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Lack of Conventional Dendritic Cells Is Compatible with Normal Development and T Cell Homeostasis, but Causes Myeloid Proliferative Syndrome

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SUMMARY

Dendritic cells are critically involved in the promotion and regulation of T cell responses. Here, we report a mouse strain that lacks conventional CD11c^{hi} dendritic cells (cDCs) because of constitutive cell-type specific expression of a suicide gene. As expected, cDC-less mice failed to mount effective T cell responses resulting in impaired viral clearance. In contrast, neither thymic negative selection nor T regulatory cell generation or T cell homeostasis were markedly affected. Unexpectedly, cDC-less mice developed a progressive myeloproliferative disorder characterized by prominent extramedullary hematopoiesis and increased serum amounts of the cytokine Flt3 ligand. Our data identify a critical role of cDCs in the control of steady-state hematopoiesis, revealing a feedback loop that links peripheral cDCs to myelogenesis through soluble growth factors, such as Flt3 ligand.

INTRODUCTION

Antigens have to be processed and presented in the form of peptides bound to major histocompatibility complex (MHC) molecules to be recognized by T cells. Antigen-presenting cells (APCs) thus play a central role in the activation and control of T cell immunity. Dendritic cells (DCs), a morphologically distinct APC described by Steinman and colleagues (Steinman and Witmer, 1978), belong to the body-wide network of mononuclear phagocytes (van Furth and Cohn, 1968) and seem to have coevolved with adaptive T cell immunity. Beyond their unique potential to stimulate naive T cells in vitro (Steinman and Witmer, 1978), in vivo antigen targeting to DCs elicits strong T cell priming and long-lived T cell help for antibody responses (Bonifaz et al., 2002; Boscardin et al., 2006). Furthermore, vaccination with antigen-pulsed DCs proved to be a potent way to stimulate T cell responses both in mouse and man with respective protocols being in clinical trials (Gilboa, 2007; Palucka et al., 2007). Finally, conditional in vivo DC ablation established that splenic conventional DCs (cDCs) are required for the initiation of naive CD4$^+$ and CD8$^+$ T cell responses to protein antigens and pathogens (Jung et al., 2002; Probst and van den Broek, 2005; Sapoznikov et al., 2007).

Beyond their role in T cell stimulation, DCs are also involved in controlling the inherent autoreactivity of the T cell compartment. DCs were reported to play a critical role in the establishment of central T cell tolerance (Brocker et al., 1997), although more recent studies highlight the contribution of medullary thymic epithelial cells (mTECs) that promiscuously express tissue-restricted self-antigens (Kyewski and Klein, 2006). Immature or resting DCs that did not encounter pathogen signatures and hence lack expression of costimulatory molecules were shown to induce peripheral tolerance, both of CD4$^+$ and CD8$^+$ T cells (Hawiger et al., 2001; Probst et al., 2005). In addition, thymic and peripheral DCs were proposed to play a critical role in the generation of T regulatory (Treg) cells that suppress effector T cell responses (Coombes et al., 2007; Fehervari and Sakaguchi, 2004; Mahnke and Enk, 2005; Yamazaki et al., 2007). Finally, peripheral DCs were reported to support homeostatic proliferation and survival of T cells (Brocker et al., 1997; Gruber and Brocker, 2005). Although the role of DCs in T cell activation has been well established through transient DC depletion and/or DC-specific antigen targeting, the study of DC functions in T cell development and homeostasis requires long-term DC elimination in the steady state. Similarly, any potential DC functions outside of bona fide antigen presentation may be revealed only after early-onset DC deletion during development. Thus, an experimental model of constitutive DC deletion is required so that the immunological and developmental in vivo functions of this critical immune cell type are fully understood.

