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Th17 Cells Induce Th1-Polarizing Monocyte-Derived Dendritic Cells

Matthew G. Davidson,* Michael N. Alonso,* Robert Yuan,* Robert C. Axtell,†
Justin A. Kenkel,* Megan M. Suhoski,* Joseph C. González,* Lawrence Steinman,† and
Edgar G. Engleman*

In chronically inflamed tissues, such as those affected by autoimmune disease, activated Th cells often colocalize with monocytes. We investigate in this study how murine Th cells influence the phenotype and function of monocytes. The data demonstrate that Th1, Th2, and Th17 subsets promote the differentiation of autologous monocytes into MHC class II+, CD11b+, CD11c+ DC that we call DC appId.

Although all Th subsets induce the formation of DC appId, activated Th17 cells uniquely promote the formation of IL-12/IL-23–producing DC appId (DC appId7) that can polarize both naive and Th17 cells to a Th1 phenotype. In the inflamed CNS of mice with Th17-mediated experimental autoimmune encephalomyelitis, Th cells colocalize with DC, as well as monocytes, and the Th cells obtained from these lesions drive the formation of DC appId that are phenotypically indistinguishable from DC appId7 and polarize naive T cells toward a Th1 phenotype. These results suggest that DC appId7 are critical in the interplay of Th17- and Th1-mediated responses and may explain the previous finding that IFN-γ–secreting Th cells become IFN-γ–secretory Th1 cells in experimental autoimmune encephalomyelitis and other autoimmune disorders. The Journal of Immunology, 2013, 191: 1175–1187.

Monocytes comprise >10% of circulating leukocytes in humans and ~4% in mice (1). These cells rapidly infiltrate inflamed tissues and are renowned for their plasticity (2–4). Upon entering inflamed tissues, monocytes readily differentiate into inflammatory dendritic cells (DC) that share multiple characteristics with conventional DC and tissue-resident macrophages including F4/80 positivity and reduced CD11c and Ly6C expression (5, 6). Our previous studies indicate that inflammatory DC arise following direct interaction with CD4+ Th cells in humans (7). However, the role these cells play in inflammation has not yet been elucidated. Experimental mouse models of disease are ideally suited to study this question, because the key disease-causing Th cells can be generated in vitro or obtained from inflamed tissue and tested for their ability to induce the differentiation of monocytes into inflammatory DC. In the current study, we sought to investigate the biology and impact of Th cell–driven DC formation in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS) in which Th cells and circulating monocytes are known to play pathogenic roles.

In EAE, transfer of myelin-reactive Th cells robustly and reliably induces a cascade of events that results in autoimmune demyelination (8). Much is known about the roles of specific Th subsets in both the induction and progression of EAE. Autoreactive IFN-γ–producing Th1 cells or IL-17–producing Th17 cells can induce disease, whereas Th2 and regulatory T (Treg) cells are believed to be protective (9). Furthermore, mice lacking the Th1 transcription factor, Tbet, or the Th17 transcription factor, retinoic acid-related orphan receptor γt, have reduced EAE symptoms compared with control mice, demonstrating that both the Th1 and Th17 pathways are involved in EAE (10, 11). Interestingly, Hirota et al. (12) recently showed that the majority of IFN-γ–expressing Th1 cells within the inflamed spinal cord previously produced IL-17A, demonstrating the plasticity of the Th17 phenotype in EAE. However, the cell types responsible for mediating conversion of Th17 cells into Th1 cells in EAE and the significance of this process in disease resolution remain unclear.

In addition to Th cells, monocytes are thought to play an essential role in the development of EAE (13). Indeed, monocytes are often found juxtaposed with Th cells in the CNS of affected mice, and the extent of monocyte infiltration correlates strongly with the severity of disease (14). Within the inflamed CNS, activated Th cells express multiple molecules, including CD40L, IFN-γ, M-CSF, and GM-CSF, that enhance the Ag presentation and differentiation potential of monocytes. In fact, bone marrow (BM) chimera mice with myeloid cells deficient in the GM-CSFR are completely protected from EAE induction with adjuvants, often referred to as active EAE induction (15). Taken together, these findings suggest that factors expressed by CD4+ T cells act on monocytes and facilitate EAE induction and progression.

Based on our previous observation that activated human Th subsets induce the formation of phenotypically distinct monocyte-derived DC (Mo-DC) (7), we hypothesized that activated murine Th subsets would behave in a similar manner. We report in this study...
that murine Th1, Th2, and Th17 cells are capable of inducing the formation of distinct Mo-DC, termed DC<sub>Th1</sub>, DC<sub>Th2</sub>, or DC<sub>Th17</sub>, respectively. These DC<sub>Th</sub> subsets have similar cell-surface phenotypes, but differ in their cytokine production and T cell–activating ability. Most strikingly, Th17 cells induce the formation of IL-12–producing DC<sub>Th17</sub> with potent Th1-polarizing capacity and the ability to convert Th17 cells into IFN-γ–expressing Th1 cells. Further, CD4<sup>+</sup> T cells taken from the inflamed spinal cords of mice with Th17-mediated EAE also induce the formation of Mo-DC that secrete IL-12p70 and drive Th1 polarization. These findings suggest that such DC serve as a critical link in determining the balance between Th17 and Th1 polarization during immunity and EAE progression.

Materials and Methods

Mice

Wild-type (WT) C57BL/6, CD40L knockout (KO), GM-CSFR KO, and IL-12p35 KO female mice were purchased from The Jackson Laboratory and used between 6 and 14 wk of age. OT-II TCR-transgenic Rag2<sup>−/−</sup> mice were purchased from Taconic Farms. All mice were housed in an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility and maintained in specific pathogen-free conditions. Animal experiments were approved and conducted in accordance with Stanford University Asia Pacific Laboratory Accreditation Cooperation #13605.

Generation of BM chimeras

Six-week-old C57BL/6 B6 mice were placed on antibiotic-supplemented chow. One week later, they received 5 Gy total body irradiation and were injected via tail vein with 10<sup>7</sup> syngeneic WT BM or BM from IL-12p35 KO mice. They were allowed to recover for 1 mo on antibiotic food before returning to a normal diet. Nine weeks later (13 wk after irradiation), reconstitution with IL-12p35 KO BM was confirmed by PCR on circulating blood cells.

