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TLR4 Signaling in Stromal Cells Is Critical for the Initiation of Allergic Th2 Responses to Inhaled Antigen

Anna M. Tan,* Hui-Chen Chen,*1 Pierre Pochard,* Stephanie C. Eisenbarth, † Christina A. Herrick,‡ and H. Kim Bottomly*2

Allergic asthma is an inflammatory lung disease driven by Th2. We have shown that both Th1 and Th2 sensitization to inhaled OVA depend on the presence and concentration of LPS, where high concentrations (LPS hi) induce Th1 and low concentrations (LPS lo), Th2. Stromal cells (SCs), such as airway SCs, exacerbate established airway disease; however, little is known about their role early during sensitization. In this study, using bone marrow chimera mice to restrict TLR4 signaling to either the SC compartment (SC+HPC) or the hematopoietic cell (HPC) compartment (SC−HPC), we report that HPC TLR4 is necessary and sufficient for Th1 sensitization to OVA-LPS hi, whereas TLR4 in both compartments is required for Th2 sensitization to OVA-LPS lo. Surprisingly, although SC−HPC mice were unable to generate a Th1 response to OVA-LPS hi, they instead mounted a robust Th2 response, indicating that in the presence of higher concentrations of LPS, SC TLR4 is sufficient for Th2 sensitization. We show that the SC TLR4 response to LPS leads to induction of Th2-inducing dendritic cells that upregulate Notch ligand Jagged-1 but not Delta-4. Furthermore, airway SCs upregulate thymic stromal lymphopoietin in response to exposure to both OVA-LPS hi and OVA-LPS lo. These studies demonstrate that SC TLR4 signaling is critically involved in Th2 but not Th1 sensitization to inhaled Ag.

airway diseases by the production of IL-4, IL-13, mucus, thymus activa-
tion-regulated cytokine, thymic stromal lymphopoietin (TSLP), and other Th2 chemotactic cytokines and lipid mediators (24, 25).

We addressed the role of TLR4 signaling in the SC and hematopoietic cell (HPC) compartments using bone marrow (BM) chimeric mice with TLR4 restricted to either the SC compartment (SC+HPC−) or the HPC compartment (SC−HPC+). We demonstrate that although HPC TLR4 is necessary and sufficient for Th1 sensitization to OVA-LPS6, TLR4 in both compartments is required for Th2 sensitization to OVA-LPS6. Surprisingly, although SC+HPC− mice were unable to generate a Th1 response to OVA-LPS6, they instead mounted a robust Th2 response, indicating that in the presence of higher concentrations of LPS, SC TLR4 is sufficient for Th2 sensitization. The data further show that stromal TLR4 signaling leads to the maturation of Th2- inducing DCs that fail to produce proinflammatory cytokines or to upregulate the Th1-inducing Notch ligand Delta-4. Following intranasal (i.n.) administration of LPS into the airways, ASCs upregulate mRNA expression of TSLP, suggesting that the SC-dependent instruction of allergic Th2 responses is driven by ASC-mediated induction of Th2-induced DCs.

Materials and Methods

Animals

BALB/c, C3H thri lpsd (TLR4d), and C3H-Tg (DO11.10)10Dlo/J (D011.10) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our facility. BALB/cAnNcr and C57Bl/6J-Pep3b (Pep3b) were purchased from the National Cancer Institute. C57Bl6J were purchased from The Jackson Laboratory and bred in our facility. TLR4-deficient (TLR4−/−) mice, provided by Dr. S. Akira (Research Institute for Microbial Diseases, Osaka, Japan), were backcrossed nine generations onto the C57Bl6J background. In all BM chimera experiments, 6- to 12-wk-old female mice were used with four to six mice per group. Male mice, 6–14 wk of age, were used as BM donors in experiments involving BM chimeric mice. Male mice were used as BM donors so that chimerism could be assessed by detecting the presence of the male Y chromosome in female BM recipients using fluorescence in situ hybridization (FISH). All animal experiments were performed in accordance with the guidelines of Yale University’s Institutional Animal Care and Use Committee.

Sensitization and challenge protocols

Mice were anesthetized with isoflurane and sensitized i.n. once daily with g OVA (grade V; Sigma-Aldrich, St. Louis, MO) with LPShi (10–15 μg) in 50 μl saline. Following sensitization, mice were randomized into treatment groups as shown in the figure legends. Mice were challenged i.n. on days 14, 15, 18, and 19 with 25 μg LPS. The challenge was administered on day 15 to two separate groups of mice. One group was challenged with 25 μg LPS, while the other group was challenged with vehicle only. Following challenge, mice were sacrificed on day 21.

Analysis of bronchoalveolar lavage

Mice were anesthetized with isoflurane and sacrificed on day 21. BALB/cJ (wild-type [WT]) and TLR4d mice were lethally irradiated on an X-rad 320 X-ray irradiator with a total of 1200 cGy total body irradiation delivered in two 600 cGy doses spaced 3 h apart. At 4 h postirradiation, mice were reconstituted with 5–8 × 10⁷ WT or TLR4d BM cells from male donors and maintained on sulfamethoxazole-trimethoprim (5 ml per 250 ml drinking water) for 2 wk, receiving autoclaved food and water. Mice were used in experiments 7–8 wk after BM transfer to allow full reconstitution of BM cells to occur. In some experiments (Supplemental Fig. 3), C57Bl6J and TLR4−/− mice were used to confirm data obtained in chimeric mice on the BALB/cJ background. These mice received one dose of 1200 cGy total body irradiation and were otherwise treated identically to BALB/cJ mice.

Analysis of chimerism in BM chimeric mice

In BM chimeras on a BALB/cJ background, the degree of chimerism of splenic B cells, T cells, CD11c+ cells, and lung CD11c+ cells was determined by FISH on the Y chromosome of male donor cells in female recipients, 7–8 wk after irradiation.

