**Batf3-Dependent CD11b\(^{low/−}\) Peripheral Dendritic Cells Are GM-CSF-Independent and Are Not Required for Th Cell Priming after Subcutaneous Immunization**

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**Abstract**

Dendritic cells (DCs) subsets differ in precursor cell of origin, functional properties, requirements for growth factors, and dependence on transcription factors. Lymphoid-tissue resident CD8\(^{α}守住 conventional DCs (cDCs) and CD11b\(^{low/−}\)CD103\(^+\) non-lymphoid DCs are developmentally related, each being dependent on FMS-like tyrosine kinase 3 ligand (Flt3L), and requiring the transcription factors Batf3, Irf8, and Idf2 for development. It was recently suggested that granulocyte/macrophage colony stimulating factor (GM-CSF) was required for the development of dermal CD11b\(^{low/−}\)/Langerin\(^+\)/CD103\(^+\) DCs, and that this dermal DC subset was required for priming autoreactive T cells in experimental autoimmune encephalitis (EAE). Here, we compared development of peripheral tissue DCs and susceptibility to EAE in GM-CSF receptor deficient (Csf2rb\(^{−/−}\)) and Batf3\(^{−/−}\) mice. We find that Batf3-dependent dermal CD11b\(^{low/−}\)/Langerin\(^+\) DCs do develop in Csf2rb\(^{−/−}\) mice, but that they express reduced, but not absent, levels of CD103. Further, Batf3\(^{−/−}\) mice lacking all peripheral CD11b\(^{low/−}\)/Langerin\(^+\) DCs show robust Th cell priming after subcutaneous immunization and are susceptible to EAE. Our results suggest that defective T effector priming and resistance to EAE exhibited by Csf2rb\(^{−/−}\) mice does not result from the absence of dermal CD11b\(^{low/−}\)/Langerin\(^+\)/CD103\(^+\) DCs.

**Introduction**

GM-CSF regulates the development and activity of several myeloid cell types and influences both the initiation and maintenance of adaptive immune responses [1]. Mice lacking GM-CSF or its receptor show decreased antigen specific T cell priming against the encephalitogenic peptide of myelin oligodendrocyte glycoprotein (MOG\(_{35−55}\)) and are resistant to EAE [2]. Beyond this role in priming adaptive immune responses, GM-CSF acts to sustain ongoing effector responses, both in EAE initiated by MOG\(_{35−55}\) peptide [2] and in collagen-induced arthritis [3]. Similarly, in a murine model of autoimmune myocarditis, GM-CSF acts during priming of Th17 responses by promoting IL-6 and IL-23 production from DCs and during ongoing autoimmunity responses by promoting survival of autoreactive CD4\(^+\) T cells [4].

Several recent studies indicate that it is GM-CSF rather than IL-17 that is the primary Th17-derived cytokine responsible for the development of EAE [5−7]. Initially, it was thought that IL-17 production would explain why Th17 cells and ROR\(γt\) were required for EAE. However, in vivo neutralization of IL-17 only reduced EAE severity but did not eliminate disease, and non-IL-17 and non-IFN-\(γ\) pathways have been implicated [8–10]. IL-23, but not IL-6 or TGF-\(β\), was found to be critical for regulating the pathogenicity of Th17 cells [11,12]. GM-CSF derived from T cells, but not other CNS-resident or peripheral immune cells, was required for development of EAE [5], and acted during the effector phase to augment pathogenicity of Th17 cells [6,7]. IL-23 induced Th17 cells to increase production of GM-CSF, which further enhanced IL-23 production by antigen-presenting cells [6]. ROR\(γt\) was shown to induce GM-CSF, which acted on infiltrating myeloid cells entering the CNS, rather than resident microglia, during the development of EAE [7]. These studies suggest a model of EAE in which pathogenic T cells secrete GM-CSF that activates myeloid cells infiltrating the CNS to produce pathogenic lesions and further amplifies IL-23 production by DCs [13].

A recent report has suggested that the role for GM-CSF in EAE was based on its requirement for the development of dermal CD11b\(^{low/−}\)/Langerin\(^+\)/CD103\(^+\) DCs [14]. Dermal CD11b\(^{low/−}\)/Langerin\(^+\)/CD103\(^+\) DCs represent one anatomic subtype of peripheral tissue CD11b\(^{low/−}\)/CD103\(^+\) DCs, all of which share with
lymphoid tissue CD8α+ cDCs developmental dependence on Batf3, Ifnb, and Id2 [15,16]. CD8α+ cDCs are cross-presenting DCs, characterized by consistent expression of DEC205, and variable expression of CD103 which can be regulated by GM-CSF, IL-3, and TGF-β1 [17–19]. Mice lacking GM-CSF or the GM-CSF receptor were reported to lack dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs in the skin and peripheral lymph nodes under steady state and inflammatory conditions, and were resistant to EAE [14]. In addition, radiation chimeras expressing the human diphtheria toxin receptor (DTR) controlled by the Langerin promoter [20] and depleted of peripheral CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs in CD103<sup>+</sup> mice were resistant to EAE and had reduced priming of MOG<sub>35–55</sub>-specific T cells [14]. This study concluded that GM-CSF was required for the initiation of EAE because it was required for the development of dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs, which were required to prime pathogenic T cells [14].

