs to activate Ag-specific T cells was consistent with lower expression of MHC-II and costimulatory molecules (Fig. 4B) and supports the conclusion that a deficiency of Lyn causes a relative impairment in DC maturation.

Reduction of Th1 responses by lyn⁻/⁻ DCs

There is growing evidence that DCs play a critical role in balancing the Th1/Th2 character of the immune response. To determine whether the defect in DC maturation in lyn⁻/- mice affected the character of their immune responses, we studied Th responses in vitro and in vivo. In vitro activated OT II T cells were restimulated by LPS-treated WT and lyn⁻/⁻ BMDCs in the presence of OVA peptide. Supernatants were collected, and IFN-γ and IL-4 were determined by ELISA. The production of IFN-γ was reduced in lyn⁻/⁻ BMDC cultures (Fig. 8A). However, the production of IL-4 was only detected after a second stimulation and was not significantly different between WT and lyn⁻/⁻ DCs cultures (WT, 26.4 ± 6.9 pg/ml; lyn⁻/⁻, 29.4 ± 5.3 pg/ml). To study T cell polarization in vivo, we transferred OVA peptide-pulsed and LPS-treated BMDCs into OT II transgenic mice. The Th responses were measured by culturing total splenocytes isolated from BMDC-immunized OT II transgenic mice or by isolating CD4 T cells and coculturing with LPS-activated WT or lyn⁻/⁻ BMDCs. Again, the production of IFN-γ was significantly less in both the total splenocyte cultures and the isolated CD4 T cell cultures from OT II mice immunized with lyn⁻/⁻ BMDCs (Fig. 8B). The production of IL-4 was only detected in splenocyte cultures (WT, 43 ± 4.5 pg/ml; lyn⁻/⁻, 53.9 ± 6.9 pg/ml). In another study we examined the immune responses in WT and lyn⁻/⁻ mice by immunizing animals with OVA in CFA vs alum (which should bias the immune response toward Th1 or Th2, respectively). As shown in Fig. 8C, the production of IFN-γ by LN T cells was less in lyn⁻/⁻ mice, whereas the production of IL-4 was slightly greater in lyn⁻/⁻ mice. Interpretation of these results can be complicated by the fact that lyn⁻/⁻ mice have different numbers of LN T cells and Ag-presenting B cells, which would influence total cytokine production in LN cell culture. To avoid this complication, we purified CD4 T cells from OVA-immunized WT and lyn⁻/⁻ mice and directly compared their responses in culture with purified BMDCs that were pulsed with OVA overnight, thus standardizing the T cell number. Using purified CD4 T cells, we observed a more significant decrease in IFN-γ production than that seen in total LN cells (Fig. 8C). The decrease was not due to less capture of OVA, because both WT and lyn⁻/⁻ DCs had similar abilities to take up FITC-albumin (Fig. 8D). In a further effort to directly compare lyn⁻/⁻ vs WT DC functions in vivo, while avoiding complications
from effects of Lyn deficiency on other cell types, we performed a direct experiment comparing immune responses in WT mice receiving OVA-loaded WT or lyn−/− BMDCs by adoptive transfer. The immune responses to OVA-pulsed BMDC challenge were determined after 7 days. Consistently, the production of IFN-γ by splenocytes was reduced in animals that received OVA-loaded lyn−/− BMDCs compared with OVA-loaded WT BMDCs (Fig. 8E), whereas the level of IL-4 production was not significantly different. From these in vitro and in vivo experiments, we conclude that Lyn-deficient DCs fail to induce strong Th1 responses in responding normal T cells.

Discussion

In this study we used lyn−/− BMDCs to understand the role of this kinase in the regulation of DC generation, maturation, and function. Our results show that Lyn negatively regulates DC generation from BM precursors, but positively controls DC maturation and activation in vitro and in vivo. Lyn−/− BM precursors produce more DCs than WT BM precursors when cultured with GM-CSF, due to the higher sensitivity of lyn−/− cells. In contrast to the enhancement of growth, lyn−/− DCs show impaired maturation in response to various innate immune stimuli. This immature phenotype results in the reduction of specific T cell activation. In addition, lyn−/− DCs produce less IL-12 after stimulation, which may contribute to reduced priming of Th1 responses.