T cell polarization and isolation

Splenocytes were depleted with biotinylated anti-CD8α Ab (clone 53.6.7; BioLegend) followed by anti-biotin microbeads (Miltenyi Biotech). The remaining cells were cultured in complete media consisting of RPMI 1640 media (Life Technologies) with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 50 µM 2-ME, and 10% FCS with 1 µg/ml anti-CD3 (BD clone 145-2C11) to generate bulk CD4<sup>+</sup> T cells or in the addition of the following polarizing mixture: Th1, 10 ng/ml IL-12 (R&D Systems); Th2, 20 ng/ml IL-4 (R&D Systems), 20 µg/ml anti–IL-12 p40 (clone C17.8; eBioscience); and 20 µg/ml anti–IFN-γ (clone R4-6A2; eBioscience); and Th17, 1 ng/ml recombinant human TGF-β1 (R&D Systems), 20 ng/ml IL-6 (R&D Systems), and 10 µg/ml anti–IFN-γ. T cells were positively selected from these cultures after 96 h with Thy1.2 magnetic beads (Miltenyi Biotech). In some experiments, IL-17A–secreting Th17 cells were sorted to high purity using an FACSaria II (BD Biosciences) and IL-17A cytokine capture reagents (Miltenyi Biotech).

Monocyte, DC, and macrophage generation

BM from femurs, tibias, and hips was prepared after cleaning the bones and storing them at −80°C. BM from femurs, tibias, and hips was prepared after cleaning the bones and femurs were washed 4 times with PBS and twice with DMEM. All mice were housed in an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility and maintained in specific pathogen-free conditions. Animal experiments were approved and conducted in accordance with Stanford University Asia Pacific Laboratory Accreditation Cooperation #13605.

Generation and activation of DC<sub>Th</sub> subsets

Polarized T cells were purified after 96 h of culture using Thy1.2 positive selection (Miltenyi Biotech) and cultured at a ratio of 1 T cell/10 monocytes in the presence of 10 ng/ml rmIL-2 (R&D Systems) for 5 d in tissue culture–treated plates (Corning). At this point, some cocultures were imaged using a Leica DM IRB inverted microscope and Metamorph software (Molecular Devices). In some experiments, cells were activated via the addition of 1 µg/ml standard LPS (TLR2 and -4 agonist), 1 µg/ml standard LPS (TLR4 agonist), 200 µg/ml PMN M-CSF (PeproTech) plus 20 ng/ml IL-4 (PeproTech) to generate DC<sub>Th1</sub> or in 50 ng/ml M-CSF (Sigma-Aldrich) to generate macrophages.

Statistical analyses

Unpaired Student t test (two-tailed) with a 95% confidence interval was performed in Prism (GraphPad) to analyze all experimental data unless otherwise stated. Error bars represent ± SEM; *p < 0.05, **p < 0.01, and ***p < 0.001.
Results
Activated Th cells promote Mo-DC formation in vitro

To determine whether activated murine Th cells can induce monocytes to differentiate into DC, we established in vitro monocyte and Th cell cocultures. Bulk CD4+ T cells were activated with an anti-CD3 Ab for 96 h (ThBulk), then isolated with anti-Thy magnetic beads, and cultured at a 1:10 ratio with freshly isolated autologous BM monocytes. Monocytes cultured alone, or with exogenous M-CSF or exogenous GM-CSF and IL-4 (GM IL-4 DC), served as controls. After 5 d of coculture, we imaged the cells with DIC microscopy (Fig. 1A).

Monocytes cultured in complete media alone displayed poor viability. However, monocytes cultured with activated bulk CD4+ T cells were viable and showed similarities to both GM IL-4 DC and M-CSF macrophages in their size, shape, and granularity. They could easily be distinguished from T cells based on their larger size and dendritic morphology (Supplemental Video 1). Monocytes cultured with activated T cells displayed more surface MHC II, CD11c, CD86, and CD40 and less Ly6C than freshly isolated monocytes (Fig. 1B).

Based on their morphology and surface phenotype, these ThBulk-induced, monocyte-derived cells resemble DC, and on this basis, we refer to them as DCTh.

**FIGURE 1.** In vitro–activated bulk Th cells promote the differentiation of monocytes into DC. (A) BM monocytes were cultured at a ratio of 10:1 with activated bulk CD4+ T cells (ThBulk) for 5 d in the presence of 10 ng/ml IL-2 (DCThBulk) or as controls, monocytes were cultured alone, with GM-CSF and IL-4, or with M-CSF. The cells were then imaged with DIC microscopy. Scale bars, 50 μm. (B) Cell-surface phenotype of DAPI2, Thy1.22, CD11b+ cells after 5 d of culture with GM IL-4 (solid gray), with ThBulk cells and IL-2 (solid black) or fresh after isolation (gray line). Median fluorescence intensity for each marker is shown in the table above. Data are representative of more than three independent experiments. (C) DCThBulk, fresh BM monocytes, and GM IL-4 DC were pulsed with 2.5 μg/ml OVA peptide and cultured at a ratio of 1 APC/15 naive T cells from OT-II RAG KO mice. (D) Cells were pulsed with 2.5 μg/ml MOG peptide and cultured at various ratios with naive T cells from 2D2 mice. Error bars represent SEM. ***p < 0.001.
We next assessed whether DC<sub>TN</sub> can present Ag and activate T cells, which is a defining property of DC. DC<sub>TN</sub> were purified from our cultures using CD11c MACS positive selection, pulsed with an MHC II–restricted OVA peptide in the presence of LPS, and cultured with TCR-transgenic responder CD4<sup>+</sup> T cells (OT-II). Consistent with their surface phenotype, DC<sub>TN</sub> were ~10-fold more potent at T cell activation than fresh monocytes. However, DC<sub>TN</sub> were far less potent at stimulating T cell proliferation than GM IL-4 DC (Fig. 1C). To verify that DC<sub>TN</sub>-stimulatory ability was not restricted to OVA peptide presentation, we pulsed DC<sub>TN</sub> and fresh monocytes with an Ag relevant to EAE, MOG peptide (aa 35–55), and tested their T cell–activating capacity across much broader APC/T cell ratios in an Ag-specific T cell proliferation assay with naive MOG-reactive T cells from 2D2 mice (Fig. 1D). Under these conditions, DC<sub>TN</sub> induced T cell proliferation at low APC/T ratios and were superior to monocytes at all ratios tested. These experiments confirm that activated CD4<sup>+</sup> T cells are capable of inducing the differentiation of monocytes into functional DC.