To test whether loss of GM-CSF receptor protects from EAE by eliminating dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs, we asked if Batf3<sup>−/−</sup> mice, which also lack this DC subset, are also protected from EAE. We find that Batf3<sup>−/−</sup> mice develop EAE after immunization and have increased numbers of MOG<sub>35–55</sub> peptide-specific T cells, excluding a requirement for either peripheral CD11b<sup>low/-</sup>CD103<sup>+</sup> DCs or lymphoid tissue-resident CD8α+ DCs in EAE development. Direct comparison of Batf3<sup>−/−</sup> and GM-CSF receptor deficient mice revealed that GM-CSF signaling was not required for the development of dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs, but rather regulated the expression level of CD103 on all peripheral DCs.

### Results

**Batf3<sup>−/−</sup> mice develop EAE following MOG immunization**

To compare Batf3<sup>−/−</sup> mice with mice lacking the GM-CSF receptor (Csf2rb<sup>−/−</sup>) on the same genetic background, we backcrossed Batf3<sup>−/−</sup> mice onto the C57BL/6 background for 10 generations. Csf2rb<sup>−/−</sup> mice lack the common β-chain for signaling through receptors for GM-CSF, IL-5, and IL-3. However, mice have a second β-chain, Csf2rb<sup>+</sup>, which selectively pairs with the IL-3 receptor α-chain. Thus, Csf2rb<sup>−/−</sup> mice lose responsiveness to GM-CSF and IL-3, but not to IL-5 [21,22]. Wild type (C57BL/6), Batf3<sup>−/−</sup>, and Csf2rb<sup>−/−</sup> mice were subcutaneously immunized with MOG<sub>35–55</sub> peptide as previously described and followed for clinical disease [14]. Csf2rb<sup>−/−</sup> mice remained disease free as reported [14], but Batf3<sup>−/−</sup> mice developed EAE with the same severity and kinetics as wild type C57BL/6 mice (Fig. 1A).

In draining lymph nodes, examination by ELISPOT assay showed that immunized Batf3<sup>−/−</sup> mice had an increased frequency of MOG<sub>35–55</sub>-specific T cells producing IL-17 and IFN-γ (Fig. 1B), while Csf2rb<sup>−/−</sup> mice had a significantly reduced frequency of such cells, as previously reported [14]. Thus, Batf3<sup>−/−</sup> mice, which lack all peripheral CD11b<sup>low/-</sup>CD103<sup>+</sup> DCs [16], are able to prime pathogenic CD4<sup>+</sup> T cells, and remain susceptible to EAE. These findings would disagree with the interpretation that resistance to EAE in Csf2rb<sup>−/−</sup> mice results from the absence of dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs as was recently suggested [14].

**Csf2rb<sup>−/−</sup> mice retain development of Batf3-dependent dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs**

GM-CSF was recently reported to directly regulate CD103 expression on splenic CD8α+ DCs [17–19], so conceivably Csf2rb<sup>−/−</sup> mice might retain dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs that simply lack CD103. To test this, we examined DCs from C57BL/6, Batf3<sup>−/−</sup>, and Csf2rb<sup>−/−</sup> mice for expression of markers that distinguish between Batf3-dependent and Batf3-independent DC subsets (Fig. 2A).

In skin draining lymph nodes (SDLNs), migratory DCs are evident as DEC205<sup>+</sup>EpCAM<sub>hi</sub> DCs and Langerhans cells of the epidermis (CD11b<sup>+</sup>Langerin<sup>+</sup>EpCAM<sub>lo</sub>) (Fig. 2A). However, differences were evident in the dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs between these strains. C57BL/6 mice contained the normal population of dermal Langerin<sup>+</sup> DCs and Langerhans cells of the epidermis (CD11b<sup>+</sup>Langerin<sup>+</sup>EpCAM<sub>lo</sub>) (Fig. 2A). However, differences were evident in the dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup> DCs between these strains. C57BL/6 mice contained the normal population of dermal CD11b<sup>low/-</sup>Langerin<sup>+</sup>EpCAM<sub>lo</sub> DCs, which were missing in Batf3<sup>−/−</sup> mice [16]. Notably, Csf2rb<sup>−/−</sup> mice retained this population of dermal DCs, but in this strain of mice, these DCs expressed slightly lower levels of CD103 relative to C57BL/6 mice. CD24 can also be used to distinguish between populations of dermal DCs [26], and similarly C57BL/6 mice contained a population of dermal Langerin<sup>+</sup>DC24<sup>+</sup>EpCAM<sub>lo</sub> DCs that were completely missing in Batf3<sup>−/−</sup> mice, but which were present in Csf2rb<sup>−/−</sup> mice (Fig. 2B). Csf2rb<sup>−/−</sup> mice showed reduced total...
lymph node cellularity and a reduced number of overall lymph node DCs, but normal numbers of Langerhans cells and dermal CD11b<sup>low</sup>/Langerin<sup>+</sup> DCs (Fig. 2C). Batf<sup>−/−</sup> mice showed a selective ~100-fold reduction in the number of dermal CD11b<sup>low</sup>/Langerin<sup>+</sup> DCs. These results indicate that Csf2rb<sup>−/−</sup> mice do contain dermal CD11b<sup>low</sup>/Langerin<sup>+</sup> DCs, but that in the absence of GM-CSF signaling, CD103 expression is reduced.