Splenoocytes from mechanically separated spleens were isolated on a density gradient using lymphotoxide separation medium (MP Biomedicals, Irvine, CA). For lung CD11c+ cells, cells from collagenase type IV (Sigma-Aldrich) digested lungs were passed through a 70-mm filter, and lung CD11c+ cells were isolated using anti-Ly6G conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). For lung CD11c+ cells, cells from collagenase type IV (Sigma-Aldrich) digested lungs were passed through a 70-mm filter, and lung CD11c+ cells were isolated using anti-Ly6G conjugated magnetic beads (Miltenyi Biotec, Auburn, CA). These were stained with antibodies against CD45.2, allowing distinction between host and donor derived cells in BM chimeras on the BALB/cJ background.

Analysis of naive CD4 T cells for coculture

CD4 T cells were isolated from spleens of naive D011.10 mice by negative selection using mAb to IgG2a (BioLegend, San Diego, CA). These were stained with antibodies against CD45.2, allowing distinction between host and donor derived cells in BM chimeras on the BALB/cJ background.

Isolation of DCs from mLN cells for coculture

DCs were isolated from mLN cells of naive D011.10 mice by negative selection using mAb to IgG2a (BioLegend, San Diego, CA). These were stained with antibodies against CD45.2, allowing distinction between host and donor derived cells in BM chimeras on the BALB/cJ background.

Isolation of DCs from mLN cells for coculture

For negative selection of DCs, mLN cells were preincubated with anti-FcR (2.4G2), followed by anti–Ig-coated magnetic beads (Polysciences, Warrington, PA). Highly pure (∼99%) populations of naive CD4 T cells (CD62Lhi,CD44lo) cells were obtained by labeling isolated CD4 T cells with FITC-labeled anti-CD44 (Pgp-1) and PE-labeled anti-CD62L (Mel14). These were stained with antibodies against CD45.1, CD45.2, CD3ε, B220, CD11c, FcεRⅠ, and cKit (BD Pharmingen, San Diego, CA).

Isolation of DCs from mLN cells for coculture

Restimulation of mLN cells from challenged mice

mLN cells were isolated, and single-cell suspensions were prepared and stimulated in vitro with 200 μg/ml OVA and syngeneic T cell-depleted mytymycin C (Sigma-Aldrich)-treated splenocytes (APCs). After 48 h, concentrations in the supernatant of IFN-γ, IL-4, IL-5, and IL-13 were measured using commercially available ELISA kits (R&D Systems), and IL-17 was measured using Bioplex cytokine bead array system (Bio-Rad, Hercules, CA) on the Lumex 100 IS System (Millepore, Bedford, MA) plate reader. Lower detection limits were 25.0 pg/ml (IL-4), 125.0 pg/ml (IL-5 and IL-13), 3.0 pg/ml (IL-17), and 1.9 ng/ml (IFN-γ).

Generation of BM chimeric mice

Six- to 12-wk-old female BALB/cJ (wild-type [WT]) and TLR4d mice were lethally irradiated on an X-rad 320 X-ray irradiator with a total of 1200 cGy total body irradiation delivered in two 600 cGy doses spaced 3 h apart. At 4 h postirradiation, mice were reconstituted with 5–8 × 10⁷ WT or TLR4d BM cells from male donors and maintained on sulfamethoxazole-trimethoprim (5 ml per 250 ml drinking water) for 2 wk, receiving autoclaved food and water. Mice were used in experiments 7–8 wk after BM transfer to allow full reconstitution of BM cells to occur. In some experiments (Supplemental Fig. 3), C57Bl6J and TLR4−/− mice were used to confirm data obtained in chimeric mice on the BALB/cJ background. These mice received one dose of 1200 cGy total body irradiation and were otherwise treated identically to BALB/cJ mice.

Isolation of ASCs

Perfused lungs were finely minced in Dispase and swirled 45 min at RT. HBSS/5% FCS/40 μg/ml DNase type I (Sigma-Aldrich) was added and incubated at RT at 10 min. Single-cell suspensions were prepared and RBCs were lysed. Cell pellets were resuspended in PBS plus 2 μM EDTA/0.5% FCS and labeled with predetermined optimal concentrations of biotinylated 2.4G2, anti-CD11b, and anti-CD45 mAb (clone 30-F11) 25 min

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on ice, washed with PBS plus 2 mM EDTA (no FCS), and incubated in MACS streptavidin-conjugated magnetic beads per the manufacturer’s instructions. Cells were magnetically separated. The enriched SCs (flow-through cells) contained >98% CD45.2+ cells, determined by staining the enriched cells with anti-CD45.2 (clone 104; BD Pharmingen) and performing flow cytometry (Supplemental Fig. 4). We confirmed that CD45.2 Ab is capable of binding cells labeled with biotinylated Abs to 2.4G2, CD11b, and CD45 and streptavidin-conjugated magnetic beads by staining an aliquot from each group prior to placement on the magnetic column for cell separation (prededipletion). Although the enriched fraction (post-depletion) containing SCs was negative for CD45.2, the prededipletion fraction contained cells that stained positive for CD45.2, indicating that epitone masking by the biotinylated Abs or streptavidin-conjugated beads was not occurring (Supplemental Fig. 4).

Ex vivo coculture

Enriched mLN DCs from sensitized mice (72 h after first of three daily i.n. sensitizations) were cocultured with naïve DO11.10 T cells at 1:20 (DC:T) in complete Bluffs media (C’ Bluffs) (Click’s medium plus 50 µM 2-ME plus 20 µg/ml gentamicin; Life Technologies, Carlsbad, CA) containing 10% FCS, 1% penicillin/streptavidin, and 2 mM L-glutamine. After 24 h, cells were pulsed with 25 U/ml IL-2. On day 4, cells were washed and rested 48 h in C’ Bruffs. Rested cells were restimulated at a ratio of 1:1 with APCs with or without 5 µg/ml OVA peptide (OVA323–339). Culture supernatants were collected after 48 h, and cytokine levels were analyzed with Bioplex cytokine bead array system (Bio-Rad) on the Luminex 100 IS System (Millipore) plate reader.

