Generation of Th17 cells in response to intranasal infection requires TGF-β1 from dendritic cells and IL-6 from CD301b+ dendritic cells

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The authors declare no conflict of interest.

October 13, 2015

Intranasal (i.n.) infections preferentially generate Th17 cells. We explored the basis for this anatomic preference by tracking polyclonal CD4+ T cells specific for an MHC class II-bound peptide from the mucosal pathogen Streptococcus pyogenes. S. pyogenes MHC class II-bound peptide-specific CD4+ T cells were first activated in the cervical lymph nodes following i.n. inoculation and then differentiated into Th17 cells. S. pyogenes-induced Th17 formation depended on TGF-β1 from dendritic cells and IL-6 from a CD301b+ dendritic cell subset located in the cervical lymph nodes but not the spleen. Thus, the tendency of i.n. infection to induce Th17 cells is related to cytokine production by specialized dendritic cells that drain this site.

Th17 | CD301b+ | dendritic cells | Streptococcus pyogenes

Th17 cells are a subset of CD4+ helper T cells that orchestrate protective immunity to extracellular bacterial and fungal pathogens, predominantly at epithelial surfaces (1). T-cell antigen receptor (TCR) recognition of an MHC class II-bound peptide (p:MHCII) on an antigen-presenting cell causes Th17 cells to secrete the signature cytokine IL-17A, which acts primarily by increasing chemokine production in epithelial tissues to enable the recruitment, activation, and migration of neutrophils and monocytes (1).

In vitro studies have shown that naive CD4+ T cells differentiate into Th17 cells when stimulated by p:MHCII ligands in the presence of transforming growth factor-β (TGF-β1) and IL-6 (2-4). Similarly, TGF-β1 is required for Th17 differentiation during the induction of experimental autoimmune encephalomyelitis (EAE) (5). Th17 cells are also generated during intranasal (i.n.) infection by Streptococcus pyogenes, and TGF-β1 receptor signaling and IL-6 are involved (6, 7). However, Th17 cell formation in the small intestine does not depend on TGF-β1 (8) and requires IL-1β but not IL-6 (9). Additionally, Kuchroo and colleagues reported that although Th17 differentiation was normally dependent on IL-6, it could occur without it through an IL-21–dependent pathway if regulatory T cells were absent (10).

Infection via the i.n. route may induce Th17 cells because nasal-associated lymphoid tissue (NALT) contains specialized antigen-presenting cells that preferentially produce IL-1β or IL-6 and TGF-β1. Here, we tested this idea by studying the primary immune response to i.n. infection with S. pyogenes bacteria (11). We found that S. pyogenes p:MHCII–specific Th17 cell formation depended on TGF-β1 from dendritic cells and IL-6 from a CD301b+ dendritic cell subset located in the cervical lymph nodes (CLNs) but not the spleen.

Significance

Naïve helper T cells can differentiate into several specialized subtypes that help other cells kill microbes. The processes that determine how the different T-cell subtypes form in the body are not understood. Here we identify a population of dendritic cells that is responsible for the formation of one helper T-cell type during nasal infection. These results extend knowledge about T-cell specification and could be applied to improve vaccines for nasal pathogens.

Author contributions: J.L.L. and M.K.J. designed research; J.L.L. and T.D. performed research; S.W.K., D.H.K., and P.C. contributed new reagents/analytic tools; J.L.L., T.D., and M.K.J. analyzed data; and J.L.L. and M.K.J. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1513532112/-/DCSupplemental.

Results

I.n. Infection with Sp-2W Induces a 2W-I-Aβ-Specific Th17 Response. A p:MHCII tetramer-based approach was used to track CD4+ T cells specific for an I-Aβ p:MHCII called 2W (12) in C57BL/6 (B6) mice infected i.n. with S. pyogenes expressing this peptide (Sp-2W). Sp-2W bacteria were engineered by fusing the 2W peptide to the surface-anchored M1 protein (7). This approach was used because no immunogenic I-Aβ-binding peptides have been identified from the natural S. pyogenes proteome. CD4+ T cells expressing TCRs specific for the 2W-I-Aβ epitope were detected by staining spleen and lymph node cells from individual mice with fluorochrome-labeled 2W-I-Aβ tetramers and anti-fluorochrome magnetic beads followed by enrichment of the tetramer-bound cells on magnetized columns (13, 14). Previous studies have shown that uninfected B6 mice contain about 300, primarily CD44lo, naïve 2W-I-Aβ–specific CD4+ T cells (13) and that i.n. Sp-2W infection causes these cells to proliferate to produce a large population of CD44hi 2W-I-Aβ–specific effector T cells by 7 d postinfection (7).