**Th1, Th2, and Th17 cells drive the formation of phenotypically distinct DC<sub>TN</sub>**

To determine the ability of particular Th subsets to induce DC<sub>TN</sub> formation, we polarized T cells into Th1, Th2 or Th17 cells in vitro, using standard combinations of cytokines, blocking Abs, and anti-CD3 activating Ab. T cell polarization was confirmed by intracellular staining (Fig. 2A) and ELISA of culture supernatants (Supplemental Fig. 1A). As expected, Th1 cells expressed high levels of INF-γ, whereas Th2 cells expressed IL-4, and Th17 cells expressed IL-17A/F. In contrast to IL-17A<sup>+</sup> cells in vivo (9) but in accord with IL-17A<sup>+</sup> cells generated in vitro (17), our in vitro–polarized Th17 cells produced less GM-CSF upon initial polarization and restimulation than the other Th subsets (Supplemental Fig. 1B). Similar to Th<sub>bas</sub> cells, activated Th1, Th2, and Th17 cells cocultured with monocytes promoted the formation of cells with dendritic morphology (Fig. 2B, Supplemental Video 2), which we refer to as DC<sub>TN</sub><sub>Th1</sub>, DC<sub>TN</sub><sub>Th2</sub>, and DC<sub>TN</sub><sub>Th17</sub>, respectively. All DC<sub>TN</sub> subsets upregulated MHC II, CD86, and CD40 compared with fresh monocytes (Fig. 2C). Interestingly DC<sub>TN</sub><sub>Th2</sub> had lower expression of the costimulatory molecules, CD40 and CD86, as compared with DC<sub>TN</sub><sub>Th1</sub> and DC<sub>TN</sub><sub>Th17</sub>. DC<sub>TN</sub><sub>Th1</sub>, DC<sub>TN</sub><sub>Th2</sub>, and DC<sub>TN</sub><sub>Th17</sub> were purified, pulsed with OVA peptide and LPS, extensively washed, and then cultured with OT-II T cells to evaluate their functional properties. After 3 d in culture, considerable T cell activation and proliferation were observed in the DC<sub>TN</sub><sub>Th1</sub> and DC<sub>TN</sub><sub>Th17</sub> wells, but not in the DC<sub>TN</sub><sub>Th2</sub> Wells. DC<sub>TN</sub><sub>Th1</sub> induced significantly more T cell proliferation than DC<sub>TN</sub><sub>Th2</sub> (Fig. 2D), and both DC<sub>TN</sub><sub>Th1</sub> and DC<sub>TN</sub><sub>Th17</sub> induced significantly more T cell proliferation than macrophages or fresh monocytes. Although DC<sub>TN</sub><sub>Th2</sub> and fresh monocytes induced detectable levels of T cell proliferation, it was minimal compared with DC<sub>TN</sub><sub>Th1</sub> and DC<sub>TN</sub><sub>Th17</sub>. These observations show that activated Th1, Th2, and Th17 T cells promote the development of Mo-DC capable of presenting Ag and activating T cells, but only DC<sub>TN</sub><sub>Th1</sub> and DC<sub>TN</sub><sub>Th17</sub> are potent APC, as gauged by their ability to induce T cell proliferation.

**Activated DC<sub>TN</sub><sub>Th17</sub> produce large amounts of IL-12 in response to TLR stimulation**

DC production of IL-12p70 promotes the development of IFN-γ–producing Th1 cells (18). Although a Th1 response can be beneficial in the clearance of tumors and intracellular pathogens, it can be harmful in the setting of autoimmunity (19, 20). In EAE, IL-12p70 and Th1 cells are not required to initiate disease, as IL-12–specific p35 KO mice that cannot produce biologically active IL-12p70 are still susceptible to active EAE induction (21, 22). Nonetheless, IFN-γ–producing Th1 cells can contribute to inflammation (23) and are sufficient to induce EAE on their own in transgenic T cell transfer models. Surprisingly, even though Th1 cells can drive inflammation, there is evidence to suggest that a Th1 response is also important for disease resolution in EAE (24).

To determine whether the DC<sub>TN</sub><sub>Th17</sub> subsets differ in their capacity to produce IL-12p70, we activated each DC<sub>TN</sub><sub>Th17</sub> subset with standard LPS for 24 h and then analyzed the supernatants for the presence of this cytokine (Fig. 3A). All DC<sub>TN</sub><sub>Th17</sub> subsets produced minimal IL-12p70 in the absence of stimulation. However, after stimulation with LPS, DC<sub>TN</sub><sub>Th17</sub> produced significantly more IL-12p70 than either DC<sub>TN</sub><sub>Th1</sub> or DC<sub>TN</sub><sub>Th2</sub>. Remarkably, DC<sub>TN</sub><sub>Th17</sub> produced ~10 times more IL-12p70 than commonly studied GM IL-4 DC. No intracellular IL-12p40 was detected within the Th17 cells present in the cocultures, indicating that DC<sub>TN</sub><sub>Th17</sub> were the sole source of IL-12p70 (Supplemental Fig. 1C).

To address the question of whether stimuli other than LPS can induce DC<sub>TN</sub><sub>Th17</sub> to produce IL-12p70, we stimulated DC<sub>TN</sub><sub>Th1</sub>, DC<sub>TN</sub><sub>Th2</sub>, or DC<sub>TN</sub><sub>Th17</sub> cells with a panel of TLR agonists including Pam3CSK4 (TLR1/2), HKLM (primarily TLR2), polyinosinic-polycytidylic acid (TLR3), ultra-pure LPS (TLR4), imiquimod (TLR7), or CpG ODN2336 (TLR7/9) (Fig. 3B). DC<sub>TN</sub><sub>Th17</sub> responded robustly to most of these stimuli by producing IL-12p70. Notably, Pam3CSK4 and HKLM, both TLR2 agonists, induced high levels of IL-12p70 secretion. Ultra-pure LPS, CpG, and, to a lesser extent, imiquimod also induced detectable amounts of IL-12p70. Remarkably, the ability to produce IL-12p70 was limited to DC<sub>TN</sub><sub>Th17</sub>, as DC<sub>TN</sub><sub>Th1</sub> and DC<sub>TN</sub><sub>Th2</sub> cultured with the same TLR agonists failed to respond by secreting IL-12p70, even though they clearly became activated in response to TLR stimulation (Supplemental Fig. 1D). Thus, although all DC<sub>TN</sub> subsets can respond to TLR stimulation, DC<sub>TN</sub><sub>Th17</sub> are uniquely programmed to produce high levels of IL-12p70 and do so in response to a variety of danger signals.