Lyn kinase has been shown to play a dual role in signaling through multiple receptors in hemopoietic cells (32–34). Numerous studies have illuminated the regulatory role of Lyn in myeloid cells, including monocytes (30), macrophages (37, 38), neutrophils (39, 49), eosinophils (26, 31), and mast cells (41–43). This study is the first report to characterize lyn−/− DCs. As described in other myeloid cells, Lyn negatively regulates the generation of DCs from BM precursors (Fig. 2). It has been shown that lyn−/− mice possess increased numbers of myeloid progenitors (37). However,
we demonstrate the faster generation of lyn−/− BMDCs by CFSE dilution and CD11c+ DC differentiation (Fig. 2, B and C) in addition to cell counting and survival assays. Moreover, CD11c+ DC number is enhanced in aged lyn−/− mice (Fig. 3), whereas these cells tended to have lower MHC-II expression compared with WT mice, which is consistent with the findings in BMDC cultured cells (Fig. 4). In addition, lyn−/− DCs enriched the CD8α+ population, known as myeloid DC, in agreement with the enhancement of myeloid cells in lyn−/− mice. The hypersensitive response of lyn−/− DC progenitors to GM-CSF may be due to reduction in tyrosine phosphorylation of inhibitory receptors, such as paired Ig-like receptor B (PIR-B), which, in turn, recruit phosphatases to the membrane to down-modulate signaling responses. Indeed, it is interesting to note that PIR-B-deficient BMDCs are also hypersensitive to GM-CSF stimulation, display immature phenotypes after stimulation, have reduced IL-12 production, and tend to induce a Th2-type, but not Th1-type, response (50). Given that PIR-B is a substrate of Lyn, the similarity in the DC phenotypes between these two knockout mice is logical.

DCs play a sentinel role in response to pathogens; after activation by innate immune stimuli, DCs mature into efficient APCs that guide subsequent T cell-dependent adaptive responses (7). Our data suggest that Lyn is involved in DC maturation. As shown in Figs. 4 and 5, lyn−/− DCs cannot mature appropriately after treatment with various stimuli compared with WT cells. Interestingly, despite the fact that Lyn is a negative regulator of IL-4 signaling in B lymphocytes (51), we did not observe that IL-4 enhanced maturation in lyn−/− DCs (Fig. 4A). This may be due to different cells (B cells vs DCs) and functions (class switching vs maturation). Although it has been suggested that BMDCs express only TLR4 and TLR9 (9), recent studies have shown that BMDCs can be stimulated by poly(I:C) and flagellin (52, 53). Lyn−/− BMDCs showed impaired maturation to these stimuli as well (Fig. 5). The signaling mechanism by which Lyn controls DC maturation remains to be elucidated. Given our previous evidence that the Src family kinases Hck, Fgr, and Lyn are not involved in immediate LPS signaling events (14), it seems likely that the role Lyn plays in DC maturation is probably not in the membrane-proximal signaling events from TLRs.

Given that there is a close interplay among signaling pathways affecting cell adhesion, cell proliferation, and cell maturation, the possibility that altered adhesive capacity could affect the lyn−/−...
DC phenotype is an issue. This is especially true given the hyperadhesive phenotype of lyn−/− neutrophils (39). Because the above studies were conducted with nonadherent DCs, and we observed no differences in the morphology or expression of integrins between WT vs lyn−/− DCs, we believe that our studies have avoided confounding affects from differences in adhesive capacity. Additional studies will be required to address whether altered integrin signaling may be a mechanism to explain the effect of Lyn deficiency on DC generation and maturation.

The maturation of DCs is linked to their capacity to activate T cells. We have shown that lyn−/− BMDCs induce less (in vitro) or delayed (in vivo) proliferation of Ag-specific T cell compared with WT BMDCs (Fig. 7), in agreement with their immature phenotype. Notably, the reduction of T cell activation in vitro seemed to be far

**FIGURE 8.** Reduction of Th1 responses induced by Lyn-deficient DCs in vitro and in vivo. Cytokine production was determined by ELISA. A, CD4 OT II T cells were cultured with OVA peptide-loaded LPS-activated WT or lyn−/− BMDCs for 4 days, and supernatants were collected as the first stimulation. After 1 day of rest, the activated T cells were restimulated with OVA peptide-loaded LPS-activated WT or lyn−/− BMDCs, and supernatants were collected on day 3 as the second stimulation for cytokine ELISA. B, OVA peptide-loaded LPS-activated WT or lyn−/− BMDCs were transferred into OT II transgenic mice via i.v. injection. Splenocytes were collected 4 days later, and total cells were cultured with 1 μg/ml OVA peptide, or CD4 T cells were purified and cultured with LPS-activated WT or lyn−/− BMDCs (indicated as CD4 T/BMDC). Supernatants were collected on day 3 for cytokine ELISA. C, WT and lyn−/− mice were immunized with OVA and CFA via s.c. injection (right) or with OVA and alum via i.p injection (left). Ten days later, cells were isolated from draining LNs or spleens and cultured with 100 μg/ml OVA (indicated as total LN cells and splenocytes) or CD4 T cells were purified and cultured with OVA-loaded WT or lyn−/− BMDCs (indicated as CD4 T/BMDC). Supernatants were collected on day 3 for cytokine ELISA. D, WT or lyn−/− BMDCs were incubated with CFSE-albumin. The uptake of CFSE-albumin at 4°C (□) and 37°C (black lines) was monitored by flow cytometry. E, WT mice were immunized with OVA-loaded WT or lyn−/− DCs (10⁶ cells/mouse) via i.v. injection. Seven days later, splenocytes were isolated and cultured with OVA (100 μg/ml). Supernatants were collected on day 3 for cytokine ELISA. All assays were performed in triplicate, and the results were displayed as the average ± SD. Data shown are representative of at least two independent experiments. *, p < 0.05; **, p < 0.01; no marker, no difference.
more significant than the reduction in MHC-II and costimulatory molecules. The signals for T cell activation are not only transduced from DCs to T cells, but also from T cells back to DCs. CD40L and CD28 on the T cell can influence DC function (54, 55). Thus, in addition to lower expression levels of costimulatory molecules, these signals may be not intact in lyn−/− DCs (28), which could further impair the ability of Lyn-deficient DCs to activate normal T cells. However, lyn−/− DCs still induced T cell proliferation in vivo, although it was delayed. The physiological environment could provide additional factors to support T cell activation by DCs in vivo.