Th17 cell formation was measured by assessing IL-17A production by 2W-I-Aβ–specific effector cells. B6 mice were infected i.n. with Sp-2W bacteria and 7 d later challenged with an i.v. injection of heat-killed S. pyogenes or Sp-2W bacteria. 2W-I-Aβ tetramer-based cell enrichment and direct ex vivo intracellular cytokine staining (15) was performed 3 h after the i.v. injection. None of the 2W-I-Aβ–specific effector cells present on day 7 after i.n. Sp-2W infection (Fig. 1A) expressed CD69 or produced IL-17A or IFN-γ after recall with heat-killed S. pyogenes bacteria (Fig. 1B and C). However, 30% of the 2W-I-Aβ–specific effector cells produced IL-17A after recall with heat-killed Sp-2W bacteria (Fig. 1B and C), whereas none of the cells produced IFN-γ (Fig. 1C). The failure of the majority of 2W-I-Aβ–specific effector cells to make IL-17A after challenge was not due to a lack of TCR stimulation by 2W-I-Aβ complexes, as many of the cells expressed the TCR signal-dependent CD69 molecule but did not make IL-17A (Fig. 1B). Therefore, about one-third of the
2W:I-A<sup>b</sup>-specific effector cells induced by i.n. <i>S. pyogenes</i> infection were Th17 cells.

**Anatomy of 2W:I-A<sup>b</sup>-Specific Th17 Response to i.n. Sp-2W Infection.**

The induction of Th17 cells after i.n. infection warranted investigation of the events in the secondary lymphoid organs associated with this site. The NALT was investigated because it is the primary site of infection after i.n. <i>S. pyogenes</i> inoculation (11). Naïve 2W:I-A<sup>b</sup>-specific T cells were detected in the CLNs and spleen, but not the much smaller NALT before infection (Fig. 2). Beginning at day 3 after infection, some of the 2W:I-A<sup>b</sup>-specific cells in CLNs but not the spleen had increased CD44 and became large blasts, indicating that activation began in the CLNs. By day 4, 2W:I-A<sup>b</sup>-specific T cells in the CLNs had increased dramatically in number and most were large blasts (Fig. 2). CD44<sup>high</sup> 2W:I-A<sup>b</sup>-specific T cells appeared in the spleen at this time but were smaller blasts than the ones in the CLNs. Beginning on day 5, CD44<sup>high</sup> 2W:I-A<sup>b</sup>-specific T cells that were small blasts finally appeared in the NALT and accumulated in this location to a peak number on day 7 (Fig. 2). Together, these results indicated that naïve 2W:I-A<sup>b</sup>-specific T cells were first activated in the CLNs after i.n. Sp-2W inoculation. The fact that large 2W:I-A<sup>b</sup>-specific T-cell blasts never appeared in the spleen and NALT indicated that these cells proliferated in other sites, probably the CLNs, before migrating to the spleen and NALT.

**IL-6 Is Necessary for Th17 Differentiation in Response to i.n. Sp-2W Infection.**

The cytokines that induce Th17 differentiation after <i>S. pyogenes</i> infection were next investigated. The role of IL-6 was studied in IL6<sup>−/−</sup> mice after i.n. administration of heat-killed Sp-2W bacteria. Heat-killed bacteria were used to ensure that the animals survived until completion of the experiment (7). About 20% of 2W:I-A<sup>b</sup>-specific effector cells from wild-type (WT) B6 mice primed i.n. with heat-killed Sp-2W bacteria 7 d earlier produced IL-17A 3 h after i.v. challenge with heat-killed Sp-2W bacteria and none produced IFN-γ, whereas comparable cells from IL-6<sup>−/−</sup> mice produced no IL-17A and about 10% produced IFN-γ (Fig. 3A). The lack of IL-17A production by 2W:I-A<sup>b</sup>-specific cells in IL-6-deficient mice was not accompanied by an increase in Foxp<sup>3</sup> cells as reported in the EAE model (Fig. 3B) (10). Therefore, IL-6 was necessary for Th17 cell differentiation and suppression of Th1 but not regulatory T-cell formation after i.n. Sp-2W administration.