Many recent studies have focused on the role of IL-23 in maintaining the polarization of Th17 cells. In fact, IL-23 is critical for EAE induction in the traditional MOG/CFA model, but is not necessary to maintain disease once EAE has been induced via passive transfer of T cells (25). DC<sub>TN</sub> subsets were assessed for IL-23 production by ELISA (Fig. 3C). As with IL-12p70, very little IL-23 was detected without stimulation. However, after LPS stimulation, both DC<sub>TN</sub><sub>Th1</sub> and GM IL-4 DC produced large amounts of IL-23. Taken together, these results demonstrate that DC<sub>TN</sub>, particularly DC<sub>TN</sub><sub>Th17</sub>, can respond to many different inflammatory mediators by producing both IL-12 and IL-23.

**CD40L and RANKL on activated Th17 cells are required for the formation of IL-12p70–producing DC<sub>TN</sub><sub>Th17</sub>**

We next wanted to explore why Th17 cells, but not Th1 or Th2 cells, induced DC with the ability to produce large amounts of IL-12p70. Although Th17 cells express a unique set of cytokines and effector molecules such as IL-17A, IL-17F, and IL-22, these molecules are not known to enhance IL-12p70 production. In contrast, the TNF-α family of proteins contains multiple molecules that influence cytokine production by myeloid cells (26). Two such molecules, CD40L and RANKL, are also highly expressed by activated Th cells (27, 28). We stimulated DC<sub>TN</sub><sub>Th17</sub> with LPS in the presence of blocking Abs to CD40L or RANKL or isotype control Ab (Fig. 4A). CD40L blockade resulted in a nearly complete lack of IL-12p70 expression as assayed via ELISA. RANKL blockade resulted in a highly significant partial reduction of IL-12p70 expression. We confirmed these findings with intracellular staining of the same cells using an Ab to IL-12p40 (Fig. 4B).

To determine if Th17 cells from CD40L KO mice are also impaired in their ability to generate IL-12p70 competent DC<sub>TN</sub><sub>Th17</sub>, we generated Th17 cells from WT or CD40L KO mice and cultured...
FIGURE 2. Activated Th1, Th2, and Th17 cells drive the formation of phenotypically distinct populations of DCTh. (A) CD8-depleted splenocytes were activated with 1 μg/ml anti-CD3 in the presence of various polarizing cytokines and blocking Abs to skew the T cells toward a Th1, Th2, or Th17 phenotype. After 96 h, the cells were activated and stained intracellularly for IFN-γ, IL-4, and IL-17A. Cells shown are gated on Live/Dead Blue−, Thy1.2+. (B) BM monocytes as used in Fig. 1 were cultured 10:1 with Th1-, Th2-, or Th17-polarized T cells for 5 d in the presence of 10 ng/ml IL-2. The cultures were then imaged with DIC microscopy. (C) Cell-surface phenotype of DAPI−, Thy1.2+, CD11b+ cells after 5 d of culture with Th1− (blue), Th2− (green), and Th17-polarized T cells (orange) or fresh after isolation. Median fluorescence intensity for each marker is shown in the table above. This experiment was performed at the same time and under identical conditions as Fig. 1C. Data shown are representative of more than three independent experiments. (D) DCTh1, DCTh2, DCTh17, M-CSF macrophages, or fresh monocytes were purified, pulsed with MHC II–restricted OVA peptide and 1 μg/ml LPS for 90 min, washed, and then cultured at a ratio of 1 APC/15 naive T cells from OT-II RAG KO mice in triplicate for 3 d and pulsed. (Figure legend continues)
them with monocytes for 5 d and then stimulated the cocultures with LPS overnight (Fig. 4C). In line with data from the Ab-blocking experiments, IL-12p70 levels were significantly reduced in the supernatant from monocyte/CD40L KO Th17 cocultures. Similar experiments using Th cells from RANKL KO were considered, but dysregulated lymphoid cell development in these animals would have confounded such experiments (29).

Finally, we wanted to determine whether Th17-skewed cells express more CD40L and/or RANKL compared with the Th1 and Th2 skewed cells. To test this idea, we restimulated polarized Th cultures with CD3/CD28 microbeads after a 2-d rest in IL-2, which was necessary to maintain Th1 and Th2 cell viability (data not shown). Th17 cell cultures contained ~50% more CD40L+ T cells than Th1 or Th2 cultures (Fig. 4D). In addition, 52% of the cells in the Th17 cultures were RANKL+ compared with 36% in Th1 cultures and 25% in Th2 cultures. These data demonstrate that Th17 cultures contain large numbers of CD40L- and RANKL- positive cells and that these molecules are necessary for maximal IL-12p70 expression in T cell–induced monocyte-derived DC.

DC<sub>Th17</sub> polarize Ag-specific naive T cells and previously committed Th17 cells into Th1 cells

Because stimulated DC<sub>Th17</sub> produce large amounts of IL-12p70, we hypothesized that they would be superior at inducing Th1 polarization. To test this, we pulsed the DC<sub>Th</sub> subsets with OVA peptide and LPS and, after extensive washing, cultured purified DC<sub>Th</sub> subsets at a 1:15 ratio with naive OT-II T cells. After 96 h of culture, intracellular staining for IFN-γ, IL-4, and IL-17A was performed (Fig. 5A). No IL-4 or IL-17A staining was detected above isotype control levels (data not shown). However, in accordance with the IL-12p70 production data, large numbers of T cells expressing high levels of IFN-γ were seen in the cultures containing DC<sub>Th17</sub>. IFN-γ was detected in a small percentage of the T cells cultured with DC<sub>Th1</sub>, but these cells expressed ~2.5-fold less IFN-γ on a per-cell basis than T cells cultured with DC<sub>Th17</sub>.