**C57BL/6 Batf<sup>3−/−</sup> mice contain CD8α<sup>+</sup> cDCs in SDLNs but not spleen**

In analyzing SDLNs of C57BL/6 Batf<sup>3−/−</sup> mice, we noticed an unexpected population of DEC205/CD8α<sup>+</sup> cDCs that were eliminated in the spleen and SDLNs of 129S6/SvEv Batf<sup>3−/−</sup> mice [16,27] (Fig. 2A, 2C). Therefore, we directly compared DCs from SDLNs of Batf<sup>3−/−</sup> mice on both genetic backgrounds (Fig. S1A). C57BL/6 Batf<sup>3−/−</sup> mice lacked dermal CD11b<sup>low</sup>/CD103<sup>+</sup> DCs (Fig. S1A) but had a persistence of DEC205/CD8α<sup>+</sup> cDCs in peripheral lymph nodes (Fig. S1A, S1B). In spleens, Batf<sup>3−/−</sup> mice showed a significant decrease in DEC205/CD8α<sup>+</sup> cDCs on both C57BL/6 and 129S6/SvEv backgrounds compared to controls (Fig. S1C, S1D). On the 129S6/SvEv background, DEC205/CD8α<sup>+</sup> cDCs were reduced from 4.8% of splenic cDCs in control mice to only 0.11% of splenic cDCs in Batf<sup>3−/−</sup> mice. On the C57BL/6 background, DEC205/CD8α<sup>+</sup> cDCs were reduced from 14% of splenic cDCs in control mice to only 2.5% of splenic cDCs in Batf<sup>3−/−</sup> mice (Fig. S1C, S1D). Thus, for splenic cDCs, Batf3 is non-redundant in the development of DEC205/CD8α<sup>+</sup> cDCs.

While loss of Batf3 does not eliminate the population of DEC205/CD8α<sup>+</sup> cDCs in SDLNs, these cells did not express CD103 normally (Fig. S1E). 9% of the DEC205/CD8α<sup>+</sup> cDCs in SDLNs of control mice expressed CD103, while only 1.8% expressed CD103 from Batf3<sup>−/−</sup> mice. Notably, CD103 expression was also reduced on this population from SDLNs of Csf2rb<sup>−/−</sup> mice. Conceivably, the persistence of DEC205/CD8α<sup>+</sup> cDCs in SDLNs on the C57BL/6 background could result from genetic polymorphisms that impact the redundancy of Batf3 in the development of these cells. Alternately, variable inflammation between strains could be involved, since infection with *Toxoplasma gondii* can regulate the size of splenic CD11b<sup>low</sup>/CD103<sup>+</sup> DC populations [26].

**129S6/SvEv Batf3<sup>−/−</sup> mice are susceptible to EAE**

Conceivably, persistence of DEC205/CD8α<sup>+</sup> cDCs in SDLNs of C57BL/6 Batf3<sup>−/−</sup> mice was the basis for their susceptibility to EAE, based on potentially overlapping functions for DEC205<sup>−</sup>/CD8α<sup>+</sup> cDCs and dermal CD11b<sup>low</sup>/CD103<sup>+</sup> DCs [29]. To test this, we examined Batf3<sup>−/−</sup> mice on the 129S6/SvEv background, which lack both DEC205<sup>−</sup>/CD8α<sup>+</sup> and dermal CD11b<sup>low</sup>/CD103<sup>+</sup> DCs in both spleen and SDLNs (Fig. 2D).

As with C57BL/6 Batf3<sup>−/−</sup> mice, Batf3<sup>−/−</sup> mice on the 129S6/SvEv background were equally as susceptible to EAE as wild type controls. Thus, loss of dermal CD11b<sup>low</sup>/CD103<sup>+</sup> DCs could not have explained the resistance to EAE that was observed in Csf2rb<sup>−/−</sup> mice.

**Csf2rb<sup>−/−</sup> mice retain Batf3-dependent CD11b<sup>low</sup>/peripheral tissue DCs with reduced CD103 expression**

We next compared C57BL/6, Batf3<sup>−/−</sup>, and Csf2rb<sup>−/−</sup> mice for the presence of DC subsets in peripheral tissues, specifically the lung and kidney. In the lung, DCs exist as two populations, characterized as Batf3-dependent CD11b<sup>low</sup>/Langerin<sup>+</sup>CD103<sup>+</sup> DCs and Batf3-independent CD11b<sup>low</sup>/Langerin<sup>−</sup>CD103<sup>+</sup> DCs. Lungs from Batf3<sup>−/−</sup> mice completely lacked CD11b<sup>low</sup>/Langerin<sup>−</sup>CD103<sup>+</sup> DCs (Figure 3A) as expected [16]. Importantly, lungs from Csf2rb<sup>−/−</sup> mice retained a normal population of pulmonary CD11b<sup>low</sup>/CD103<sup>+</sup> DCs, which expressed reduced levels of CD103 and undetectable levels of Langerin (Fig. 3A). Csf2rb<sup>−/−</sup> mice develop alveolar proteinosis due to defective surfactant handling by lung macrophages in the absence of GM-CSF signaling [21]. Conceivably, this inflammatory process could have influenced CD103 expression by CD11b<sup>low</sup>/lung DCs. To test this, we examined CD103 expression by DCs of the kidney, since this organ shows no pathology in Csf2rb<sup>−/−</sup> mice. Again, while Batf3<sup>−/−</sup> mice completely lacked CD11b<sup>low</sup>/CD103<sup>+</sup> kidney DCs, Csf2rb<sup>−/−</sup> mice had normal numbers of CD11b<sup>low</sup>/CD103<sup>+</sup> kidney DCs, but which expressed moderately reduced levels of CD103 (Fig. 3B). In summary, it appears that Csf2rb<sup>−/−</sup> mice retain normal development of peripheral tissue CD11b<sup>low</sup>/CD103<sup>+</sup> which express reduced levels of CD103.