The cell type that produced the IL-6 needed for Th17 differentiation was then explored. The literature suggested that hematopoietic or nonhematopoietic cells could be involved (16). These possibilities were assessed with chimeric mice produced by injecting bone marrow cells from WT B6 or IL6<sup>−/−</sup> mice into irradiated B6 or IL6<sup>−/−</sup> mice. Following hematopoietic reconstitution, chimeras were inoculated i.n. with Sp-2W bacteria. Seven days later, about 15% of the CD44<sup>high</sup> 2W:I-A<sup>b</sup>-specific T cells in chimeras produced IL-17A after transplanting WT bone marrow cells into irradiated WT recipients produced IL-17A but not IFN-γ (Fig. 3C) 3 h after i.v. challenge with heat-killed Sp-2W bacteria. The same was true for mice in which WT bone marrow cells were transplanted into IL6<sup>−/−</sup> recipients. However, very few of the 2W:I-A<sup>b</sup>-specific effector cells generated in chimeras in which IL6<sup>−/−</sup> bone marrow cells were transplanted into WT mice produced IL-17A and some produced IFN-γ (Fig. 3C). Therefore, hematopoietic cells were the source of IL-6 necessary for Th17 differentiation after i.n. administration of Sp-2W bacteria.

A cell ablation approach was then used to determine whether dendritic cells were the source of IL-6 needed for Th17 cell differentiation. Chimeras were produced by injecting irradiated B6 recipients with a 1:1 mixture of bone marrow cells from CD11c-diphtheria toxin receptor (CD11c-DTR) transgenic mice and either WT or IL6<sup>−/−</sup> mice. Administration of diphtheria toxin (DT) to these mice resulted in ablation of the CD11c-DTR-expressing dendritic cells leaving dendritic cells that can or cannot make IL-6. In DT-treated CD11c-DTR:WT chimeras, which contained dendritic cells with the capacity to make IL-6, about 15% of the 2W:I-A<sup>b</sup>-specific effector cells generated by i.n. administration of Sp-2W bacteria produced IL-17A, whereas very few made IFN-γ (Fig. 3D). Similar results were obtained for non-DT-treated control mice.
TGF-β1 from Dendritic Cells Is Necessary for Th17 Differentiation in Response to i.n. Sp-2W Infection. The role of TGF-β1 in Th17 differentiation after i.n. Sp-2W inoculation was also assessed. Chimeric mice were produced by injecting a mixture of bone marrow cells from CD4-Cre Tgfb1+/+ and CD4-Cre/Tgfb1+/− mice into irradiated B6 CD45.1+ mice. Half of the T cells in these mice expressed TGF-β1 receptor II after reconstitution, whereas the other half did not. About 12% of the WT 2W:I-Aβ-specific cells induced by i.n. Sp-2W infection produced IL-17A following challenge with heat-killed Sp-2W, whereas the TGF-β1 receptor II-deficient T-cell population lacked IL-17A–producing cells and instead contained a population of IFN-γ–producing cells (Fig. 4A). TGF-β1 signaling in CD4+ T cells is therefore necessary for Th17 differentiation after i.n. S. pyogenes infection.

Radiation bone marrow chimeras were then used to identify the cellular source of TGF-β1. Bone marrow cells from WT B6 or Tgfb1+/− mice were injected into irradiated B6 or Tgfb1+/− mice to produce chimeras. About 20% of the 2W:I-Aβ-specific effector cells induced by i.n. Sp-2W infection in chimeras produced by transplanting WT bone marrow cells into irradiated WT or TGF-β1–deficient recipients produced IL-17A, whereas very few produced IFN-γ (Fig. 4B). In contrast, very few of the 2W:I-Aβ–specific effector cells in chimeras produced by transplanting TGF-β1–deficient bone marrow cells into irradiated WT mice produced IL-17A and about 10% produced IFN-γ. Therefore, hematopoietic cells were the source of TGF-β1 required for Th17 differentiation after i.n. Sp-2W infection.

Other studies suggested that either the CD4+ T cells (17) themselves or dendritic cells (18) could be sources of TGF-β1 for Th17 differentiation. The latter possibility was tested in CD11c-Cre/Tgfb1+/− mice in which the Tgfb1 gene was disrupted in CD11c+ cells. As expected, about 30% of the 2W:I-Aβ–specific effector cells induced by i.n. Sp-2W infection produced IL-17A and few if any produced IFN-γ in control mice (Fig. 5A). The population of 2W:I-Aβ–specific T cells in mice lacking the Tgfb1 gene in CD11c+ cells contained significantly fewer IL-17A–producing cells than the control mice and also contained some IFN-γ-producing cells (8%), indicating that CD11c+ cells were important contributors of TGF-β1 for Th17 differentiation in this system. Some Th17 differentiation still occurred in mice lacking TGF-β1 in dendritic cells. This result indicated that other sources of this cytokine, perhaps activated T cells, must also contribute.