Gene expression analysis by quantitative PCR

RNA was extracted using TRIzol Reagent (Invitrogen) and gene expression determined by quantitative PCR (qPCR) on Stratagene Thermocycler (Stratagen, La Jolla, CA). Expression was normalized to β-actin or HPRT. Primers: 5′-GAGATCCCCCTACCTCCCCCA-3′ and 5′-GGGCATGGACGGCGACA-3′; HPRT, 5′-CCACGACAGCTTGCAACCTTAACCA-3′ and 5′-GTAATGATCACTCAACGGGGGAC-3′; TSLP, 5′-CGACAGCATGGTCTCTTCCTCA-3′ and 5′-ATTTGTGCACTGGTACCC-3′; DI4, 5′-AGGTGCCACTTCGGTTACACAG-3′; TSLP, 5′-AGGTGCCACTTCGGTTACACAG-3′ and 5′-CAATCCCCACTGCC-3′; and Jag1, 5′-AGTAGAAGGCTGTCACC-AAGCAAC-3′. Forward and reverse primers for Delta-4 (Dil4) and Jagged-1 (Jag1) were described previously (8).

DC migration analysis

Analyses were performed using 100 µg FITC-OVA (Sigma-Aldrich), 50 µg OVA-LPShi (Molecular Probes, Eugene, OR), or 50 µg OVA peptide (OVA323–339) (ASPEAQGALANIA VDKA). Eo peptide presentation on I-Ab was detected using the biotin-conjugated anti-I-Ab/Eo complex-specific Ab, YAE (ebiScience, San Diego, CA) as described previously (28). Labeled OVA or PBS was administered on days 0, 1, and 2 to naïve BALB/c mice with LPSb or LPSs in 50 µl PBS. Seventy-two hours after the first i.n. sensitization, mice were sacrificed, and mLN cells were isolated. Cells were preincubated on ice with anti-PCR (2.4G2), labeled with MAb specific for CD11c (HL3), CD40 (HMS0-3), I-Ad (AMS-32.1) (BD Pharmingen), CD86 (GL-1; eBioscience), and biotin-conjugated YAE mAb (eBioscience) (for Eo-peptide experiments) to detect Eo peptide presentation on I-Ab and then analyzed by flow cytometry.

Statistical analysis

Data are presented as means ± SEM, indicated by error bars. Statistical differences between groups in in vivo experiments were calculated using the Mann-Whitney U test in GraphPad Prism, version 4.0. Statistical differences between groups in vitro experiments were calculated using the Student t test. All values of p ≤ 0.05 were considered statistically significant.

Results

Low and high concentrations of LPS drive distinct Th cell responses

We have previously published that C.C3H trlr4 lpsd (TLR4d) mice, in which functional TLR4 signaling is absent because of a spontaneous point mutation in the signaling domain of TLR4, fail to be sensitized to inhaled OVA and do not mount an immune response to OVA challenge (10). Furthermore, OVA-LPSs sensitization induces a Th2 response, whereas OVA-LPSb drives Th1. Fig. 1A shows a representative BAL cellular distribution of these previously published results.

Both low and high concentrations of LPS induce DC migration

We compared the ability of DCs to migrate to the mLN in OVA-LPSbs and OVA-LPSbs-sensitized mice. Following 3 d of once daily exposure to inhaled OVA and LPS, or PBS alone, the percentage of CD11c+ DCs in isolated mLNs was determined by flow
cytometric analysis of CD11c-labeled cells, and the total number of DCs in the mLNs was determined. The total number of DCs in the mLN was similar between OVA-LPS\textsuperscript{lo} and OVA-LPS\textsuperscript{hi} groups, and both were significantly increased compared with saline control mice (Fig. 1B). Similar results have also been obtained at the earlier time points of 24 and 48 h (unpublished data).

**Both low and high concentrations of LPS induce DCs to upregulate costimulatory molecules**

To determine whether low and high concentrations of LPS induce similar levels of costimulatory molecule expression on DCs in the mLN, we analyzed CD40 and CD86 expression on the total DC population and on the specific population of DCs that had captured and processed labeled Ag, in addition to the total DC population.

We found that, 72 h after the first i.n. sensitization, mLN DCs from mice sensitized with DQ-OVA-LPS\textsuperscript{hi} or with DQ-OVA-LPS\textsuperscript{lo} expressed increased levels of CD40 and CD86 compared with DCs from PBS-treated mice. More DCs expressed high levels of CD40 in DQ-OVA-LPS\textsuperscript{lo}-sensitized mice, and the peak of CD86 expression was brighter, although a similar percentage of DCs in the DQ-OVA-LPS\textsuperscript{lo} group also expressed high levels of CD86 (Fig. 1C). These findings are consistent with patterns of costimulatory molecule expression observed on lung DCs 48–72 h following an identical sensitization protocol (unpublished data). DCs that had captured Ag (CD11c\textsuperscript{+}FL-1\textsuperscript{+} cells; Fig. 1D, Supplemental Fig. 1B) from OVA-LPS\textsuperscript{hi} and OVA-LPS\textsuperscript{lo}-sensitized mice expressed similar levels of CD40 and, in both groups, upregulated CD86; however, as was observed in the total DC population, FL-1\textsuperscript{+} DCs from OVA-LPS\textsuperscript{hi} mice expressed brighter levels of CD86 (Fig. 1E).

In OVA-LPS\textsuperscript{lo}-sensitized mice, FL-1\textsuperscript{+} DCs represented 4.2% of all CD11c\textsuperscript{+} cells, compared with 1% in OVA-LPS\textsuperscript{hi}-sensitized mice (Fig. 1D, Supplemental Fig. 1B). The percentage of FL-1\textsuperscript{+} DCs in OVA-LPS\textsuperscript{hi} was surprisingly low, at just under twice background fluorescence (PBS control, 0.6%; Fig. 1D, Supplemental Fig. 1B).