Under our experimental conditions, DC<sub>Th</sub> subsets did not induce Th17 polarization from naive CD4+ T cells. However, we hypothesized that DC<sub>Th</sub> may influence the stability of the Th17 phenotype in polarized cells. To investigate this possibility, we generated Th17 cells from OT-II RAG KO mice under polarizing conditions, rested the cells for 2 d in media in the absence of IL-2, and then cultured them with peptide-pulsed or unpulsed DC<sub>Th1</sub> or DC<sub>Th17</sub>. After 4 d, the T cells were stimulated with PMA and ionomycin in the presence of BFA and then stained intracellularly for IL-17A (Fig. 5B and quantified in Supplemental Fig. 1E). Compared to Th17 cells cultured alone, IL-17A production capacity by Th17 cells was better maintained when the Th17 cells were cocultured with DC<sub>Th1</sub> or DC<sub>Th17</sub>, and this effect was further

with [³H]thymidine for the last 18 h. The dotted line shows the mean proliferation of the T cells in the absence of APC. Results are representative of two independent experiments. Error bars represent SEM.

**FIGURE 3.** DC<sub>Th17</sub> produce large amounts of IL-12 in response to TLR stimulation. DC<sub>Th</sub> were generated by monocyte–T cell coculture for 5 d as in Fig. 2. (A) On day 5, 1 µg/ml of LPS was added, or cells were not additionally treated. IL-12p70 in cell-free supernatants collected 24 h later was measured by ELISA. A representative experiment performed in triplicate is shown. Results are representative of more than five similar experiments. (B) On day 5, various TLR agonists were added to DC<sub>Th1</sub>, DC<sub>Th2</sub>, DC<sub>Th17</sub>, or control cell cultures. IL-12p70 in cell-free supernatants collected 24 h later was measured by ELISA. Done in triplicate. (C) Supernatants as in (A) were tested by ELISA for IL-23. A representative experiment performed in triplicate is shown. Results are representative of two similar experiments. Error bars represent SEM.
enhanced in cultures treated with LPS. An identical trend was observed when the Th17 cells were rested in the presence of TGF-β, which has been reported to be necessary for Th17 maintenance in vitro (data not shown) (30). Interestingly, the addition of OVA peptide to the DCTh resulted in the appearance of IFN-γ–producing T cells in the DCTh17 culture, but not in the DCTh1 culture, suggesting that DCTh17 can promote reprogramming of Th17 cells into Th1 cells in an Ag-specific manner.

The possibility exists that the IFN-γ–producing cells in our cocultures were derived from holdover naive cells that did not become polarized to a Th17 phenotype during the initial coculture. To address this possibility, we polarized OT-II cells toward a Th17 phenotype and then FACS purified the IL-17A–secreting Th17 cells by using cytokine-capture cell sorting. These highly purified cells, which by definition produced IL-2 for 2 d and then restimulated with CD3/CD28 microbeads. Two hours after restimulation, the cells were FC blocked and then stained for CD40L. Six hours after restimulation, the cells were FC blocked and then stained for RANKL. Cells shown were gated on CD4+; Thy1.2+; DAPI− cells. Shown with an isotype control for each Ab and cell type. Representative of two independent experiments.

FIGURE 4. Th17 cells induce IL-12 expression in DCTh17 in a CD40L- and RANKL-dependent manner. (A) Monocytes were cultured for 5 d with Th17 cells in the presence of 10 μg/ml of functional-grade blocking Ab to either CD40L or RANKL. A coculture with 10 μg/ml of each functional-grade isotype was included as a control. After 5 d, 1 μg/ml of LPS was added to the cultures, and the next day, the cell-free supernatant was harvested for IL-12p70 ELISA. Done in triplicate. (B) DCTh17 were prepared as in (A), but after 12 h of stimulation with LPS, the cells were treated with BFA for 4 h. The cells were then FC receptor blocked and intracellularly stained for IL-12p40. The cells shown were previously gated on Live/Dead Aqua−, Thy1.2+, CD11b+ cells. (C) DCTh17 were generated with WT Th17 or CD40L KO Th17 cells. After 5 d, the cells were stimulated with 1 μg/ml of LPS. Twenty-four hours later, the cell-free supernatant was tested for IL-12p70 with ELISA. Done in triplicate. (D) Th1, Th2, or Th17 cultures were rested in the presence of IL-2 for 2 d and then restimulated with CD3/CD28 microbeads. Two hours after restimulation, the cells were FC blocked and then stained for CD40L. Shown with an isotype control for each Ab and cell type. Representative of two independent experiments.
**FIGURE 5.** DC<sub>Th17</sub> polarize naive T cells to Th1 cells and can convert Th17 cells into Th1 cells in the presence of specific Ag and LPS. (A) DC<sub>Th</sub> subsets or control APCs were purified with magnetic microbeads and then pulsed with 2.5 μg/ml MHC II–restricted OVA peptide and 1 μg/ml LPS for 90 min. The cells were washed twice and then cultured at a ratio of 1 APC/15 naive T cells from OT-II RAG KO mice. On day 4 of culture, the cells were restimulated with PMA/ionomycin and BFA for 4 h prior to intracellular staining. Cells shown are gated on Live/Dead Blue<sup>2</sup>, Thy1.2<sup>+</sup> cells. Data are representative of two independent experiments. (B) Th17 cells were rested in media for 2 d and then cultured 1 DC/5 T cells with DC<sub>Th1</sub> or DC<sub>Th17</sub> that had been pulsed with PBS, LPS alone, or LPS and MHC II–restricted OVA peptide for 90 min. After 3 d of coculture, the T cells were activated with PMA/ionomycin and BFA for 4 h and then intracellularly stained for IL-17A and IFN-γ. Cells were gated on Live/Dead Blue<sup>2</sup>, Thy1.2<sup>+</sup> cells. One replicate for each condition is shown. Data are representative of two independent experiments. (C) IL-17A–secreting OT-II T cells were sorted from in vitro Th17 cultures, rested in the absence of IL-2 for 3 d, and then cocultured with ISQ- and LPS-pulsed DC<sub>Th1</sub> or DC<sub>Th17</sub>. After 4 d, the ex-IL-17A<sup>+</sup> cells were evaluated for IL-17A and IFN-γ expression by intracellular cytokine staining. Done in duplicate. (D) The percentage of IFN-γ and IL-17A single-positive T cells in each condition are shown. Error bars represent SEM.
Th cells, monocytes, and DC colocalize in EAE lesions