A potentially confounding factor to this experiment is that some T cells probably delete the Tgfb1 gene in CD11c-Cre/Tgfb1+/− mice due to transient expression of CD11c at some point during T-cell development (19). Thus, it was possible that TGF-β1 deficiency in T cells caused the defects in Th17 differentiation observed in CD11c-Cre/Tgfb1+/− mice. Adoptive transfer of WT

**Fig. 3.** Dendritic cell–derived IL-6 is necessary for 2W:1-Aβ–specific Th17 differentiation after i.n. Sp-2W infection. (A, C, and D) Cytokine production by 2W:1-Aβ–specific T cells from 2W:1-Aβ tetramer–enriched samples from WT B6, 104 Il6−/−, or chimeric mice infected i.n. 7 d earlier with Sp-2W bacteria, 3 h after i.v. challenge with heat-killed Sp-2W bacteria. In C, the bone marrow donor is indicated before the → symbol and the recipient after. The chimeras in D were produced with a 1:1 mixture of bone marrow from the indicated donors and treated with DT on day −1, 2, and 5 relative to infection. Contour plots from representative samples and scatter plots with values from individual mice are shown. Numbers on the contour plots indicate the percentage of cells in the indicated gate. ***P < 0.0005, *P < 0.05; ns, not significant (unpaired two-tailed Student’s t test). Data are representative of three independent experiments.

However, in DT-treated CD11c-DTR:Il6−/− chimeras, which lacked dendritic cells that could make IL-6, very few of the 2W:I-Aβ–specific effector cells produced IL-17A and some produced IFN-γ (Fig. 3D). Therefore, CD11c+ cells, likely dendritic cells, were the source of IL-6 necessary for Th17 differentiation after i.n. inoculation with Sp-2W bacteria.

**Fig. 4.** Hematopoietic cell–derived TGF-β1 is necessary for 2W:1-Aβ–specific Th17 cell differentiation after i.n. Sp-2W infection. (A and B) Cytokine production by 2W:1-Aβ–specific T cells from 2W:1-Aβ tetramer–enriched samples from mice infected i.n. 7 d earlier with Sp-2W bacteria, 3 h after i.v. challenge with heat-killed Sp-2W bacteria. (A) Contour and scatter plots from chimeras containing a 2:1 mixture of congenically marked CD4-Cre (CD45.1/CD45.2) and CD4-Cre/Tgfb1−/− (CD45.2) T cells, showing 2W:1-Aβ–specific T cells derived from the CD4-Cre bone marrow or the CD4-Cre/Tgfb1−/− bone marrow donor. (B) Contour and scatter plots from chimeras produced by transplanting bone marrow cells from the donors indicated before the → symbol into irradiated recipients of the type indicated after the → symbol. The scatter plots show the percentage of the 2W:1-Aβ–specific T cells from the indicated source that produced the indicated cytokine in individual animals. Numbers on the contour plots indicate the percentage of cells in each quadrant. ***P < 0.0005, **P < 0.005, *P < 0.05 (unpaired two-tailed Student’s t test).
CD4+ T cells was performed to address this issue with the assumption that these T cells should exhibit defective Th17 differentiation in CD11c-Cre/Tgfb1f/f mice if dendritic cells are the major source of TGF-β1. CD4+ T cells were purified from CD45.1+ B6 mice and transferred into control CD11c-Cre mice or CD11c-Cre/Tgfb1f/f mice. Each recipient mouse was estimated to receive about 20% of the CD4+ T cells suggested that dendritic cells, not T cells, were critical for Th17 differentiation in this system.