Similar studies were carried out using FITC-labeled OVA (to characterize Ag uptake and migration; Supplemental Fig. 1A) or Exo-conjugated OVA (to characterize Ag presentation on surface MHC molecules using YAe mAb (Supplemental Fig. 1C). As was observed with DQ-OVA (Fig. 1D, Supplemental Fig. 1B), whereas similar total numbers of DCs were present in the mLNs of LPS\textsuperscript{hi} and LPS\textsuperscript{lo} groups (FITC-OVA: 96.2 × 10\textsuperscript{3} [LPS\textsuperscript{lo}] versus 123.7 × 10\textsuperscript{3} [LPS\textsuperscript{hi}]; Ex-OVA: 82.99 × 10\textsuperscript{3} [LPS\textsuperscript{lo}] versus 73.8 × 10\textsuperscript{3} [LPS\textsuperscript{hi}]; unpublished data), there was an increased percentage of FITC\textsuperscript{+} (Ag-bearing) and YAe\textsuperscript{+} (Ag-presenting) DCs in LPS\textsuperscript{lo} groups compared with LPS\textsuperscript{hi} groups (FITC-OVA: 7.55% [LPS\textsuperscript{hi}] versus 17.2% [LPS\textsuperscript{lo}]; Ex-OVA: 2.4% [LPS\textsuperscript{hi}] versus 6.0% [LPS\textsuperscript{lo}]) (Supplemental Fig. 1A, 1C).

CD40 and CD86 expression levels on all mLN DCs and on Ag-presenting (YAe\textsuperscript{+}) DCs were evaluated (Supplemental Fig. 1D), and the costimulatory molecule expression profiles were similar to those in Ag-containing DCs (DQ-OVA\textsuperscript{+} DC; Fig. 1C, 1E). In conclusion, exposure to both low and high levels of LPS in the airways was sufficient for DCs to capture and process Ag, migrate to the mLN, upregulate costimulatory molecules, and present Ag. Furthermore, these experiments show that OVA-LPS\textsuperscript{lo} sensitized mice reproducibly had increased percentages of Ag-containing mLN DCs.

**LPS dose-dependent function of Th1- and Th2-inducing DCs**

To evaluate the ability of DCs exposed to OVA and LPS\textsuperscript{lo} or LPS\textsuperscript{hi} to activate and polarize naive, Ag-specific CD4 T cells, we set up an ex vivo assay to coculture OVA-specific naive D011.10 TCR transgenic CD4 T cells (D011.10 CD4 T cells) with isolated mLN DCs that had captured Ag in vivo (i.e., DCs were not pulsed with any additional Ag following isolation).

Cell suspensions were made from the mLNs of WT mice that had been sensitized i.n. with OVA-LPS\textsuperscript{lo} or OVA-LPS\textsuperscript{hi} for 3 d, and DCs were enriched by positive magnetic selection of CD11c\textsuperscript{+} cells, followed by cell sorting to further enrich the DCs (>97%). mLN cells that were depleted of DCs were also tested in the coculture assay to determine whether the DCs in the lymph node (LN) population were required for both Th1 and Th2 differentiation of naive CD4 T cells. The highly enriched DCs, or mLN cells that were depleted of DCs, were cocultured with D011.10 CD4 T cells at increasing ratios (DC:T cell) from 1:160 to 1:10, and T cell proliferation (\textsuperscript{[\textit{3}H]thymidine} incorporation) was measured. As expected, T cells cultured alone did not proliferate (Fig. 2A). mLN cells isolated from OVA-LPS\textsuperscript{lo}– or OVA-LPS\textsuperscript{hi}-sensitized mice that were depleted of DCs, or from mice sensitized with LPS\textsuperscript{hi} only (no Ag), did not stimulate D011.10 CD4 T cell proliferation, indicating that DCs were absolutely required for T cell activation and that this was an Ag-dependent mechanism of CD4 T cell activation (Fig. 2A). Significant levels of T cell proliferation were observed when T cells were cocultured with enriched DCs isolated from either OVA-LPS\textsuperscript{hi}– or OVA-LPS\textsuperscript{lo}–sensitized mice (Fig. 2A). However, mLN DCs from OVA-LPS\textsuperscript{lo}–sensitized mice induced increased proliferation of D011.10 CD4 T cells compared with OVA-LPS\textsuperscript{hi} (Fig. 2A).

We next asked whether mLN DCs isolated from OVA-LPS\textsuperscript{hi}– or LPS\textsuperscript{lo}-sensitized mice were sufficient to induce Th1 and Th2...
differential, respectively. Naive (CD62L<sup>hi</sup>CD44<sup>hi</sup>) DO11.10 CD4 T cells were isolated from the spleens and enriched to $>98\%$ purity by cell sorting for coculture with mLN DCs. Following 4 d of coculture and 2 d of rest in fresh media, we restimulated DO11.10 CD4 T cells with mytomycin C-treated, T-depleted splenocytes (APCs) pulsed with OVA peptide and measured the cytokines present in the culture supernatant after 48 h. mLN DCs from naive mice, or from LPS<sup>hi</sup>-only sensitized mice (no Ag), did not induce CD4 T cells to produce Th1 or Th2 cytokines (unpublished data and Fig. 2B), which is consistent with the failure of these DCs to induce CD4 T cell proliferation. DCs isolated from OVA-LPS<sup>lo</sup>-sensitized mice induced naive CD4 T cells to secrete significantly higher concentrations of the Th2 cytokines IL-4, IL-5, and IL-13. A significantly decreased concentration of IFN-γ was detected, compared with OVA-LPS<sup>hi</sup>-sensitized mice. DO11.10 CD4 T cells cocultured with DCs from OVA-LPS<sup>hi</sup>-sensitized mice produced high levels of IFN-γ and low levels of Th2 cytokines (Fig. 2B). These data demonstrate that DCs that have captured Ag in the lung and migrated to the mLN are sufficient for instructing naive CD4 T cell differentiation into both Th1 and Th2 effector cells, depending only on the concentration of LPS.