We induced EAE using a Th17-skewed passive-transfer model that enables investigation of the biology of EAE in the absence of confounding factors such as CFA and pertussis toxin. In this model, lymphoid cells from actively immunized mice are cultured in MOG and IL-23 to skew the cells toward a Th17 phenotype (Supplemental Fig. 1F), and then the polarized cells are transferred to naive recipients to induce EAE. Spinal cords were harvested from diseased mice at various time points, and the surface phenotype of the infiltrating cells was analyzed by flow cytometry. Other than resident microglia, immune cells were largely absent from the spinal cords of presymptomatic mice, but rapidly accumulated at disease onset and remained abundant throughout the course of disease (Fig. 6A). As expected, numerous CD4+ T cells infiltrated the spinal cord at the onset of disease, and these cells remained numerous throughout disease progression (Fig. 6B). CD45hi, Ly6g−, CD11b+, CD11c− monocytes were present at onset, increased at peak disease, and then decreased at recovery. CD45hi, CD11b+, CD11c− DC increased after onset and remained at high frequency during EAE remission. Interestingly, Ly6g−/CD11b− neutrophils accumulated with increased disease severity and then rapidly dissipated with recovery.

To determine if monocytes, DC, and T cells are associated with one another in the EAE lesions, we performed immunofluorescence staining on spinal cord tissue from Th17-skewed passive-transfer EAE at peak disease or healthy control mice (Fig. 6C). No immune cells were visualized in the healthy WT control spinal cord tissue. In contrast, multiple perivascular lesions were seen in the white matter of the EAE spinal cords containing CD11b+ cells (granulocytes and monocytes), CD11b+/CD11c− cells (DC), and CD3+ T cells (T cells). Upon further magnification (Fig. 6D), all three cell types could be seen in direct contact with one another. These data show that monocytes, T cells, and DC colocalize in EAE lesions and are consistent with the hypothesis that activated T cells drive the formation of DCTh in vivo.

Infiltrating Th cells from EAE lesions drive Mo-DC formation

To determine if Th cells from EAE spinal cords can promote DCTh formation, we purified CD4+ T cells from Th17 passive-transfer EAE mice and cultured them with BM monocytes from healthy mice for 4 d. As a control, we used splenic CD4+ T cells from healthy mice, as almost no T cells are present in healthy spinal cords and because splenocytes from EAE mice may contain recirculating encephalitogenic T cells. We performed this coculture in the absence of specific Ag or other stimuli, as the CNS-infiltrating T cells were already activated. However, as with the in vitro–activated Th cells, the addition of exogenous IL-2 was necessary to maintain T cell survival (data not shown). We observed by microscopy that monocytes cultured alone or with splenic T cells retained a rounded morphology and displayed poor viability (Fig. 7A). In contrast, monocytes cultured with EAE CD4+ T cells not only remained viable but also became considerably larger and more granular and developed numerous active dendrites, reminiscent of GM IL-4 DC and DCp, which could be visualized with time-lapse microscopy (Supplemental Video 3). The monocytes cultured with EAE CD4+ T cells also upregulated their surface expression of CD11b, CD11c, MHC II, CD40, and CD86 compared with monocytes cultured with healthy splenic CD4+ T cells (Fig. 7B). The cells also expressed CD209, a marker of Mo-DC found in inflammatory settings (6). Interestingly, the cells retained high Ly6C expression, reminiscent of Mo-DC recovered from the CNS in transfer experiments (13). This phenotype closely resembled that of DCTh and on this basis, we refer to these EAE Th cell–infiltrated cells as DCThEAE.

Th cell production of GM-CSF has been implicated as a driver of pathogenesis in EAE (15, 31). In line with these studies, CNS-infiltrating Th cells produced GM-CSF ex vivo when cultured with monocytes and IL-2 (Supplemental Fig. 1G). To determine whether GM-CSF was involved in the formation of DCThEAE, we cultured CNS-infiltrating Th cells with monocytes from WT or GM-CSFR KO mice. Unexpectedly, signaling through the GM-CSFR on monocytes was not necessary for the induction of a DCTh phenotype (Supplemental Fig. 1H).

To determine if DCThEAE could take up and present Ag to naive T cells, we used these cells in an Ag-specific T cell proliferation assay (Fig. 7C). As their phenotype would suggest, Mo-DC generated by EAE Th cells, but not monocytes cultured with splenic Th cells, were capable of robustly stimulating naive OT-II T cells when pulsed with OVA peptide. As EAE was induced by Th17-skewed cells, we wanted to determine if DCThEAE behave like DCTh17 in their ability to promote a Th1 response. To address this possibility, we purified DC from monocyte/EAE CD4+ T cell cocultures and examined IL-12p70 production following stimulation with ultra-pure LPS. Similar to DCTh17, DCThEAE produced substantial amounts of IL-12p70 upon stimulation (Fig. 7D). IL-12p40+ DC were also detected in the spinal cords of EAE mice by IC staining in the absence of exogenous stimulation (data not shown). Moreover, when pulsed with LPS and OVA peptide and cultured with naive OT- II T cells, DCThEAE-polarized naive T cells to an IFN-γ Th1 phenotype as indicated by ELISA (Fig. 7E). Neither IL-17A nor IL-17A/F was detected in these same cultures (data not shown). Thus, encephalitogenic CD4+ T cells from the inflamed spinal cord of Th17-skewed passive transfer EAE mice induce the formation of Mo-DC with Th1-polarizing capacity.

To investigate the role of myeloid cell–derived IL-12 in vivo during EAE, we used BM chimeric mice. EAE was induced in IL-12p35 KO and WT BM chimeras via passive transfer of Th17-skewed encephalitogenic cells (Fig. 7F). Both groups of mice began to show signs of tail paralysis by day 10. However, recipients of IL-12p35 KO BM displayed milder symptoms early (days 13–15), yet were impaired in their ability to recover from peak disease (days 17–20). Although these trends did not reach statistical significance due to the low numbers of mice used (n = 4 to 5), the data support the hypothesis that myeloid-derived IL-12 contributes to disease pathology but is also important for suppressing a pathogenic Th17-mediated response in favor of a milder Th1-mediated response.