Chimeras were then produced by injecting a 1:1 mixture of bone marrow from CD45.1+ mice and either WT or Tgfb1f/f mice into irradiated WT recipients to determine if dendritic cells were the source of TGF-β1. Administration of DT to these mice results in ablation of the CD11c-DTR-expressing dendritic cells leaving dendritic cells that can or cannot make TGF-β1. In DT-treated CD11c-DTR:WT chimeras, which contained dendritic cells with the capacity to make TGF-β1, about 10% of the 2W:I-Ab-specific effector cells generated by i.n. administration of Sp-2W bacteria produced IL-17A after challenge, whereas very few cells made IFN-γ (Fig. 5C). However, in DT-treated CD11c-DTR:Tgfb1f/f chimeras, which lacked dendritic cells that could make TGF-β1, very few 2W:I-Ab-specific effector cells produced IL-17A, and some cells produced IFN-γ. Therefore, CD11c+ cells, likely dendritic cells, were the source of TGF-β1 necessary for Th17 differentiation after i.n. infection with Sp-2W bacteria.

**CD301b+ Dendritic Cell-Produced IL-6 Is Necessary for Th17 Differentiation in Response to i.n. Sp-2W Infection.** The results suggested that the i.n. route to induce Th17 cells was related to the presence of IL-6 and TGF-β1-expressing dendritic cells in the CLNs. CLN contains three MHCI intermediate tissue-derived dendritic cell populations that migrate from the skin or subcutaneous-epidermal Langhans cells, CD207+ dermal dendritic cells, and CD11b+ dermal dendritic cells, along with three MHCI intermediate blood-derived resident populations (20, 21). Quantitative RT-PCR was used to determine whether migratory or resident dendritic cells were the main sources of Il6 or Tgfb1 mRNA. The analysis was performed on cells from B6 mice before, or 3 d after, i.n. or i.v. administration of Sp-2W bacteria (Fig. 6A). Migratory dendritic cells (MHCI intermediate) from the CLNs contained large amounts of Il6 mRNA and intermediate amounts of Tgfb1 mRNA with or without infection. In contrast, MHCI intermediate CLN-resident dendritic cells as well as CD11c+ cells in the NALT or spleen of naïve and Sp-2W–inoculated mice contained low amounts of Il6 mRNA and large amounts of Tgfb1 mRNA. Therefore, migratory dendritic cells in the CLNs constitutively produced the largest amounts of IL-6 and some TGF-β1, whereas other dendritic cells produced very little IL-6 and large amounts of TGF-β1.

The robust IL-6 production by migratory dendritic cells warranted further investigation of this population. The radiation chimera results shown in Fig. 3 indicated that the relevant dendritic cell was radiosensitive, which ruled out epidermal Langerhans cells because these cells are radioresistant (22). CD207+ dermal dendritic cells were also ruled out because S. pyogenes-induced Th17 induction was normal in Baf3 γ– mice (Fig. 6B), which lacked this population (23). We therefore focused on CD11b+ dendritic cells. CD11b+ dendritic cells encompass a heterogeneous population, containing subsets that are dependent on Notch2. Th17 generation was normal in CD11c-Cre/Notch2f/f mice (24) after Sp-2W infection, demonstrating that Notch2-dependent CD11b+ dendritic cells were not involved (Fig. 6C). We next focused on CD11b+ CD301b+ dermal dendritic cells, which are involved in Th2 cell responses in skin (21). The approach relied on a transgenic mouse expressing a DTR-eGFP fusion protein under control of the CD301b promoter (Mgl2DTR) (21). Surprisingly, DT treatment lead to a wasting syndrome in Mgl2DTR mice associated with anorexia. A promoter (Mgl2DTR) (21). Surprisingly, DT treatment lead to a wasting syndrome in Mgl2DTR mice associated with anorexia.
CD301b+ migratory dendritic cell-derived IL-6 is necessary for 2W:I-Aβ-specific Th17 cell differentiation after i.n. Sp-2W inoculation. (A) Quantitative RT-PCR for Il6 (Left) or Tgfb1 (Right) mRNA relative to Hprt mRNA from magnetically enriched, then FACS-sorted CD11c+ cells from the indicated tissues of uninfected mice or mice infected i.n. with Sp-2W 3 d earlier. Each sample was pooled from three mice. (B) and (C) Cytokine production by 2W:I-Aβ-specific T cells from 2W:I-Aβ tetramer-enriched samples from (B) WT or Batf3−/− or (C) CD11c-Cre or CD11c-Cre/Notch2−/− mice infected i.n. 7 d earlier with Sp-2W bacteria, 3 h after i.v. challenge with heat-killed Sp-2W bacteria. (D) Contour plots of CD19+ CD90+ CD45.1+ cells from CLNs of 1:1 Mgl2DTR-eGFP:WT or Mgl2DTR-eGFP:IL6−/− chimeric mice inoculated i.n. with heat-killed Sp-2W bacteria 7 d earlier and treated with or without DT. Gates used to identify CD11c+ MHCIIhigh migratory dendritic cells along with the percentage of cells in these gates are shown in the Top row. The Bottom row shows CD301b (MgII) expression on CD11c+ MHCIIhigh migratory dendritic cells in each group. (E) Total number of 2W:I-Aβ-specific T cells in the spleen and lymph node (Left panel) of DT-treated chimeric mice produced with a 1:1 mixture of Mgl2DTR-eGFP and WT or IL6−/− bone marrow and inoculated i.n. 7 d earlier with heat-killed Sp-2W bacteria and cytokine production by CLN cells 3 h after in vitro culture with phorbol 12-myristate 13-acetate and ionomycin (Middle and Right panels). Pharmacologic stimulation of T cells was used for cytokine recall in this case because heat-killed bacteria do not reach the CLNs after i.v. injection. **P < 0.005; ns, not significant (unpaired two-tailed Student’s t test).