Here, we report the generation and characterization of a binary transgenic mouse model that constitutively lacked conventional CD11c^{hi} DCs. cDC-less mice were born at normal Mendelian frequencies and showed unimpaired development. The lack of

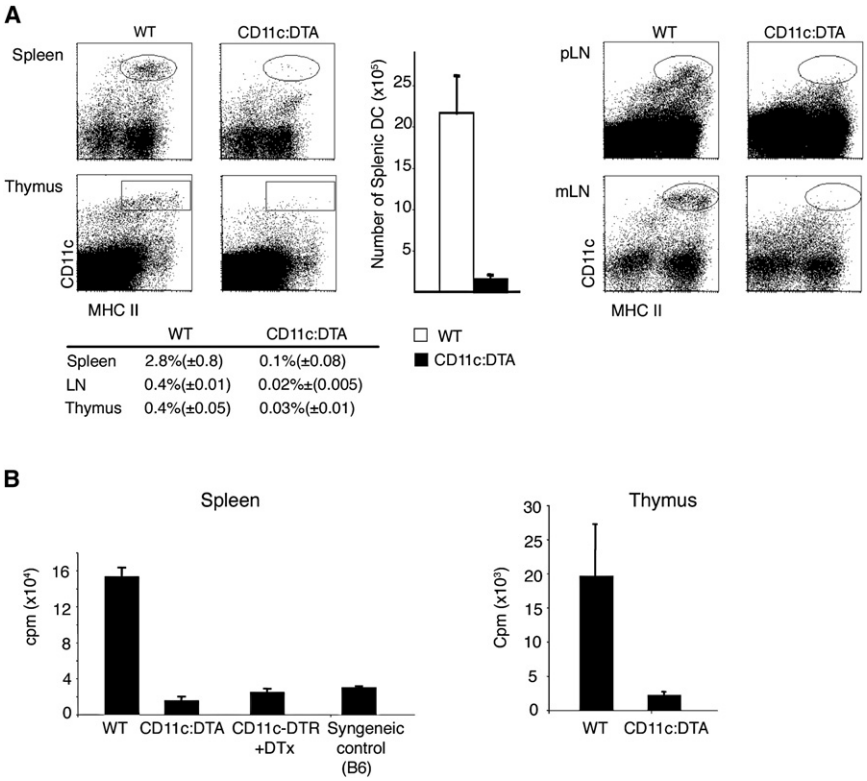

Figure 1. Constitutive Dendritic Cell Depletion in the CD11c:DTA Mice

(A) Flow-cytometry analysis of cells isolated from spleen, LNs, and thymi of CD11c:DTA mice or littermate controls. DCs were identified as CD11c^{hi} MHC-II^{hi} cells. Percentages refer to CD11c^{hi} cDCs out of total cells. n = 3 for each group. $p < 0.001$. The bar diagram summarizes numbers of cDCs in CD11c:DTA mice and littermate controls.

(B) Mixed leukocyte reaction with 5×10^5 splenocytes (left) or thymocytes (right) isolated from CD11c:DTA and littermate mice (C57BL/6 background, H2^b), cultured with 10^5 allo-reactive BALB/c CD4$^+$ T cells for 72 hr, after which thymidine incorporation was measured. The bar graph represents means ± SD (n = 3). Controls include T cells cocultured with splenocytes isolated from DTx-treated DTR mice and syngeneic splenocytes (BALB/c, H2^d). One representative experiment out of three is shown. p values are < 0.001.

cDCs resulted in a deficiency to mount efficient and antivirally protective T cell responses. Surprisingly, however, the steady-state T cell compartment and T cell homeostasis remained largely unaffected by the absence of DC, as did thymic negative selection and the generation of natural T regulatory cells. Finally, we report the unexpected finding that cDC-less mice developed a myeloid proliferative syndrome by triggering a hitherto-unknown hematopoetic feedback regulation linking peripheral cDC to myelogenesis through soluble growth factors, such as Flt3 ligand. Importantly, our finding might explain other DC-deficiency-associated myelo-proliferative disorders, such as the one reported for IRF8-deficient mice.