The relative immature phenotype of lyn−/− DCs may contribute to their inability to fully prime Th1 T cell responses. In addition, the impaired IL-12 production by lyn−/− DCs could reduce Th1 polarization, although the production of IL-6 and TNF-α by these cells was not diminished (Fig. 6B). This would suggest that lyn−/− mice should show defects in Th1-type responses. It has been previously reported that lyn−/− mice develop exaggerated disease in an experimental autoimmune encephalomyelitis model, which is classically thought of as a Th1-type immune response. However, even in this report the investigators found that Th1 responses were reduced in total LN cells from lyn−/− animals (56). It is very likely that the effects of Lyn deficiency in other cell types besides DCs contribute to enhanced disease in the experimental autoimmune encephalomyelitis model. For example, the increased sensitivity of B lymphocytes or the dramatic expansion of myeloid cells (macrophages and neutrophils) in lyn−/− mice may be sufficient to produce exaggerated inflammatory responses even when the DCs are driving a lower level of Th1 immunity. Consistent with increased inflammatory response in lyn−/− mice, we observed significantly more severe foot pad swelling in lyn−/− mice (1.2 ± 0.1 mm in lyn−/− mice vs 0.7 ± 0.2 mm in WT mice) after OVA/CFIA immunization, even when the Th1 responses were reduced in the very same lyn−/− animals (Fig. 8C). We confirmed the reduction of Th1 responses by culturing CD4 T cells purified from draining LNs with BMDCs, avoiding the problem of different T and B cell numbers between WT and lyn−/− mice when using total LN cells. More directly, when WT mice were immunized with OVA-loaded WT or lyn−/− BMDCs, the lyn−/− cells consistently induced a lower level of Th1 response (Fig. 8E). In contrast, a recent report showed the IgE-dependent allergic responses, which is Th2-type immune response, were enhanced in lyn−/− mice (42). Increased mast cell numbers, circulating histamine, expression of FceRI, and eosinophilia were observed in lyn−/− animals, which may have also influenced allergic responses independently of Th1 vs Th2 T cells priming. Although immature DCs have recently been demonstrated to induce Th2 responses (57), suggesting that immature lyn−/− DCs may skew toward a Th2 phenotype in these animals (Fig. 8, C and E), the expansion of other inflammatory cells, such as mast cells and eosinophils, may have a greater effect on the allergic response. Collectively, these studies highlight the difficulty of studying DC function in lyn−/− mice in vivo, because deficiency of the kinase affects so many other immune cells. The exaggerated inflammatory responses, potentially caused by expansion of various myeloid lineages in lyn−/− animals may overcome the defect in DC maturation in vivo. Ultimately, it will require a DC lineage-restricted deficiency of Lyn to formally test whether the loss of this kinase in this cell type alters Th1 vs Th2 polarization in disease models.

DCs express four members of Src family kinases, including Lyn, Hck, Fgr, and Src (Fig. 1). In our preliminary data, BMDCs from various Src family kinase-deficient mice (hck−/−, fgr−/−, hck−/−fgr−/−, and hck−/−fgr−/−lyn−/−) have shown different phenotypes from lyn−/− BMDCs. It has been reported that Hck, Fgr, and Lyn are not obligatory for LPS-initiated signal transduction in macrophages (14). However, Hck and Fgr have negative regulatory roles in neutrophil and DC chemokine signaling (46). These results suggest that the balance between stimulatory and inhibitory regulation of Src family kinases is complicated and may make interpretation of DC phenotypes in multikinase-deficient mice difficult. This same argument applies to studies using Src family kinase inhibitors, such as PPI and PP2, to study the contributions of these enzymes to DC function (15–20, 22–24). However, our results support the model that lyn plays unique roles in DC generation and maturation, and that other Src family kinases cannot compensate for its function.

In conclusion, we provide evidence that Lyn plays both negative and positive regulatory roles in DC generation and maturation, respectively. It has been suggested that Lyn could be a target in DC preparation for immunotherapy and control of autoimmunity. Recently, some Lyn-specific inhibitory strategies have been reported in cancer research (58, 59). It may be interesting to apply these strategies to the modulation of DC function.

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