These results indicated that CD301b+ dendritic cells are important sources of IL-6 for Th17 generation during i.n. infection.

**Discussion**

Our results suggest that IL-6 and TGF-β1 are required for Th17 differentiation induced by i.n. S. pyogenes infection. Although these results support early in vitro studies (2–4) and one study showing that TGF-β1 was required for the initiation of EAE (14), they are in contrast to in vivo studies that showed that IL-6 is not required for microbially-induced Th17 cell formation (9) and that TGF-β1 is not required for Th17 differentiation during EAE induction (8). The fact that IL-1β or IL-23 was important for Th17 cell differentiation in the cases where IL-6 or TGF-β1 was not suggests the existence of several independent Th17 cell differentiation pathways (8, 9). The IL-6– and TGF-β1–dependent pathway induced by i.n. S. pyogenes infection also suppressed Th1 cell differentiation. Interestingly, however, effector T cells induced by i.n. S. pyogenes infection did not become Foxp3+ cells in the absence of IL-6 as observed in the EAE model (10). Therefore, exposure of pMHCIIC-activated T cells to TGF-β1 without IL-6 does not necessarily lead to regulatory T-cell differentiation.

CD11c+ dendritic cells were the main sources of IL-6 and major sources of TGF-β1 for Sp-induced Th17 formation. Ablation of TGF-β1 production by CD11c+ cells, however, did not completely inhibit S. pyogenes-induced Th17 formation, indicating that other cells of hematopoietic origin are involved. CD4+ T cells, perhaps regulatory T cells, were good candidates based on work in the EAE model (17). Nonetheless, S. pyogenes pMHCIIC-specific naive CD4+ T cells did not become Foxp3+ regulatory T cells during S. pyogenes inoculation, and we found no role for TGF-β1 production by CD4+ T cells in S. pyogenes-driven Th17 differentiation. It is possible that TGF-β1-producing regulatory T cells form during EAE because of constant TCR signaling in response to constitutively expressed self-pMHCIIC ligands. This may not occur after S. pyogenes inoculation because the TCR stimulus is rapidly cleared from the infected tissue.

We found that CD301b+ dermal dendritic cells were a major source of the IL-6 required for S. pyogenes-induced Th17 differentiation. It is likely that the CD301b+ dermal dendritic cells provided IL-6 to developing Th17 cells in the CLNs, as this was the site in which T-cell activation first occurred after S. pyogenes infection. These dendritic cells may have originated in the NALT and then migrated to the CLNs (25) after taking up S. pyogenes bacteria or may have been in the CLNs at the time of infection. Our results indicate that the i.n. route of infection may be better than the i.v. route for induction of Th17 cells because of potent IL-6–producing CD301b+ dendritic cells in the CLNs but not the spleen. A previous study showed that IL-6 is necessary for Th17 differentiation in sites that drain mucosal and cutaneous tissues because it overcomes Th1 inhibitory retinoic acid production from CD103+ DCs that exist in these tissues, but not the spleen (26).