RESULTS

Generation of Mice that Constitutively Lack Conventional DCs

In order to probe for potential functions of cDCs during the development of the vertebrate organism and subsequent homeostasis, we generated a mouse model that constitutively lacks CD11c^{hi} cells. To this end, we crossed *CD11c*-Cre BAC transgenic mice (Caton et al., 2007) to mice that harbor a conditional diphtheria toxin A (DTA) transgene in the constitutively active Rosa26 locus (Brockschnieder et al., 2006). Cre-recombinase-mediated deletion of the loxP signal-flanked transcriptional STOP cassette in these mice results in specific toxin activation in Cre-expressing cells. DTA inhibits protein synthesis (Holmes, 2000) and CD11c-expressing cells of *CD11c*-Cre;R26-DTA animals (called "CD11c:DTA mice" hereafter) are thus expected to undergo spontaneous apoptosis. CD11c:DTA double-transgenic mice were born at normal Mendelian frequencies. Flow-cytometry analysis of CD11c:DTA mice revealed the essential absence of MHC II$^+$ CD11c^{hi} cells from spleens, peripheral and mesenteric LNs, thymi, and nonlymphoid tissues of CD11c:DTA mice (Figure 1A and Figures S1A and S1B available online). Histological analysis confirmed the lack of CD11c^{hi} cells in lymphoid organs of CD11c:DTA mice (Figures S2A and S2B). Importantly, the bulk of plasmacytoid DCs (PDCs) and epidermal Langerhans' cells (LCs) were still present in CD11c:DTA mice (Figures S1C and S1D). Classical in vitro DC depletion experiments have shown that splenic DCs are of critical importance as stimulators in a primary mixed leukocyte reaction (MLR) (Steinman and Witmer, 1978). Moreover, conditional in vivo cDC ablation results also in the inability of splenocytes to prime alloreactive T cells (Jung et al., 2002). To obtain a functional confirmation for the absence of cDCs in CD11c:DTA mice, we therefore assayed cell suspensions of splenocytes and thymocytes in an MLR. When the respective cells were isolated from CD11c:DTA mice, they failed to stimulate alloreactive responder T cells, as compared to single-transgenic littermate controls (Figure 1B). Taken together, our results show that CD11c:DTA mice lack CD11c^{hi} conventional DCs (cDCs), but develop normally, suggesting that during development the cells are not required for processes beyond their immune functions.

cDC-less Mice Are Immunodeficient

The requirement of cDCs in the MLR reaction highlights their role as unique APCs in the priming of naive T cell responses. To investigate the in vivo immune status of cDC-less mice, we next tested their ability to respond to antigen and pathogen challenge. Ovalbumin (OVA)-specific TCR transgenic CD4$^+$ and CD8$^+$ T cells (OT-I and OT-II [Barnden et al., 1998; Hogquist et al., 1994]) were transferred into CD11c:DTA mice or littermate controls and the recipients were challenged by intravenous OVA injection (10 μg). Both CD4$^+$ and CD8$^+$ T cell responses were impaired in the spleens of CD11c:DTA mice (Figure S3A).