**Basal CD103 expression by CD8α<sup>+</sup>-equivalent cDCs requires Batf3 but not GM-CSF signaling**

While CD103 has been used as a marker for the dermal CD11b<sup>low</sup>/Langerin<sup>+</sup> DC lineage [14], recent work shows that CD103 expression can be dynamically regulated by cytokines [17,18]. Therefore, we wished to re-examine the relationships between CD103 expression, Batf3, and GM-CSF signaling. We selected the Flt3L-induced DC differentiation from bone marrow cultures for this purpose since it has allowed the analysis of CD103 expression as dependent on both Batf3 and GM-CSF [17,18] (Fig. 4).

Previously, CD24<sup>high</sup> SIRPα<sup>low</sup> DCs were considered an “equivalent” of CD8α<sup>+</sup> cDCs in Flt3L-treated bone marrow cultures [30], despite the fact that only a subpopulation of these cells express CD103 (Fig. 4). The CD103<sup>+</sup> fraction of CD24<sup>high</sup> SIRPα<sup>low</sup> DCs was more potent than the CD103<sup>+</sup> fraction for cross-presentation [17], suggesting a correlation between CD103 expression and CD8α<sup>+</sup> cDC function. We find that addition of...
CD11b<sup>low</sup> Peripheral DCs Are GM-CSF-Independent

**A**

- C57BL/6
- Batf3<sup>−/−</sup>
- Csf2rb<sup>−/−</sup>

**B**

- C57BL/6
- Batf3<sup>−/−</sup>
- Csf2rb<sup>−/−</sup>
GM-CSF during day 7 through 9 of Flt3L culture induces CD103 expression on all CD24<sup>high</sup>SIRP<sub>α<sub>2</sub></sup><sup>low</sup> DCs (Fig. 4), in agreement with recent reports which showed that such GM-CSF-induced cells exhibit robust cross-presentation [17,18]. However, while addition of IL-3 and TGF-β1 to Flt3L cultures can both induce expression of CD103, only IL-3 induced the capacity for cross-presentation [17], dissociating CD103 expression from cross-presentation. Likewise, we found that GM-CSF treatment induced CD103 expression on all CD24<sup>high</sup>SIRP<sub>α<sub>2</sub></sup><sup>low</sup> DCs, even in bone marrow cultures from Batf<sub>3<sub>−/−</sub></sub> mice (Fig. 4), which completely lack CD103<sup>+</sup> DCs in vivo [16], further dissociating CD103 expression from the properties of true CD8<sup>a</sup> cDC equivalents. In agreement, a recent report found that GM-CSF-induced CD103<sup>+</sup>CD24<sup>high</sup>SIRP<sub>α<sub>2</sub></sup><sup>low</sup> Flt3L-derived DCs from Batf<sub>3<sub>−/−</sub></sub> mice lacked the capacity for cross-presentation [18]. Finally, Csf2rb<sub>−/−</sub> bone marrow generated a subpopulation of CD103<sup>−/−</sup>CD24<sup>high</sup>SIRP<sub>α<sub>2</sub></sup><sup>low</sup> DCs, indicating development that is independent of GM-CSF signaling, although whether these arise from the actions of cytokines such as IL-3, or arise independently of soluble factors present in the culture remains unknown. In summary, these results indicate that CD103 expression can be regulated by GM-CSF, independently of Batf3, and may not represent a static marker of Batf3-dependent DC lineages.

**Discussion**

Our study makes two new observations. First, we show that CD11b<sup>low</sup> non-lymphoid DCs develop in the absence of GM-CSF signaling. Second, we show that Batf3<sup>−/−</sup>-dependent CD11b<sup>low</sup> non-lymphoid DCs are not required for T effector cell priming or clinical EAE after subcutaneous immunization. King et al. recently concluded that mice deficient for GM-CSF or GM-CSF receptor were resistant to EAE because they lacked dermal CD11b<sup>low</sup>-Langerin<sup>−</sup>DC103<sup>+</sup> DCs, which they proposed were required for priming effector CD4 T cells with MOG<sub>35-55</sub> [14]. Dermal CD11b<sup>low</sup>-Langerin<sup>−</sup>DC103<sup>+</sup> DCs are a conventional DC subset that requires the transcription factor Batf3 for their development [16]. Here, we directly compared Csf2rb<sub>−/−</sub> with Batf3<sub>−/−</sub> mice for their susceptibility to EAE and for their development of various DC subsets using a broad panel of DC markers, including Langerin, CD11b, EpCAM, CD24, and CD103. We confirm that the dermal CD11b<sup>low</sup>-Langerin<sup>−</sup>DC103<sup>+</sup> DC subset is absent in Batf3<sub>−/−</sub> mice, but surprisingly find that Csf2rb<sub>−/−</sub> mice harbor normal numbers of this DC subset in SDLNs as a CD11b<sup>low</sup>-Langerin<sup>−</sup>EpCAM<sup>−</sup>CD24<sup>−/−</sup> cell that expresses reduced, but not absent, levels of CD103. Moreover, in other peripheral tissues, Csf2rb<sub>−/−</sub> mice have similar CD11b<sup>low</sup>-Langerin<sup>−</sup>DC103<sup>+</sup> DCs that express reduced, but not absent, levels of CD103.