Expression of Notch ligands in mLN DCs

To determine whether high and low concentrations of LPS administered with OVA differentially induced Notch ligands in DCs activated in vivo, we isolated mLN DCs from OVA-LPS<sup>hi</sup>- and OVA-LPS<sup>lo</sup>-sensitized mice at 0, 24, and 48 h after sensitization and determined the expression of Delta-4 and Jagged-1 over time by qPCR. At 48 h after the first i.n. sensitization, we observed a $>10$-fold increase in the Delta-4/Jagged-1 ratio in mLN DCs from OVA-LPS<sup>hi</sup>-sensitized mice but no increase in the Delta-4/Jagged-1 ratio in DCs from OVA-LPS<sup>lo</sup>-sensitized mice (Fig. 2C). In OVA-LPS<sup>hi</sup>-sensitized mice, the Delta/Jagged ratio was significantly lower than the ratio in mLN DCs isolated from OVA-LPS<sup>lo</sup>-sensitized mice at 48 h. The Delta/Jagged ratio was low because Delta-4 mRNA expression was only weakly upregulated in OVA-LPS<sup>lo</sup> mLN DCs (Fig. 2D), while concurrently, Jagged-1 mRNA expression was upregulated following sensitization and maintained at elevated expression at 48 h (Fig. 2E). The upregulation of Jagged-1 in both OVA-LPS<sup>hi</sup> and OVA-LPS<sup>lo</sup> DCs indicated that expression to even low levels of LPS is sufficient to lead to the upregulation of Jagged-1. Taken together, these results indicate that the Delta-4/Jagged-1 ratio is an indicator of the Th1- or Th2-inducing capacity of an mLN DC, and this striking difference demonstrates that other factors beside the ability to produce proinflammatory cytokines distinguish these functionally distinct, Th1- and Th2-inducing mLN DCs.

Generation of BM chimeras

To test whether TLR4 signaling in DCs is required for both Th1 and Th2 sensitization, we restricted TLR4 signaling to either the hematopoietic or stromal compartment using BM chimeric mice on the BALB/c background (Fig. 3A). Some experiments were also carried out using mice on the C57BL/6 background (Supplemental Fig. 3).

To restrict TLR4 signaling to the HPC compartment, we lethally irradiated recipient TLR4<sup>ld</sup> mice and reconstituted them with WT BM. In these mice, referred to as SC HPC<sup>lo</sup>, only HPCs derived from transferred WT BM are TLR4 sufficient (+), whereas the radioresistant SCs derived from the recipient TLR4<sup>ld</sup> strain do not express functional TLR4 (−). Conversely, in SC HPC<sup>hi</sup> mice, we restricted TLR4 signaling to only the SC compartment by lethally irradiating WT mice and reconstituting the HPC compartment with TLR4<sup>ld</sup> BM. As controls, WT or TLR4<sup>ld</sup> mice were lethally irradiated and reconstituted with their own BM type (SC HPC<sup>lo</sup> and SC HPC<sup>hi</sup>, respectively). Seven to 8 wk following BM reconstitution, chimeric mice were sensitized with OVA and LPS<sup>hi</sup> or LPS<sup>lo</sup>, and 2 wk later, their responses to i.n. OVA challenge were evaluated.

Evaluation of chimerism

Mice on the BALB/c background that were congenic for a cell surface marker expressed on all HPCs (e.g., CD45.2 (Ly5.2)), as required for assessment of chimerism of many different cell lineages, were not available to us. Thus, we opted to generate BM chimeras using female mice as recipients and male mice as BM donors. We then determined chimerism by FISH to detect the Y chromosome of male donor cells in female recipients. An exemplary FISH analysis on lung DCs from male (i), female (ii), and chimeric (iii) lung CD11c<sup>+</sup> cells is shown in upper panels of Fig. 3B. Using FISH, we determined that full chimerism (>95%) of lung CD11c<sup>+</sup> cells (Fig. 3B) and splenic B cells, T cells, and DCs (Fig. 3D) had been established.

To determine chimerism of BALB/cJ lung mast cells, which we find to be present at a very low frequency (<0.05%) in the lungs of naive mice, we transferred BM from BALB/cJ129-II4tm1lky/J (4get) mice or from BALB/c mice (as negative control) to BALB/cJ mice. Mast cells from 4get mice are constitutively GFP positive (29) and can be detected by flow cytometric analysis of auto-fluorescence (AF) low FceRα<sup>+</sup>,cKit<sup>+</sup> cells. In two experiments, it was determined that 7 wk following lethal irradiation, mast cells were 88–89% BM donor derived (Fig. 3C), compared with the positive control (4get FceRα<sup>+</sup>cKit<sup>+</sup> BM). Background GFP fluorescence was detected in 4% of negative control cells.

To facilitate analysis of chimerism in C57BL/6J mice, whose HPCs express CD45.2, CD45.1 congenic mice were used as BM donors for irradiated C57BL/6J and TLR4<sup>ld</sup> mice. In chimeric mice, CD45.1<sup>+</sup> cells derived from the donor BM are distinguished from any remaining recipient CD45.2<sup>+</sup> HPCs by flow cytometric analysis. Individual chimeric mice, four per group, were analyzed 7 wk after lethal irradiation, and the degree of chimerism (%) was determined to be as follows: (97.8 ± 0.6 [lung mast cells]), 95.6 ± 0.6 [lung DCs], 81.0 ± 0.9 [splenic T cells], 95.5 ± 0.8 [splenic DCs], and 97.8 ± 0.6 [splenic B cells] (Fig. 3E–G).

Th2 responses can be generated in the absence of DC TLR4 signaling

OVA-LPS<sup>lo</sup> sensitization. Upon OVA challenge, the OVA-LPS<sup>hi</sup>-sensitized positive control group (SC HPC<sup>+</sup>) exhibited typical, Th2-type, eosinophilic BAL cell differentials, whereas SC HPC<sup>−</sup> chimeras had very low numbers of BAL cells (Fig. 4A). When TLR4 signaling was restricted to either the hematopoietic or the stromal compartment (SC HPC<sup>−</sup> and SC HPC<sup>−</sup> respectively), the total number of BAL cells was significantly reduced ($p < 0.001$) to levels similar to the negative control (SC HPC<sup>−</sup>) (Fig. 4A).