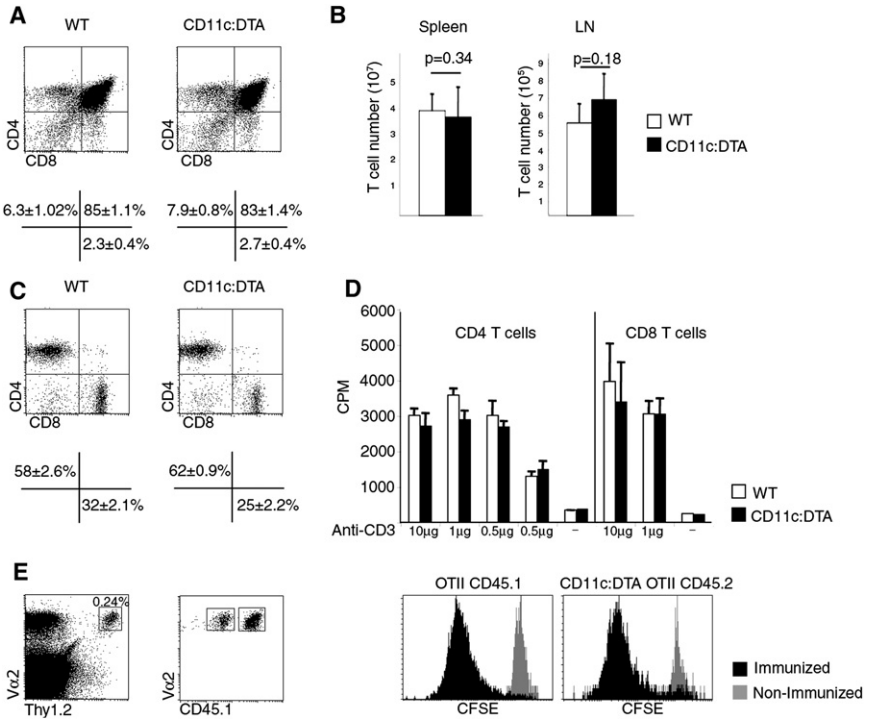

Figure 2. Unimpaired Steady-State T Cell Compartment in CD11c:DTA Mice

(A) Flow-cytometric analysis of T cells isolated from thymi of CD11c:DTA mice and WT LM controls, stained for CD4 and CD8. Numbers in quadrants show mean percentage of respective cells ± SD (n = 5).

(B) Flow-cytometric analysis of T cells isolated from spleens of CD11c:DTA mice and WT LM controls, stained for CD4 and CD8. Numbers in quadrants show mean percentage of respective cells out of CD3-positive splenocytes ± SD (n = 5).

(C) Bar graph represents means ± SD (n = 5) of T cell numbers in the spleen and inguinal LNs of CD11c:DTA mice and LM controls. T cells are identified as CD3-positive cells. Results are representative example of three independent experiments.

(D) In vitro proliferation assay of $CD4^+$ and $CD8^+$ T cells isolated from spleens and LNs of CD11c:DTA mice and littermate controls. Cells were exposed to plate-bound anti-CD3 after coating with indicated concentrations. Bar graphs represent means ± SD (n = 3).

(E) Flow-cytometric analysis of cotransferred CFSE-labeled $CD4^+$ T cells (2×10^6 each) isolated from OT-II;CD11c:DTA mice (Thy1.1 $CD45.2^+$) and OT-II LM controls (Thy1.1 $CD45.1^+$), respectively into Thy1.2 WT B6.Cg-$Igh^aThy1^aGpi1^a$/J recipients. The recipients were analyzed 3 days after immunization with soluble OVA or without immunization. Histograms show splenocytes analyzed for presence of the grafted cell populations identified by their allotypic markers as shown in the dot blots. n = 4 for each group.

This result confirms earlier reports that splenic $CD4^+$ and $CD8^+$ T cell responses depend on the presence of $CD11c^{hi}$ DCs (Jung et al., 2002; Sapoznikov et al., 2007). Noteworthy, however, was that $CD4^+$ T cell responses in the LNs of CD11c:DTA mice persisted (Figure S3B), as reported for cDC-depleted CD11c-DTR mice (Sapoznikov et al., 2007).