Our data agree with recent reports that GM-CSF directly regulates CD103 expression on Flt3L-derived CD8<sup>a</sup> equivalent cDCs and splenic DEC205<sup>−</sup>CD8<sup>a</sup> cDCs [17–19]. Beyond this, we show that GM-CSF also regulates levels of CD103 expression on peripheral tissue-resident CD11b<sup>low</sup> DCs, which were not examined in the previous studies. Notably, a separate report has suggested that GM-CSF signaling is required for the development of small intestinal lamina propria CD11b<sup>+</sup>CD103<sup>+</sup> DCs [31], although their data could be interpreted as simply representing reduced CD103 expression by these DCs. In that report, Csf2rb<sub>−/−</sub> mice had half as many lamina propria CD11b<sup>+</sup>CD103<sup>+</sup> DCs as wild type mice, but 1.7-fold more CD11b<sup>−</sup>CD103<sup>−</sup> DCs than wild type mice, consistent with a reduction of CD103 expression as an explanation for the “missing” subset.

King et al. also found decreased T cell priming to subcutaneous immunization with MOG<sub>35-55</sub> peptide and partial EAE resistance after in vivo depletion of peripheral Langerin<sup>−</sup>DC103<sup>+</sup> DCs using radiation chimeras reconstituted with bone marrow from transgenic mice expressing DTR under the control of the Langerin promoter [20]. The use of radiation chimeras allowed the selective depletion of dermal CD11b<sup>low</sup>-Langerin<sup>−</sup> DCs, avoiding depletion of epidermal CD11b<sup>+</sup>Langerin<sup>−</sup> Langerhans cells, because the latter are radiation-resistant, and were therefore of host origin and did not express the DTR. Our results indicate that Csf2rb<sub>−/−</sub> mice are not deficient in dermal CD11b<sup>low</sup>-Langerin<sup>−</sup> DCs, but rather that these cells have reduced CD103 expression. However, Batf3<sub>−/−</sub> mice lack this dermal DC subset, and yet are susceptible to EAE induction and display increased, not decreased, T cell priming to MOG<sub>35-55</sub> peptide immunization. It is unclear why DTR-mediated depletion of donor-derived, radiation-sensitive Langerin<sup>−</sup> DCs reduces priming and delays EAE, when genetic ablation of these cells in Batf3<sub>−/−</sub> mice leaves priming and EAE intact. Their use of DTR-mediated depletion involved irradiation of mice prior to immunization, which may have had unintended consequences. Furthermore, a study by Henri et al. has identified additional dermal DC subsets [26], including one that is Langerin<sup>+</sup> but CD103<sup>−</sup>, although it’s Batf3-dependence was not characterized. Conceivably, the depletion of this subset by diphtheria toxin treatment could contribute to deficiencies between our results and those of King et al. Notably, DTR-mediated depletion reduced the severity of EAE, but with a profound decrease only in MOG<sub>35-55</sub>-specific IFN-γ-secreting T cells, with minimal change in the number of IL-17-secreting T cells. In contrast, MOG<sub>35-55</sub>-immunized Csf2rb<sub>−/−</sub> mice had profound reductions in antigen-specific IFN-γ-secreting and in IL-17-secreting T cells, suggesting that Csf2rb<sub>−/−</sub> mice differ from mice in which Langerin<sup>−</sup> DCs are depleted using DTR.

Our results suggest that the absence of dermal CD11b<sup>low</sup>-Langerin<sup>−</sup> DCs leads to augmented T cell priming of both Th17 and Th1 responses. Igyártó et al. have recently shown that skin infection of Batf3<sub>−/−</sub> mice with recombinant Candida albicans led to reduced Th1 but increased Th17 responses compared to control mice [32]. These authors demonstrated that dermal Langerin<sup>−</sup> DCs expressed IL-12, explaining their ability to promote Th1 cell responses, and IL-27, a cytokine known to inhibit Th17 cell responses [33,34]. While we found increased Th17 responses in Batf3<sub>−/−</sub> mice immunized with MOG<sub>35-55</sub> and CFA, we also observed increased Th1 responses compared to control...
Figure 4. GM-CSF induces CD103 expression on Flt3L-derived CD8α-equivalent DCs. FACS analysis of developing DCs in bone marrow cells from C57BL/6, Batf3−/− (C57BL/6 background), and Csf2rb−/− mice cultured with Flt3L for 9 days, with or without the addition of GM-CSF to the culture on day 7. The left column is gated on live single cells, the second on CD11c−CD45RA+ cDCs, the third on I-Ab+cDCs, and the fourth on CD24hiSirpαlo cells. Numbers represent the percentage of cells within the indicated gates. Data are representative of bone marrow cells from two mice per genotype over two experiments.