Discussion

It is well documented that monocytes infiltrate inflamed tissues and differentiate into inflammatory DC (5, 6, 32). However, the physiologic mechanisms responsible for their formation and subsequent function during immunity and inflammatory disease are poorly understood. The present study demonstrates that activated Th cells can govern inflammatory DC formation and function. The present study also demonstrates that activated CD4+ T cells found within EAE spinal cords elicit the differentiation of monocytes into DC, which suggests that this system is active in autoimmune disease. This finding is consistent with published studies of EAE indicating that T cell–derived molecules including CD40L, GM-CSF, M-CSF, FLT3 ligand, and CCL2 acting on infiltrating myeloid cells are key drivers of disease (13, 15, 33–35). Further, the phenotype and functions of DCTh are influenced by the type of Th cells in the inflammatory milieu. Our data show that DCTh and DCTh17 are proficient APCs capable of presenting Ag and stimulating naive T cells. Interestingly, although Th2 cells caused the
differentiation of monocytes into cells that resembled DCTh, the resulting DCTh2 actually had very little T cell–stimulating capacity, which might be explained by their lower CD86 expression. These findings support previously published work showing that only Th1 and Th17, but not Th2 cells, can induce EAE (9).

The cytokines produced by the various DCTh subsets differed substantially. In contrast to DCThBulk, DCTh17 produced large amounts of IL-12 and IL-23 after LPS stimulation. Importantly, IL-12 was secreted from DCTh17 after stimulation with a variety of different TLR agonists, suggesting that these cells produce IL-12 as a generalized response to danger. The induction of IL-12 in DCTh17 was highest when the cells were stimulated with TLR2 and -4 agonists. This may be important in the context of autoimmunity because multiple endogenous TLR ligands, including

FIGURE 6. T cells, monocytes, and DC colocalize in EAE lesions. (A) Leukocytes infiltrating the CNS were obtained by density gradient separation and then analyzed by flow cytometry at the indicated times after passive transfer of Th17-skewed encephalitogenic cells. Representative FACS plots (n = 2 to 3 mice/group) of DAPI+, CD45+ cells isolated from a single spinal cord are shown. (B) Frequency of viable CD4+ T cells, granulocytes, monocytes, and DC from (A). Error bars represent SEM. (C) Representative spinal cord tissue from a healthy WT mouse (top panel) or an EAE mouse at peak disease with a score of 4 (bottom panel) was stained for CD11b (green), CD11c (red), CD3 (pink), and DAPI (blue), and the resulting images are shown at low power. (D) The tissue outlined by the white box in (C) is shown at high power.
FIGURE 7. Infiltrating EAE Th cells induce the formation of Th1 skewing Mo-DC. (A) CD4+ T cells from EAE mice spinal cords or a healthy WT spleen cells were cultured 1:10 with monocytes alone or monocytes in the presence of 10 ng/ml IL-2 for 4 d and then imaged with bright-field microscopy. Scale bars, 50 μm. The results are representative of four independent experiments. (B) Cells from (A) were gated on CD4+ , CD11b+, DAPI+ and analyzed by flow cytometry. Median fluorescence intensity for each marker is shown in the table above. Results are representative of three independent experiments. (C) Cells from (A) were harvested, pulsed with MHC II–restricted OVA peptide, cultured in triplicate at a ratio of 1 APC/33 naive T cells from OT-II RAG KO mice for 3 d, and then pulsed with [3H] thymidine for 20 h. The dotted horizontal line represents the mean proliferation of OT-II T cells in the absence of APC. The solid horizontal line represents the mean proliferation of the DC'ThEAE without OT-II T cells. Cultures were performed in triplicate. (D) Cells prepared as in (A) were stimulated overnight with 1 μg/ml of LPS. The cell-free supernatant was assayed in duplicate by ELISA for IL-12p70. Data shown are representative of two independent experiments. (E) CD11b+ DC'ThEAE selected via MACS were pulsed with or without 1 μg/ml LPS and 2.5 μg/ml OVA peptide for 90 min, washed, and then cultured at a ratio of 1 APC/5 naive T cells from OT-II RAG KO mice for 5 d. Supernatant was collected and assayed via ELISA for IFN-γ. Done in triplicate. (F) BM chimeric mice were generated using WT or IL-12p35 KO BM. Ten weeks later, the mice were injected with Th17-skewed encephalitogenic cells to induce EAE, and mice were monitored for signs of disease on a daily basis. n = 4 to 5 mice/group. Data are presented as means ± SEM, with significance determined by a two-tailed Mann–Whitney U test. Day 17, p = 0.27; day 18, p = 0.36; day 19, p = 0.37; day 20, p = 0.45. ND, Not detected.
products released from necrotic cells, have been shown to signal through these receptors (36, 37).

The capacity of Th17 cells to recruit and activate neutrophils is well established and can lead to the destruction of pathogens as well as injury to peripheral tissue (38). Our time-course analysis of the CNS in Th17-skewed passive transfer EAE shows that recovery from disease correlates strongly with a reduction of infiltrating neutrophils. A possible explanation for this reduction is the rise of DCTh17, which through their production of IL-12 would be expected to inhibit the generation of Th17 cells. The rapid shutdown of IL-17 production in Th17 cells after exposure to IL-12 suggests a possible mechanism for generation of Th17 cells. The rapid shutdown of IL-17 production in Th17 cells after exposure to IL-12 would be expected to inhibit the generation of Th17 cells that previously produced IL-17 cytokines within the inflamed spinal cord of EAE mice (12). Similar IFN-γ-producing T cells have also been described in other Th17-mediated disease such as colitis (39) and arthritis (40), so it will be important to determine if DCTh17 play a role in these disorders as well.

Our data demonstrate that CD40L and RANKL are necessary for maximal IL-12p70 production by DCTh17. After restimulation, Th17 cells more frequently express these molecules than either Th1 or Th2 cells, which may contribute to their ability to enhance IL-12p70 production in DCTh. These molecules are expressed rapidly after T cell activation, which might be expected to result in the up regulation of IL-12 shortly after a Th17-mediated response. Other molecules differentially expressed by Th17 cells following activation may play a role in endowing Th17 cells with this unique ability to induce IL-12p70 in DCTh.