To test the impact of the absence of cDCs on T cell-mediated protection against pathogens, we challenged CD11c:DTA mice and littermate controls with the noncytopathic lymphocytic choriomeningitis virus (LCMV). LCMV protection strictly depends on a rapidly developing cytotoxic T lymphocyte (CTL) response (Kagi et al., 1994). CD11c:DTA mice failed to generate efficient virus-specific CTL responses, thereby resulting in impairment of viral clearance (Figure S4A). To test the ability of cDC-less mice to cope with a cytopathic viral infection, we challenged the CD11c:DTA mice with a mouse hepatitis virus (MHV) A59 strain. The defense against the MHV requires type I IFN-producing PDCs (Cervantes-Barragan et al., 2007) but also involves a CTL component, as indicated by the impaired MHV clearance in MHC class I deficient ($B2m^{-/-}$) mice (Figure S4B). Although CD11c:DTA mice mounted a considerable response of MHV-specific IFN-γ-secreting effector T cells in the liver, this response was impaired in the spleen (Figure S4C). Moreover, three out of five MHV-infected CD11c:DTA mice failed to clear the MHV from the liver, although they did not manifest MHV-mediated liver damage (Figure S4C). These data are consistent with the key role of PDCs in the response to MHV (as PDCs are present in CD11c:DTA mice), but also demonstrate an important role of cDCs in anti-viral CTL priming in the spleen. Collectively, our results support the prominent role of cDCs in the stimulation of T cell immunity, though cells other than cDCs can contribute to CTL response initiation during viral infection.

cDC-less Mice Have an Unimpaired T Cell Compartment

T cells not only require MHC-expressing peripheral APCs for the triggering of adaptive T cell immunity, but also for the maintenance of homeostasis and steady-state survival, ensuring the conserved size of the peripheral naive T cell pool. Given their prominent role in T cell priming, DCs are prime candidates for this activity because they are known to interact with T cells even in the absence of antigen (Bousso and Robey, 2003). Interestingly, thymic morphology and organization into medulla and cortex were unimpaired in CD11c:DTA mice (Figure S5A). Moreover, percentages of thymic T cell subpopulations were unchanged, with both immature $CD4^+CD8^+$ double positive (DP) and mature single positive (SP) cells present in similar frequencies and undisturbed $CD4^+/CD8^+$ T cell ratios (wild-type [WT] littermate: 2.72 ± 0.55; CD11c:DTA 2.91 ± 0.54) (Figure 2A). Spleens of cDC-less mice generally showed a well-preserved segregation into red and white pulp, as well as T and B cell zones (Figure S5B and S5C). Furthermore, when compared to littermate controls, CD11c:DTA mice exhibited normal T cell numbers in peripheral lymphoid organs (Figure 2B). Within the peripheral T cell compartment, the ratio between $CD4^+$ and $CD8^+$ T cells was found to be slightly elevated (WT littermate: 1.8 versus CD11c:DTA: 2.4) (Figure 2C).

To probe the functionality of T cells developed in the absence of cDCs, we investigated their ability to respond to in vitro stimulation and in vivo antigen challenge after transfer into WT mice. As shown in Figure 2D, $CD4^+$ and $CD8^+$ T cells isolated from

cDC-less mice and littermate controls responded equally well to anti-CD3-driven in vitro stimulation. For the in vivo assay of T cell function, we generated TCR transgenic CD11c:DTA mice by crossing the OT-II transgene, encoding the OVA-specific TCR (Barnden et al., 1998) onto the CD11c:DTA background. We then isolated $CD4^+$ T cells from the OT-II;CD11c:DTA mice and littermate OT-II controls harboring cDCs, labeled them with CFSE, and cotransferred the cells into WT recipient mice. The use of the respective allotypic CD45 markers allowed us to identify the two grafted cell populations in the host (Figure 2E). Upon antigen challenge, OVA-specific $CD4^+$ T cells isolated from the cDC-less mice responded in this competitive assay as well as the respective cells isolated from DC-proficient animals. Collectively, these data indicate that, despite their deficiency in T cell priming, cDC-less mice have a predominantly unimpaired T cell compartment with functionally intact T cells.

cDCs Are Dispensable for Thymic Negative Selection and the Generation of Natural T Regulatory Cells