To further establish that the Th2 response was abrogated in SC HPC<sup>−</sup> and SC HPC<sup>−</sup> chimeric mice, we evaluated the draining LN response following challenge. The total number of cells in the mLN was only moderately increased compared with the negative control group (SC HPC<sup>−</sup> chimeric mice) in either group with compartment-restricted TLR4 signaling, confirming that sensitization to OVA in these chimeric mice was diminished compared with the WT control (Fig. 4B). The data shown in Fig. 4B are representative of the average number of cells in each group (mLN from all mice were pooled before cell suspensions were made and cells counted) in each of three experiments. Upon restimulation with OVA-pulsed APCs in vitro, mLN cells isolated from SC HPC<sup>−</sup> mouse produced Th2 cytokines, as is typical of WT Th2 responses. Consistent with the BAL data indicating that no Th2...
FIGURE 3. Chimerism analysis. A, Table of BM chimeric groups and ability to respond to LPS; + or − in group name indicates presence or absence of TLR4 signaling in each compartment. B and D, degree of chimerism in female BALB/cJ mice that received male BM was assessed by FISH to detect the Y chromosome. B, representative fluorescent images of FISH on lung CD11c+ cells from positive control (male) (“+Con.”) (i); negative control (female) (−Con) (ii); and cells pooled from three chimeric mice are shown (magnification ×63) (Chim.) (iii). The degree of chimerism, expressed as percent (%), positive, are shown for two experiments. C, the percentage of GFP+ mast cells (AF low, FcεRIα+cKit+ cells) pooled from five chimeric WT mice that had received BM from BALB/c.129-Il4tm1lky/J (4get) mice (4get chim.), relative to the positive control (4get mast cells) (+Con.), was determined. D, splenic...
FIGURE 4. Role of TLR4 signaling in stromal and hematopoietic compartments. BM chimeric mice were sensitized with three daily i.n. doses of OVA and LPSoh (A–C) or LPSkl (D–G). Following challenge 2 wk later, the responses were analyzed as follows: (A, D) BAL cell differential, average number per mouse: , eosinophils; gray bar, neutrophils; and , lymphocytes. Data are pooled from three independent experiments and expressed as mean ± SEM (n = 15), *p < 0.05; **p < 0.01 compared with SC+HPC control. Statistical significance was determined using the Mann-Whitney U test. B and E, Representative plot of average number of mLN cells per mouse following challenge from three experiments. Five mice per group were used for analysis. C and F, Cytokines produced in vitro by mLN cells isolated from challenged BM chimeric mice following restimulation with APCs and OVA. In each experiment, mLN cells were pooled from five mice per group for culture. G, Representative lung sections stained with H&E or PAS shown at ×100 and ×200, respectively, from OVA-LPSoh-sensitized BM chimeric mice following OVA challenge. Arrows indicate areas of peribronchiolar cellular infiltrate (H&E) or positive mucus staining (PAS). Data representative of three or more (A–F) and two (G) similar experiments.

response was initiated, LN cells in SC+HPC− and SC+HPC+ mice produced decreased levels of Th2 cytokines compared with the positive control (Fig. 4C). Although some IFN-γ was detected in the SC+HPC+ and SC+HPC− groups, the levels were very low overall (compared with the IFN-γ concentration associated with a Th1 response).

**OVA-LPSoh sensitization**

We next asked whether, following Ag challenge, chimeric mice sensitized with OVA-LPSoh would also exhibit a dual requirement for TLR4 signaling in both the stromal and hematopoietic compartments for the initiation of Th1 responses. As expected, the SC+HPC− control group did not respond to Ag challenge, whereas the total number of BAL cells and the BAL cell differential in SC+HPC+ chimeras were characteristic of a WT Th1 response (Fig. 4D). SC+HPC− mice exhibited a Th1-like BAL cell differential comprising neutrophils and very few eosinophils, although the total BAL cell number was reduced by ∼50% compared with SC+HPC+ mice, suggesting that Th1 induction and/or recruitment of cells to the lung following Ag challenge was impaired in these mice (Fig. 4D). Strikingly, SC+HPC− mice mounted a robust BAL response to OVA-LPSoh (Fig. 4D). The response induced by stromal TLR4 was Th2 driven, as the BAL made up >50% eosinophils. This experiment was also carried out in mice on the C57BL/6 background, using TLR4−/− mice, with identical results, indicating that this finding was not limited to a single mouse strain (Supplemental Fig. 3).

Analysis of the total numbers of cells in the mLN revealed that high numbers of cells were present in SC+HPC+, SC+HPC−, and SC+HPC− mice, indicating that LN response to challenge was robust in all groups, except the negative SC−HPC− control group (Fig. 4E). Like mLN cells from SC+HPC+ mice, upon restimulation with OVA-pulsed APCs, LN cells from SC+HPC− produced IFN-γ but low levels of Th2 cytokines. IFN-γ production in SC+HPC− mice was reduced compared with SC+HPC+ mice, which is consistent with our observation that the BAL responses in SC+HPC+ chimeras were reduced by ∼50% in total cell number but were still characteristic of a Th1-driven inflammatory response. The mLN cells from SC+HPC− mice, in contrast, produced dramatically elevated levels of the Th2 cytokines IL-4, IL-5, and IL-13 and failed to produce IFN-γ (Fig. 4F).

We also determined the expression levels of IL-17. In WT control mice (SC+HPC−), IL-17 was detected at the highest level in the OVA-LPSoh group but was detected at or near background levels (of SC+HPC− and PBS control groups) in OVA-LPSoh groups, indicating that high amounts of LPS are required for IL-17...
production in our system (Supplemental Fig. 2). In the OVA-LPShi groups, IL-17 was also detected in both the SC-HPC+ and SC+HPC- groups, indicating that TLR4 signaling in either compartment was sufficient for IL-17 production; however, the levels of production were lower in SC’HPC- mice, suggesting that HPC TLR4 signaling induces a more robust IL-17 response.

To further confirm our novel finding that stromal TLR4 signaling is sufficient to induce Th2 responses to OVA-LPShi, we performed H&E and PAS staining of fixed lung tissue isolated from these chimeric mice. H&E staining revealed that peribronchiolar inflammation was present in the lungs of SC’HPC+, SC’HPC-, and SC+HPC- mice, whereas mucus production, indicated by positive PAS staining, was present only in SC’HPC- mice (Fig. 4G).