One clinically important question in MS is the role of Th subsets in established disease. Whereas Th1 cells can clearly cause EAE under experimental conditions, Th17 cells have been implicated as the initiating cells in MS and EAE, based on their unique ability to traffic through the choroid plexus in a CCR6-dependent manner prior to CNS inflammation (41, 42). Th1 cells are believed to arise later, potentially as a safeguard against excessive Th17-mediated immunity. Th1 cells, which infiltrate the CNS later than Th17 cells, are considered to be more responsive to Treg-mediated immunosuppression than Th17 cells and thus drive less autoimmune pathology (43). This idea is supported by data showing that Th1, but not Th17, cells require IL-2 for their survival (44, 45). Because Treg cells regulate IL-2 levels via expression of the high-affinity IL-2R (CD25), they would be poised to control Th1 inflammation, but would be less effective at controlling Th17 inflammation (46).

The findings presented in this study are consistent with the view that although Th1 cells may contribute to EAE pathology, these cells ultimately have a moderating effect on autoimmune disease. This is supported by the fact that Th1 cells are the major T cell subset present in the spinal cord just prior to the recovery phase in Th17-initiated EAE (43). Specifically, our findings suggest a model in which Th17 cells initiate disease and then induce the formation of DCTh17 with potent Th1-polarizing capacity. In the presence of Ag and danger signals, these DCTh17 promote the conversion of Th17 cells already present in the inflamed CNS, as well as naive T cells, into IFN-γ+ Th1 cells. These Th1 cells may contribute to some CNS pathology, before ultimately being suppressed by Treg cells.

Our experiment with IL-12p35 BM chimera mice, along with work by others demonstrating that mice lacking IFN-γ, IL-12p35, or IL-12Rβ2 develop more severe EAE than control mice, reinforces this hypothesis (22, 47, 48). Because infiltrating DC produce the majority of the IL-12 in the inflamed spinal cord (49), their influence on recovery from EAE may be critical. Additionally, EAE mice lacking the IFN-γ receptor in BM-derived cells have persistent neutrophil-rich foci in the CNS, suggesting that sensitivity to IFN-γ is necessary for modulating a neutrophil response (50). Most importantly, in an EAE model similar to our own in which disease was induced by passive transfer of IL-23–treated cells, the addition of an IFN-γ-neutralizing Ab resulted in significantly worse disease (51). Intriguingly, IFN-β, which is widely used in the treatment of MS, requires intact IFN-γ signaling for efficacy in EAE (16).

Monocytes and T cells respond to many of the same chemotactic gradients including CCL2 (52), CXCL9–11, and CCL3–5 (2), so it is not surprising that inflamed tissues in multiple autoimmune diseases contain monocytes and activated T cells in direct contact (7). The induction of Mo-DC formation by activated Th cells likely has implications that extend beyond CNS inflammation. In this regard, previous work from our group investigating the skin of patients with psoriasis and atopic dermatitis showed that Th cells induce the formation of DCTh17 from monocytes at sites of inflammation in humans (7). Taken as a whole, our findings raise the exciting possibility that activated T cells drive the formation of DCTh17 in a diverse set of autoimmune diseases and that DCTh17 specifically act to bridge Th1 and Th17 responses.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figures:

A) CD8-depleted splenocytes were cultured under Th1, Th2 or Th17 polarizing conditions for 96 hours. The cell-free supernatant was then assayed in triplicate using ELISA for IFNγ, IL-17A/F and IL-4. Error bars represent SEM.

B) ThBulk, Th1, Th2 and Th17 cells were generated by culturing splenocytes under polarizing conditions for 72 hours. The cells were then rested for 48 hours in the presence of IL-2. The cells were then restimulated for 6 hours with CD3/CD28 beads to induce cytokine secretion. Supernatants were collected at all three time points and evaluated for GM-CSF via ELISA. Done in duplicate.

C) DCTh17 or GM-CSF and IL-4-derived DC were stimulated with LPS. 12 hours later BFA was added and the cells were further incubated for 4 hours. The cells were then stained intracellularly for IL-12p40 and gated on viable CD11b+ DCTh17 or Thy1.2+ Th17 cells. Specificity was confirmed by staining GM IL-4 DC with an unconjugated anti-IL-12p40 antibody followed by staining with the fluorochrome-labeled antibody.

D) 1μg/ml ultra-pure LPS was added to DCTh cultures for 24 hours or the cells were left unstimulated. The cells were then FC blocked and stained for CD40. The cells shown were gated on dapi−, Thy1.2+, CD11b+ cells.

E) The percentages of IFNγ and IL-17A single positive T-cells present after culture with DCTh1 or DCTh17 are shown. This is a quantification of the data presented in Figure 5B. Done in duplicate. Error bars represent SEM.

F) Splenocytes and lymph node cells from mice actively immunized with MOG/CFA were cultured in triplicate with MOG only or MOG and 10ng/ml IL-23 for 72 hours. The cell-free supernatant was assayed in triplicate for IL-17A/F via ELISA. Error bars represent SEM.

G) CD4+ T-cells were purified from the inflamed spinal cord of EAE mice or the spleen of healthy control mice and cultured with monocytes in the presence of IL-2 for 4 days. The cell-free supernatant was assayed for GM-CSF by ELISA. Run in triplicate. Representative of 2 independent experiments.

H) CD4+ T-cells were purified from the inflamed spinal cord of EAE mice and cultured with monocytes from either WT or GM-CSFR KO mice in the presence of IL-2 for 4 days. Monocyes cultured with WT CD4+ T-cells in the presence of IL-2 were included as a negative
control. Dapi, Thy1.2, CD11b+ cells are shown with MFI for each marker. Representative of 2 independent experiments.

**Supplemental Movies**

A) Fresh BM monocytes, cultured monocytes, DC_{ThBulk}, GM IL-4 DC or M-CSF macrophages were cultured on a cover slip on the bottom of tissue culture dishes. After 5 days, the dishes were placed in a 37°C incubated chamber and imaged with DIC microscopy. Images were taken every 10 seconds for 10 minutes. Movies are shown at 10 frames per second.

B) DC_{Th1}, DC_{Th2}, DC_{Th17}. Images were acquired as in Supplemental Movie A.

C) DC_{ThEAE} were imaged in tissue culture dishes at room temperature with bright field microscopy at 40x.
A

B

C

D

E

F

G

H