To obtain a global view on the TCR repertoire of CD11c:DTA mice, we next determined the Vβ chain usage of their thymic and peripheral T cells. As shown in Figure 3A, the absence of cDCs did not affect the $CD4^+$ or the $CD8^+$ T cell repertoire, which remained similar to WT littermate controls. Notably, certain Vβ chains, such as Vβ5, Vβ11, and Vβ12, are underrepresented in C57BL/6 mice ($H2^b$) because of the presence of superantigens (SAg) encoded by endogenous MMTV proviruses (Scherer et al., 1993). The fact that CD11c:DTA mice exhibited unchanged Vβ distributions thus suggested that cDCs are dispensable for this SAg-induced T cell depletion in the thymus. This notion is further supported by that fact that introduction of an MHC $H2^b$ allele, through intercross with BALB/c WT mice, resulted in a comparable MMTV SAg-induced reduction of Vβ3-positive cells in cDC-proficient and cDC-deficient mice (Figure 3B). To directly test the role of cDCs in thymic negative selection, we resorted to a transgenic system that involves the expression of a de novo self-antigen (RIPmOVA mice [Kurts et al., 1996]). We generated mixed bone marrow (BM) chimeras by transferring either WT OT-II or CD11c:DTA OT-II BM into lethally irradiated WT or RIPmOVA recipient mice. In these chimeras, the OVA antigen is in the thymus exclusively expressed by medullary epithelial cells (mTECs). Recent studies had indicated that negative selection of $CD4^+$ T cells in such chimeras requires crosspresentation of the mTEC-derived antigen by BM-derived cells, presumably DCs (Gallegos and Bevan, 2004). [OT-II > RIPmOVA] chimeras displayed as reported (Gallegos and Bevan, 2004) a substantial reduction of $V\alpha2\ V\beta5^+$ OVA-reactive T cells in their thymus and periphery, as compared to [OT-II > WT] control chimeras (Figure 3C). More importantly, antigen-induced deletion of OVA-reactive $CD4^+$ T cells was also observed in [OT-II;CD11c:DTA > RIPmOVA] chimeras and was thus independent of graft-derived thymic or peripheral DCs.

To directly test for the potential existence of autoreactivity in the T cell compartment of CD11c:DTA mice, we exposed $CD4^+$ T cells isolated from cDC-deficient (CD11c:DTA, $H2^b$) and cDC-sufficient (LM, $H2^b$) mice to syngeneic ($H2^b$) and allogeneic ($H2^d$) splenocytes. In both cases, T cell proliferation was restricted to the H2-mismatched stimulator cells (Figure 3D). T cell autoreactivity was further assayed as recently reported (Luckashenak et al., 2008) by transfer of CFSE-labeled polyclonal $CD4^+$ and $CD8^+$ T cells from CD11c:DTA mice and littermate controls into CD45.1-congenic WT mice. Analysis of the recipients 10 days after transfer revealed the presence of both grafted populations at similar frequencies; moreover, neither of them had undergone substantial proliferation (Figure 3E). Taken together, these results demonstrate the absence of overt autoreactivity from the peripheral $CD4^+$ and $CD8^+$ T cell compartment of cDC-less mice.

In addition to their role in thymic selection, cDCs were proposed to play a critical role in the generation of Treg cells, both in the thymus and the periphery (Coombes et al., 2007; Fehervari and Sakaguchi, 2004; Mahnke and Enk, 2005; Yamazaki et al., 2007). Notably, however, the frequency of $CD4^+$ T cells with Treg phenotype ($FoxP3^+$) was similar in thymi and spleens of CD11c:DTA mice and of littermate controls (Figure 3F). Moreover, when subjected to a characteristic functional in vitro assay, these cells suppressed the proliferative response of $CD25^-$ responder $CD4^+$ T cells as did Treg cells isolated from littermate controls (Figure 3G). Collectively, these data establish that cDCs are neither required to establish thymic negative selection nor required for the generation of functional natural Treg cells.