Our results add to a growing body of evidence that CD301b+ dendritic cells are important drivers of T-cell differentiation. Iwasaki and colleagues found that CD301b+ dendritic cells were involved in Th2 differentiation after immunization with protein antigen plus alum adjuvant or worm infection (21). These stimuli may cause CD301b+ dendritic cells to produce Th2-inducing cytokines, whereas other stimuli such as i.n. S. pyogenes bacteria or IL-23 was important for Th17 generation in these experiments (8, 9). The IL-6– and TGF-β1–dependent pathway induced by i.n. S. pyogenes infection also suppressed Th1 cell differentiation. Interestingly, however, effector T cells induced by i.n. S. pyogenes infection did not become Foxp3+ cells in the absence of IL-6 as observed in the EAE model (10). Therefore, exposure of pMHCIIC-activated T cells to TGF-β1 without IL-6 does not necessarily lead to regulatory T-cell differentiation. CD11c+ dendritic cells were the main sources of IL-6 and major sources of TGF-β1 for Sp-induced Th17 formation. Ablation of TGF-β1 production by CD11c+ cells, however, did not completely inhibit S. pyogenes-induced Th17 formation, indicating that other cells of hematopoietic origin are involved. CD4+ T cells, perhaps regulatory T cells, were good candidates based on work in the EAE model (17). Nonetheless, S. pyogenes pMHCIIC-specific naive CD4+ T cells did not become Foxp3+ regulatory T cells during S. pyogenes inoculation, and we found no role for TGF-β1 production by CD4+ T cells in S. pyogenes-driven Th17 differentiation. It is possible that TGF-β1-producing regulatory T cells form during EAE because of constant TCR signaling in response to constitutively expressed self-pMHCIIC ligands. This may not occur after S. pyogenes inoculation because the TCR stimulus is rapidly cleared from the infected tissue.

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as CD301b+ dermal dendritic cells account for a large fraction of the CD11b+ migratory dendritic cell population. The involvement of CD11b+ dendritic cells in T-cell differentiation sheds additional light on the function of this population, which we showed carries antigens from s.c. infection sites to the draining lymph node and stimulates delayed-type hypersensitivity (29).

**Materials and Methods**

**Mice and Inoculations.** All mice were housed in specific pathogen-free conditions in accordance with guidelines of the University of Minnesota and National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Minnesota approved all animal experiments. Mice were given 2 × 10^5 live or heat-killed S. pyogenes or Sp-2W bacteria i.n. or i.v. as previously described (7). Additional details, including mice strains and radiation bone marrow chimeras are provided in SI Materials and Methods.

**Induction and Detection of Cytokine Production by 2WJ–Aβ–Specific Effector Cells.** For all experiments except those showed in Fig. 6E, cytokine production was elicited by i.v. injection of heat-killed Sp-2W bacteria. Sp-2W bacteria were grown in THB-Neo media to an optical density at 600 nm of 0.5, washed once with PBS, centrifuged to a pellet, suspended in PBS, and then heat-killed by incubation at 60 °C for 30 min. Bacterial killing was confirmed by plating out a sample on a blood agar plate. Heat-killed Sp-2W bacteria were stored at −20 °C until use. To induce cytokine production in vivo, 5 × 10^6 heat-killed Sp-2W bacteria in 100 μL of PBS were injected i.v. through the tail veins of mice that were previously infected with S. pyogenes bacteria. Mice were killed after 1 h, and single-cell suspensions were made in cEHAA medium supplemented with brefeldin A (10 μg/mL; Sigma). For the experiments showed in Fig. 6E, spleen and lymph node cells from Sp-2W–infected mice were stimulated in vitro with phorbol 12-myristate 13-acetate (50 nM) and ionomycin (200 nM) in the presence of brefeldin A. Cytokine production by 2WJ–Aβ–specific T cells from mice challenged with heat-killed Sp-2W bacteria or in vitro cultures was determined after tetramer-based cell enrichment as described below.

**Cell Transfer.** Spleen and lymph node cells were isolated from naive CD45.1+ B6 mice and purified by negative selection (CD4+ T-cell isolation kit, Miltenyi). One mouse equivalent of CD4+ T cells was then injected i.v. into each CD45.2+ recipient.Recipient mice were then injected 1 d later i.v. with 2 × 10^6 Sp-2W bacteria. Seven days after, the phenotype of 2WJ–Aβ–specific T cells was determined after tetramer-based cell enrichment as described above.

**Statistical Analysis.** Differences between two datasets were analyzed by a paired or unpaired two-tailed Student’s t test with Prism (Graphpad) software.