SC TLR4 signaling conditions Th2-inducing DCs

To determine whether SCs instruct lung DCs to become Th2-inducing, we characterized DC phenotype and function in SC’HPC+, SC’HPC-, and SC+HPC- mice, examined the production of proinflammatory cytokines in the lungs of sensitized chimeric mice, and determined the expression of Delta-4 and Jagged-1 in mLN DCs isolated from sensitized BM chimeric mice. First, to ascertain whether stromal TLR4 signaling induces DC migration, we determined the number of DCs that migrated to the mLN following i.n. sensitization with OVA-LPShi in SC’HPC+, SC’HPC-, and SC+HPC- mice. Increased numbers of DCs were observed in the mLN of SC’HPC+ and SC+HPC- mice compared with SC’HPC- mice (Fig. 5A). We have also observed increased numbers of DCs that had captured FITC-labeled Ag in SC’HPC- mice (unpublished data), further indicating that stromal TLR4 signaling induces a high number of DCs in the lung to mature and migrate. We observed similar levels of CD86 expression on SC’HPC+ and SC’HPC- mice, confirming that the TLR4d DCs in SC’HPC- mice were being indirectly activated by SC TLR4 signaling (Fig. 5B).

To evaluate whether proinflammatory cytokines were produced in the lung in the absence of HPC TLR4 signaling, we measured the level of proinflammatory cytokines present in the BAL fluid (BALF) of OVA-LPShi–sensitized SC’HPC+, SC’HPC-, and SC+HPC- chimeric mice. We detected significant levels of IL-1α, IL-1β, IL-6, TNF-α, MIP-1β, and MCP-1 in the BALF of SC’HPC+ mice but not in the BALF of SC’HPC- or SC+HPC- mice, indicating that HPC TLR4 signaling is required for the production of detectable levels of these proinflammatory cytokines (Fig. 5C). This was also a further confirmation that 7–8 wk postirradiation, LPS-responsive HPCs, which are capable of secreting high levels of these cytokines, are not present in the lungs of lethally irradiated WT mice reconstituted with TLR4d BM. IL-25 was not detected in the BALF of any of these groups.

To determine whether the SC TLR4 response to LPS can stimulate DCs to become Th2-inducing, we used an ex vivo coculture assay to assess the ability of freshly isolated DCs that had captured Ag in vivo to polarize naive, Ag-specific CD4 T cells. Highly enriched mLN DCs isolated from OVA-LPShi–sensitized BM chimeric mice were cocultured with naive (CD62LhiCD44lo) D011.10 CD4 T cells. mLN DCs from sensitized SC’HPC- chimeric mice induced the differentiation of polarized CD4 T cells that, upon restimulation, produced decreased levels of IFN-γ and increased levels of IL-4 compared with CD4 T cells that were cocultured with DCs isolated from the sensitized SC’HPC+ chimeric mice (Fig. 5D).

To further confirm that stromal TLR4 signaling led to the conditioning of Th2-inducing DCs, we determined the ratio of expression of Delta-4 and Jagged-1 in mLN DCs from BM chimeric mice sensitized with OVA-LPShi. Similar to Th1-inducing WT DCs, the Delta/Jagged ratio in DCs from sensitized SC’HPC- mice was significantly increased 48 h after the first of two i.n. sensitizations (Fig. 5E). Conversely, mLN DCs from sensitized SC’HPC- mice failed to upregulate the Delta/Jagged ratio, exhibiting a ratio that was >10-fold lower than that of SC’HPC+ DCs.

Airway exposure to LPS induces expression of TSLP in ASCs

To identify a potential mechanism of Th2 induction by stromal TLR4 signaling, we examined the expression of the known, pro-Th2, proallergic cytokine TSLP in ASCs. WT or TLR4d mice were sensitized i.n. with OVA-LPS or PBS alone. Three hours following sensitization, mice were sacrificed, and ASCs were isolated and enriched to >98% purity by negative depletion of CD45+, CD11b+, and FcRγ HPCs (Supplemental Fig. 4). RNA was isolated from the purified stromal fraction, and the expression of TSLP mRNA was determined by qPCR and normalized to HPRT expression. WT mice significantly upregulated TSLP gene expression after exposure to OVA-LPS compared with the PBS control, with significantly increased expression...
in the OVA-LPS\textsuperscript{hi} group compared with OVA-LPS\textsuperscript{lo}. TLR4\textsuperscript{d} mice did not upregulate TSLP in any of the groups (Fig. 6). Thus, exposure to LPS in the airways, even at low concentrations, is sufficient to stimulate a pro-Th2 response in ASCs that includes the upregulation of TSLP mRNA, strongly suggesting that SCs of the airways can condition Th2-inducing DCs.

**Discussion**

The critical factor driving both Th1 and Th2 adaptive immune responses to inhaled Ag is the ability to detect and respond to the presence of pathogen-associated molecular patterns (4, 30). We show that for sensitization to inhaled OVA-LPS\textsuperscript{lo}, TLR4 signaling in both the HPC and SC compartments is required for Th2 sensitization, whereas, surprisingly, SC TLR4 signaling was sufficient for Th2 sensitization in the presence of high concentrations of LPS. We found that SC TLR4 signaling led to DC Ag uptake, maturation, and migration to the mLNs. Furthermore, we found that mLNs DCs from SC:\textsuperscript{HPC} mice were capable of inducing naive CD4 T cells to differentiate into IL-4-producing Th2 cells in vitro.

In a recent study using an endotoxemia model to study the role of TLR4 signaling in the HPC and SC compartments, the authors oppositely concluded that stromal TLR4 signaling did not lead to the induction of DC maturation and migration (31). We note that the authors studied splenic DCs. Considerable differences exist between mucosal and nonmucosal DCs, and this may be particularly important for how DCs respond to signals from their microenvironment (32). Thus, our contrasting findings may be explained by the difference in the types of DCs studied. Furthermore, we studied migration of DCs from the lung to the mLNs, whereas the authors of this study evaluated migration of resident splenic DCs within the spleen.