cDC-less Mice Develop a Myeloid-Proliferative Disorder

Young CD11c:DTA mice (up to 5 weeks of age) showed no difference in size and behavior when compared with littermate controls. However, with time the mice developed sporadic alterations of secondary lymphoid organs and by the age of 3 months, all CD11c:DTA mice displayed lymphadenopathies and their spleens weighed approximately three times that of littermate controls (WT, 78.6 g ± 11.31; CD11c:DTA, 280.53 g ± 63.35 [n = 5]). Correlating with the increased organ size, spleen and LN cell numbers were significantly elevated in 3-month-old CD11c:DTA mice compared to age-matched littermate controls. However, BM cellularity remained essentially unaffected by the absence of cDCs (Figure 4A). Flow-cytometric analysis of CD11c:DTA spleens and LNs revealed a dramatic increase in the numbers of $CD11b^+$ myeloid cells comprising $Gr1^{int}$ monocytes and $Gr1^{hi}$ neutrophils (Figure 4B). Furthermore, we observed a minor but significant elevation of these cells in the BM (Figure 4C). Blood counts of CD11c:DTA mice revealed a myeloid shift toward neutrophils, monocytes, and eosinophils (Figure 4D), which progressed with age (Figure 4E). Morphologically, leukocytes of CD11c:DTA mice appeared normally (data not shown). Myeloid cell infiltration was also observed in peripheral nonlymphoid organs of 3-month-old CD11c:DTA mice, such as liver and kidney (Figure S6A). However, interestingly we did not find increased polymorphnuclear or mononuclear myeloid infiltrates over littermate controls in the thymi of CD11c:DTA mice (Figure S6B). In accordance with the phenotype of other mice harboring myelo-hyperproliferation (Holtschke et al., 1996), the number of splenic erythrocytes, detected by Ter119 expression, was significantly elevated in 3-month-old CD11c:DTA mice, whereas their BM, which appeared anemic exhibited lower numbers of these cells (Figure 4F). Also, histological examination of CD11c:DTA BM revealed myeloid hyperplasia and a reduction of erythroid precursors (Figure S7).

To investigate the effect of the constitutive cDCs' absence on hematopoesis, we performed colony-forming unit (cfu)