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mice. Conceivably, the different Th1 responses between our study and Igyärő et al. could arise from differences in the form of immunization, relying on CFA versus fungal infection. Notably, blood-derived inflammatory monocytes have been reported to serve as a critical source of IL-12 after CFA-based subcutaneous immunization [53]. While we observed between a 2 to 3-fold higher frequency of IL-17-producing T cells in MOG35-55 immunized Batf3<sup>−/−</sup> mice relative to controls, the clinical EAE scores were similar between these groups. Conceivably, the frequency of IL-17-producing T cells is unrelated to the severity of EAE when Th17 cells are above some threshold frequency. Alternatively, IL-17 itself may not be the limiting factor in regulating the intensity of EAE, since GM-CSF also recently was implicated as the major Th17-derived cytokine responsible for driving pathogenic lesions in this model [5–7]. In fact, however, we do observe a slightly more rapid onset of EAE in Batf3<sup>−/−</sup> mice, which was more pronounced in the 129S6/SvEv genetic background (Fig. 2D), which could relate to increased Th17 development.

If a lack of peripheral CD11b<sup>low</sup>/− DCs does not abrogate priming of T effector cells after subcutaneous immunization, why then does this priming defect occur in Gsf2b<sup>−/−</sup> mice? One possibility is that other DC subsets besides dermal CD11b<sup>low</sup>/− DCs can prime CD4 T cells [35], but that the ability of these other DCs to prime requires that they receive a GM-CSF signal. Indeed, GM-CSF is essential for IL-6 and IL-23 production by DCs during the priming phase of autoimmune myocarditis [4] and is required for inflammatory DC development from monocytes in a model of repeated immunization with methylated BSA and CFA [36]. T cells do not express GM-CSF receptor, so an intrinsic T cell defect seems unlikely. While the source of GM-CSF after subcutaneous immunization is still unclear, antigen-specific T cells can produce GM-CSF [5–7] and may augment responses by DCs during initial priming of T effector cells.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Studies Committee of Washington University (#20090320).

Mice

Wild-type 129S6/SvEv and C57BL/6 mice were purchased from Taconic. Batf3<sup>−/−</sup> mice on a 129S6/SvEv background were previously generated in our laboratory [27], and were backcrossed for 10 generations to the C57BL/6 background. Gsf2b<sup>−/−</sup> mice on the C57BL/6 background were purchased from Jackson Laboratory. Experiments were performed with sex-matched mice at 8–20 weeks of age. Mice were bred and maintained in our specific pathogen-free animal facility according to institutional guidelines.

Induction of EAE

EAE was induced as previously described [14]. Briefly, mice were immunized subcutaneously with 100 micrograms MOG35-55 peptide (Sigma Genosys) emulsified in complete Freund’s adjuvant (CFA) made with 5 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (BD Difco) in incomplete Freund’s adjuvant (BD Difco). Pertussis toxin (List Biological Laboratories) was injected intraperitoneally (300 ng) on days zero and two. Mice were observed for signs of EAE and graded on a standard 0–5 scale as described [37].

T cell priming and ELISPOT assays

Mice were immunized subcutaneously in the hind footpads with 10 nanomoles MOG35-55 peptide (Sigma Genosys) emulsified in complete Freund’s adjuvant (CFA) (BD Difco). Popliteal lymph nodes were collected at day 7, and single cell suspensions were used in ELISPOT assays with the IFN<sub>y</sub> ELISPOT antibody pair from BD Biosciences on Multiscreen Filter Plates from Millipore. An IL-17 ELISPOT was designed using anti-IL17A capture (clone TC11-18H10) and detection (clone TC11-18H11) antibodies (BD Biosciences). Cells were plated at 1 × 10<sup>6</sup> cells/well, in duplicate, and stimulated with either no antigen, or 10 micromolar MOG35-55 peptide. Plates were developed after 16 hours of stimulation at 37 °C, and spots were counted on an Immunospot counter (Cellular Technology Ltd.).

Antibodies

The following antibodies were purchased from BD Biosciences: PE-Cy7 anti-CD11b (M1/70), PerCP-Cy5.5 anti-CD8α (53-6.7), V500 anti-CD8α (53-6.7), PE anti-CD45RA (14.8), biotin anti-CD24 (30F1), FITC CD24 (M1/69), APC anti-CD172a/SIRPα (P84). FITC anti-CD11b (M1/70), APC eFluor 780 anti-CD11c (N418), PE anti-CD103 (2E7), biotin anti-CD103 (2E7), eFluor450 anti-CD317/BST2 (eBio927), eFluor450 anti-MHCII (I-A/I-E) (M5/114.15.2), APC anti-CD45.2 (104), and streptavidin-eFluor450 were purchased from eBioscience. PE anti-CD205/DEC205 (NLDC-145) and APC anti-CD205/DEC205 (NLDC-145) were purchased from Milteny. Alexa Fluor 488 anti-CD207/Langerin (929F3.01) was purchased from ImageX, PE anti-CD326/EpCAM (G8.8) was purchased from Biolegend.