Another group published that TLR4 signaling in lung structural cells was necessary and sufficient to induce a Th2 response following intratracheal exposure to house dust mite (HDM)/LPS and suggested that Th1 and/or Th17 sensitization to OVA-LPS was also dependent on SC TLR4 (33). With respect to the latter conclusion, the authors reported that SC TLR4 signaling was necessary for production of IFN-\(\gamma\) and IL-17 by restimulated mLN cells from OVA-LPS\textsuperscript{hi}–sensitized mice. In contrast, we found that SC TLR4 is neither sufficient nor necessary for Th1 sensitization and show that both IFN-\(\gamma\) and IL-17 are produced by mLN cells from SC:\textsuperscript{HPC} mice. We note that IFN-\(\gamma\)- and IL-17-producing cells were consistently detected in the SC:\textsuperscript{HPC} group compared with SC:\textsuperscript{HPC} but were still increased compared with the negative control. We found no decrease in IL-17 production in the SC:\textsuperscript{HPC} group. Furthermore, other parameters were consistent with a Th1 and/or Th17 response, such as neutrophilic BALs and similar levels of OVA-specific serum IgG2a (unpublished data) in SC:\textsuperscript{HPC}\textsuperscript{+} and SC:\textsuperscript{HPC}\textsuperscript{+} mice.

We note that the authors of the HDM study adoptively transferred naive OVA-specific transgenic CD4 T cells to chimeric mice and examined cytokine production by mLN cells only 5 d after Ag sensitization, whereas we evaluated cytokine production by mLN cells isolated from mice 21 d after the first sensitization and 2 d after the last of four airway Ag challenges. This full sensitization and challenge protocol allows full differentiation and expansion of effector T cells and, in our view, is a more accurate determinant of the type of effector response generated by the sensitization conditions.

The authors’ finding that SC TLR4 is necessary and sufficient for Th2 sensitization is in contrast to our data in OVA-LPS\textsuperscript{lo}–sensitized SC:\textsuperscript{HPC\textsuperscript{+}} mice showing that HPC TLR4 signaling is also required. However, their finding is consistent with our data in OVA-LPS\textsuperscript{hi}–sensitized mice. The authors stated that HDM contains only very low levels of endotoxin that, alone, are insufficient to induce a Th2 response. They therefore attributed the SC TLR4-dependent Th2 sensitization to HDM. HDM is known to have cysteine protease activity, which has been shown to drive Th2 sensitization in the lung (34). The cysteine protease activity may directly activate SCs, or have other nonspecific effects in the lung that lead to maturation of lung DCs and Th2 sensitization. The authors also proposed that the Der p 2 allergen may enhance the response to endotoxin by acting as an MD2-like chaperone that promotes TLR4 signaling (35). This protease activity and/or TLR4 signal-enhancing property of HDM, coupled with the low levels of endotoxin present in the extract, may have provided a sufficient SC-activating signal to drive the response independently of HPC TLR4 signaling.

We propose a model in which SCs require a threshold level of TLR4 activation to independently drive Th2 sensitization. When this threshold level is not met, endotoxin sensitivity is increased by TLR4-expressing cells of the HPC compartment, thereby permitting Th2 sensitization to low levels of LPS. This model is supported by our finding that although TSLP mRNA expression in ASCs was induced by both low and high levels of LPS, significantly increased TSLP expression was detected in LPS\textsuperscript{lo}–exposed ASCs. Thus, in SC:\textsuperscript{HPC} mice, we propose that only LPS\textsuperscript{hi}-induced levels of TSLP were sufficient to drive an HPC TLR4-independent Th2 response. Overexpression of TSLP (i.e., high levels of TSLP) in the airways is known to lead to Th2-mediated inflammation (36).

Furthermore, we propose that despite TSLP upregulation following OVA-LPS\textsuperscript{lo} sensitization in WT mice, a Th2 response is not induced because the Th2-inducing signal provided by TSLP is overcome by the Th1-favoring HPC response to LPS\textsuperscript{hi}. Our data show that Delta-4 expression is induced in TLR4-dependent DCs following sensitization with OVA-LPS\textsuperscript{lo} but not OVA-LPS\textsuperscript{hi}. We note that Delta-4 has been shown to inhibit Th2 cell differentiation by inhibiting IL-4 signaling (37). Thus, in OVA-LPS\textsuperscript{hi}–sensitized WT mice, upregulation of Delta-4 by DCs may allow the DCs to overcome the pro-Th2 signal provided by TSLP.

Our data further demonstrated that Th2-inducing mLN DCs upregulate Jagged-1 but not Delta-4. Upregulation of Jagged-1 but not Delta-4 was also observed in mLN DCs from OVA-LPS\textsuperscript{lo}–sensitized SC:\textsuperscript{HPC}\textsuperscript{+} mice, strongly suggesting that SCs induce DCs to upregulate Jagged-1, thereby conditioning DCs to drive Th2 sensitization. Further studies should be carried out to directly assess the mechanism of SC-driven Th2 sensitization and regulation of DC function.

In conclusion, we show that TLR4 signaling in both the HPC and SC compartments is critical for Th2 sensitization to OVA and low levels of LPS; however, in the presence of high concentrations of

**FIGURE 6.** TSLP mRNA expression in lung SCs. TSLP mRNA expression in lung SCs isolated by negative depletion from BALB/c (WT) and C3H tlr4 lps-d (TLR4\textsuperscript{d}) mice sensitized with OVA and LPS\textsuperscript{lo} or LPS\textsuperscript{hi}, or saline control (PBS), was determined by qPCR and normalized to HPRT expression. \(* p < 0.05\) using the Student t test. Representative of three similar experiments.
LPS, stromal TLR4 signaling is sufficient for the induction of Th2 responses. Our data further suggest that ASCs condition DCs to drive allergic Th2 responses by the expression of the pro-Th2 cytokine TSLP.

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Disclosures

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