**ACKNOWLEDGMENTS.** We thank J. Walter and R. Speier for technical assistance and T. Martin and the University of Minnesota School of Veterinary Medicine cell culture facility for cell sorting. This work was supported by National Institutes of Health Grants R01-AI39614, R37-AI27998, and R01-AI0103760 (to M.K.J.) and T32-AI07313 (to J.L.L.).
Supporting Information

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SI Materials and Methods

**Mice.** Seven- to 8-wk-old C57BL/6 (B6), B6.SJL-Ptprca Pep3b/BoyJ (CD45.1), Il6<sup>−/−</sup> (30), 129;CF-1-Rag2m1FwaTgfb1tm1Doel (Tgfb1<sup>−/−</sup>) (31), C57BL/6-J-Tg(Ifgax-cre)-EGFP<sup>*</sup>4097AchJ (CD11c-Cre) (32), B6.Cg-Tg(Cd4-cre)IcwibfluJ (CD4-Cre) (33), 129S-Tgfb1<sup>−/−</sup> (Tgfb1<sup>−/−</sup>) (31), B6.Cg-Tg(Ifgax-cre)I-1ReizJ (CD11c-Cre), and B6.129S-Notch2tm3Grid<sup>−/−</sup> (Notch2<sup>f/f</sup>) (24) mice were obtained from the Jackson Laboratory or the National Cancer Institute. CD11c-Cre/Notch2<sup>f/f</sup> mice were produced by crossing B6.Cg-Tg(Ifgax-cre)I-1ReizJ (CD11c-Cre) with Notch2<sup>f/f</sup> mice. CD11c-Cre/Tgfb1<sup>f/f</sup> mice were produced by crossing C57BL/6-J-Tg(Ifgax-cre)-EGFP<sup>*</sup>4097AchJ (CD11c-Cre) with Tgfb1<sup>f/f</sup> mice (5) obtained from D. Kaplan, University of Minnesota, Minneapolis. Mgl2DTR-eGFP mice were obtained from D. Kaplan with permission from A. Iwasaki, Yale University, New Haven, CT. CD4-Cre/TgfbrII<sup>f/f</sup> mice were produced by crossing B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ mice with TgfbrII<sup>f/f</sup> mice (35) obtained from S. Jameson, University of Minnesota, Minneapolis.

**Radiation Bone Marrow Chimeras.** Bone marrow cells were harvested from femurs, tibias, and humeri of donor mice. T cells were depleted from bone marrow cell suspensions with anti-Thy1.2 (30-H12, Bio X Cell) and low-toxicity rabbit complement (Cedarlane Laboratories). In some cases, CD45.1<sup>+</sup> CD45.2<sup>+</sup> WT bone marrow cells were mixed with bone marrow cells from other strains in a 1:1 ratio, except when CD4-Cre/TgfbrII<sup>f/f</sup> bone marrow was mixed with CD4-Cre bone marrow in a 2:1 ratio. We injected 5–10 × 10<sup>6</sup> total bone marrow cells into lethally irradiated (1,000 rads) CD45.1<sup>+</sup> mice. Chimeras containing cells derived from CD11c-DTR or Mgl2DTR-eGFP bone marrow cells were injected with 1 μg of DT on days −1, 2, and 5 relative to inoculation to ablate the DTR-expressing population and given daily injections of Lactated Ringer’s solution with dextrose to prevent dehydration and anorexia. Ablation was assessed in CLN samples stained with fluorochrome-labeled antibodies specific for CD45.1, CD19, CD90.2, CD11c, or MHCII (I-A<sup>b</sup>) and analyzed by flow cytometry. CD11c versus MHCII was displayed for CD45.1<sup>−</sup>CD19<sup>−</sup>CD90.2<sup>−</sup> cells. Ablation was considered to be effective if CD11c<sup>f</sup> or eGFP<sup>f</sup> cells were absent.

**Quantitative RT-PCR.** Tissues were isolated from naïve or Sp-2W-infected mice and digested with Collagenase D (Roche). CD11c<sup>+</sup> cells were enriched over magnetic columns (CD11c<sup>+</sup> microbeads, Miltenyi) and sorted using a FACSAria (Becton Dickinson) cell sorter to >98% purity. RNA was isolated (RNeasy column; Qia-gen), DNase digested (Turbo DNA-Free Kit; Ambion), and cDNA was made (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Quantitative RT-PCR was run using taqman primers (Taqman gene expression assays, Il6-Mm00446190_m1, Tgfbr1-Mm01178820_m1, Hprt-Mm00446968_m1; Taqman Universal Master Mix II; Applied Biosystems).