Flow cytometry

Inguinal lymph nodes and spleens were minced and digested in 5 ml Iscove’s modified Dulbecco’s media +10% FCS (cIMDM) with 250 µg/ml collagenase B (Roche) and 30 U/ml DNase I (Sigma-Aldrich) for 1 h at 37 °C with stirring. Lungs and kidneys were perfused with 10 ml DPBS via injection into the right ventricle after transection of the lower aorta. Dissected lungs and kidneys were minced and digested in 5 ml of cIMDM with 4 mg/ml collagenase D (Roche) for 1 h at 37 °C with stirring. EDTA (5 mM final concentration) was added to cell suspensions, and cells were incubated on ice for 5 min. Cells were passed through an 80 µm strainer before red blood cells lysis with ACK lysis buffer. Cells were counted on a ViCELL analyzer, and 2–5 × 10<sup>5</sup> cells were used per antibody staining reaction.

Staining was performed at 4 °C in the presence of Fc Block (clone 2.4G2, BD Biosciences or BioXcell) in FACS buffer (DPBS+0.5% BSA+2 mm EDTA+0.02% sodium azide). For experiments involving intracellular anti-Langerin staining, cells were surface antibody stained prior to fixation in 4% formaldehyde (Thermo Scientific) for 15 minutes at room temperature. Cells were subsequently permeabilized (DPBS+0.1% BSA+0.5% saponin) for 5 min at 4 °C and stained in the same permeabilization buffer with anti-Langerin antibody. Cells were washed twice in permeabilization buffer before returning them to FACS buffer. Flow cytometry was performed on a FACS CantoII (BD Biosciences), and data was analyzed with FlowJo software (Tree Star, Inc.).

Bone marrow cultures

Bone marrow cells from femurs and tibias were collected and red blood cells were lysed in ACK lysis buffer. Cells were cultured
in four ml cIMDM in six-well plates at 2 × 10^6 cells/ml containing 160 ng/ml murine Flt3L (Peprotech). At day seven of culture, murine GM-CSF (Peprotech) was added to some wells at 20 ng/ml. Non-adherent cells were collected at day nine for flow cytometry.

Supporting Information

Figure S1 Flow cytometric analyses of DCs in Batf3^−/− mice. (A) FACS analysis of SDLN (inguinal) DCs from Batf3^+/+ and Batf3^−/− mice on both the C57BL/6 and 129S6/SvEv backgrounds. Left plots from mice of each background are gated on CD11c^BST2^+ cDCs. Right plots from mice of each background are gated on migratory (DEC205^CD8α^+) DCs. Numbers represent the percentage of cells within the indicated gates. Data are representative of at least six mice per genotype and background obtained in several independent experiments. (B) Absolute cell numbers of DEC205^CD8α^+ DCs per individual inguinal lymph nodes from C57BL/6 and Batf3^−/− mice (C57BL/6 background) calculated from the FACS analysis performed in Figure S2A. Horizontal bars represent the geometric mean, p value by unpaired student’s t test. (C) and (D) FACS analysis of splenic DCs from Batf3^+/+ and Batf3^−/− mice on both the (C) C57BL/6 and (D) 129S6/SvEv backgrounds. Plots are gated on CD11c^+I-Α^+ cDCs. Numbers represent the percentage of cells within the indicated gates. Data are representative of at least six mice per genotype and background obtained in several experiments. [E] FACS analysis of SDLN (inguinal) DCs from C57BL/6, Batf3^−/− (C57BL/6 background), and C3H/HeJ^−/− mice. Left plots are gated on CD11c^+^CD8α^+ cells. Right plots are gated on DEC205^CD8α^+ cDCs. Numbers represent the percentage of cells within the indicated gates. Data are representative of eight to ten individual inguinal lymph node from four to five mice per genotype obtained in two experiments. Data is depicted in these plots are derived from analysis of the same mice used in Figure 2A, but gated to analyze CD103 expression on DEC205^CD8α^+ cDCs. (TF)

Author Contributions

Conceived and designed the experiments: KMM BTE ERU TLM JHR. Performed the experiments: BTE TRB WKC KH JWH JS. Analyzed the data: BTE KMM TLM ERU JHR. Contributed reagents/materials/analysis tools: BTE KMM. Wrote the paper: BTE KMM.

References

1. Hamilton JA (2008) Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol 8: 533–544.
2. McQuilter JL, Darwiche R, Ewing C, Onuki M, Kay TW, et al. (2001) Granulocyte macrophage-colony stimulating factor: a new putative therapeutic target in multiple sclerosis. J Exp Med 194: 573–882.
3. Cook AD, Braine EL, Campbell IK, Rich MJ, Hamilton JA (2001) Blockade of collagen-induced arthritis post-onset by antibody to granulocyte-macrophage-stimulating factor (GM-CSF): requirement for GM-CSF in the effector phase of disease. Arthritis Res 3: 293–301.
4. Sondereregger I, Iezzi G, Maier R, Schmitz N, Kurrer M, et al. (2008) GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. J Immunol 178: 39–48.
5. El Behi M, Ciric B, Dai H, Yan Y, Cullimore M, et al. (2011) The Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmunity. Cell Immunol 273: 123–130.
6. Hofstetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, et al. (2005) Therapeutic potential of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. Cell Immunol 237: 123–130.
7. Baranowski ED, Shiver LP, Marek K, Fedorov-Vasconcelos J, Verheyen D, et al. (2007) GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. J Immunol 178: 39–48.
8. Hofstetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, et al. (2005) Therapeutic potential of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. Cell Immunol 237: 123–130.
9. Lovett-Racke AE, Yang Y, Racke MK (2011) Th1 versus Th17: are T cell cytokines relevant in multiple sclerosis? Biochim Biophys Acta 1812: 246–251.
10. McQualter JL, Darwiche R, Ewing C, Onuki M, Kay TW, et al. (2001) Granulocyte macrophage-colony stimulating factor: a new putative therapeutic target in multiple sclerosis. J Exp Med 194: 573–882.
11. Cook AD, Braine EL, Campbell IK, Rich MJ, Hamilton JA (2001) Blockade of collagen-induced arthritis post-onset by antibody to granulocyte-macrophage-stimulating factor (GM-CSF): requirement for GM-CSF in the effector phase of disease. Arthritis Res 3: 293–301.
12. Sondereregger I, Iezzi G, Maier R, Schmitz N, Kurrer M, et al. (2008) GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. J Immunol 178: 39–48.
13. El Behi M, Ciric B, Dai H, Yan Y, Cullimore M, et al. (2011) The Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmunity. Cell Immunol 273: 123–130.
14. Hofstetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, et al. (2005) Therapeutic potential of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. Cell Immunol 237: 123–130.
15. Baranowski ED, Shiver LP, Marek K, Fedorov-Vasconcelos J, Verheyen D, et al. (2007) GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. J Immunol 178: 39–48.
16. Hofstetter HH, Ibrahim SM, Koczan D, Kruse N, Weishaupt A, et al. (2005) Therapeutic potential of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. Cell Immunol 237: 123–130.
17. Baranowski ED, Shiver LP, Marek K, Fedorov-Vasconcelos J, Verheyen D, et al. (2007) GM-CSF production by autoreactive T cells is required for the activation of microglial cells and the onset of experimental autoimmune encephalomyelitis. J Immunol 178: 39–48.
18. Jackson JT, Hu Y, Liu R, Masson F, D’Amico A, et al. (2011) Id2 expression delineates differential checkpoints in the genetic program of CD11b(+) and CD103(+) dendritic cell lineages. EMBO J 30: 2700–2704.
19. Zhan Y, Carrington EM, van Neervenhuizen B, Bedoui S, Sehay S, et al. (2011) GM-CSF increases cross presentation and CD103 expression by mouse CD11b(+) spleen dendritic cells. Eur J Immunol In press.
20. Kessenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, et al. (2005) Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. Immunity 22: 643–654.
21. Robb L, Drinkwater CG, Metzelf D, Li RL, Konigsen F, et al. (1995) Hematopoietic and Lung Abnormalities in Mice with a Null Mutation of the Common Beta-Subunit of the Receptors for Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-3 and Interleukin-5. Proc Natl Acad Sci U S A 92: 9565–9569.
22. Nicola NA, Robb L, Metzelf D, Gary D, Drinkwater CG, et al. (1996) Functional inactivation in mice of the gene for the interleukin-3 (IL-3)-specific receptor beta-chain: implications for IL-3 function and the mechanism of receptor transmodulation in hematopoietic cells. Blood 87: 2665–2674.
23. Burc US, Wang J, Iyagari B, Kessenpfennig A, Malissen B, et al. (2007) Identification of a novel population of Langerin(+) dendritic cells. J Exp Med 204: 3147–3156.
24. Ginhoux F, Collin MP, Bogunovic M, Abel M, Lebon F, et al. (2007) Blood-dendritic cells. Numbers represent the percentage of cells within the indicated gates. Data are representative of at least six mice per genotype and background obtained in several independent experiments. [E] FACS analysis of SDLN (inguinal) DCs from C57BL/6, Batf3^−/− (C57BL/6 background), and C3H/HeJ^−/− mice. Left plots are gated on CD11c^+^CD8α^+ cells. Right plots are gated on DEC205^CD8α^+ cDCs. Numbers represent the percentage of cells within the indicated gates. Data are representative of eight to ten individual inguinal lymph node from four to five mice per genotype obtained in two experiments. Data is depicted in these plots are derived from analysis of the same mice used in Figure 2A, but gated to analyze CD103 expression on DEC205^CD8α^+ cDCs. (TF)
T helper cells during chronic inflammation of the central nervous system. Nat Immunol 7: 937–945.

34. Diveu C, McGeachy MJ, Boniface K, Stumhofer JS, Sathe M, et al. (2009) IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells. J Immunol 182: 5748–5756.

35. Nakano H, Lin KL, Yanagita M, Charbonneau C, Cook DN, et al. (2009) Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. Nat Immunol 10: 394–402.

36. Kamphorst AO, Guermourez P, Dufziak D, Nussenzweig MC (2010) Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes. J Immunol 185: 3426–3435.

37. Lee J, Sin J, Russell JH (2010) Encephalitogenic T-cells increase numbers of CNS T-cells regardless of antigen specificity by both increasing T-cell entry and preventing egress. J Neuroimmunol 220: 10–16.

38. Guth AM, Jansen WJ, Bosio CM, Crouch EC, Henson PM, et al. (2009) Lung environment determines unique phenotype of alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 296: L296–L306.