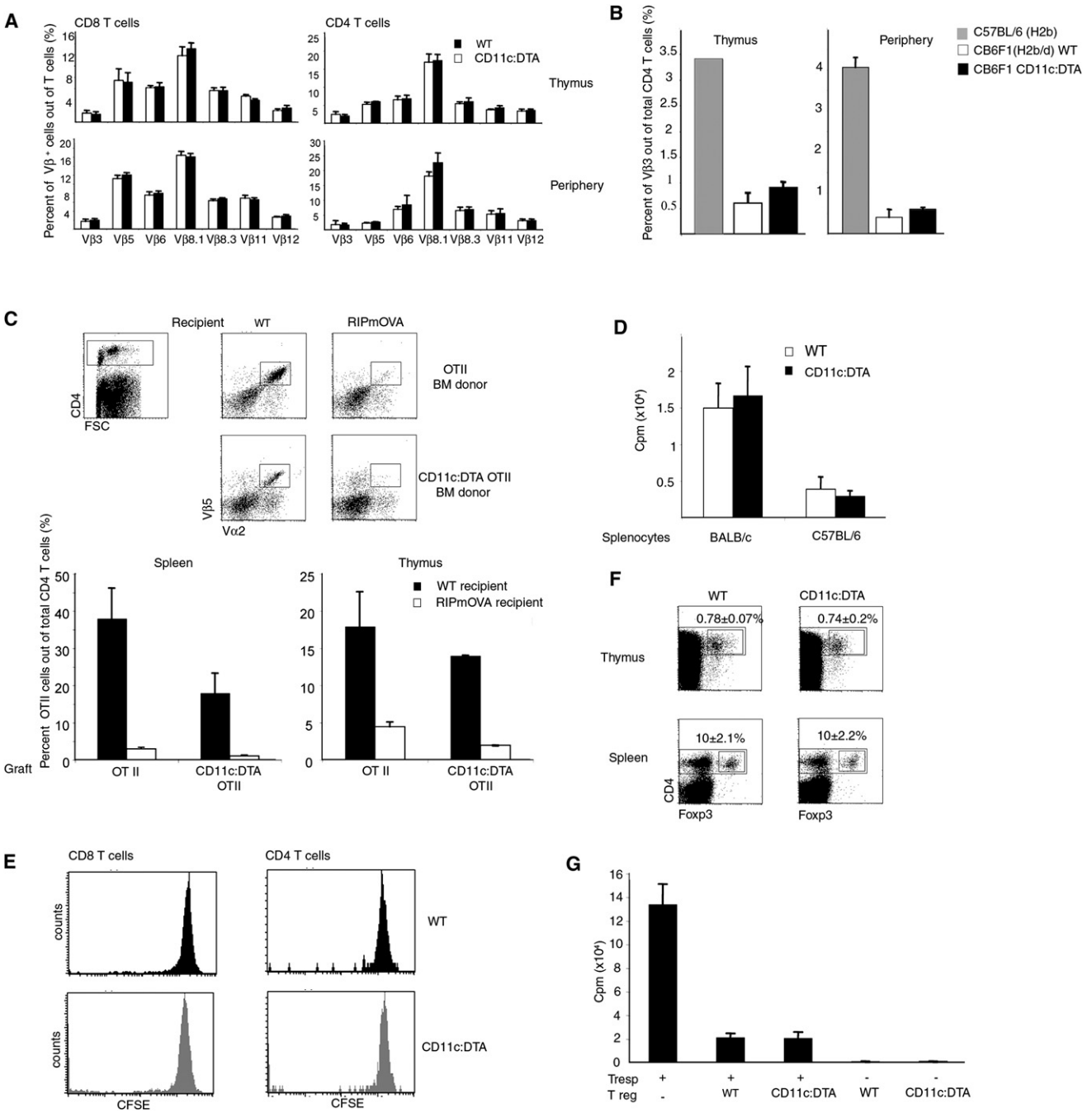

Figure 3. Unimpaired Repertoire Distribution, Thymic Negative Selection, and Treg Cell Generation in cDC-less Mice

(A) Bar graph summarizing means ± SD of flow-cytometric analysis (n = 4) for Vβ distribution among (left) total CD8$^+$ T cells and (right) CD4$^+$ T cells in the thymus and spleen of CD11c:DTA mice and LM controls.

(B) Bar graph summarizing means ± SD (n = 4) of Vβ3 distribution among total CD4$^+$ T cells in the thymus and spleen of C57BL/6 CD11c:DTA mice (gray), (C57BL/6 × BALB/c) F1 mice (white), and (C57BL/6 × BALB/c) F1 CD11c:DTA mice (black).

(C) Unimpaired thymic negative selection of OVA-reactive CD4$^+$ T cells in CD11c:DTA mice. BM cells were isolated from OT-II and CD11c:DTA;OT-II donors and transferred into irradiated WT or RIPmOVA recipients for generation of BM chimeras. Dot blots represent flow-cytometric analysis of Vα2$^+$Vβ5$^+$-specific T cells isolated from BM chimeras. Bar graphs summarize means of percentages ± SD (n = 3) of OT-II T cells out of total CD4$^+$ T cells in the thymus and spleen of the chimeras.

(D) Mixed-leukocyte reaction with CD4$^+$ T cells isolated from CD11c:DTA mice or littermate controls and allogeneic and syngeneic stimulator splenocytes. The bar graph shows mean of triplicates ± SD. Results are representative of two independent experiments.

(E) Flow-cytometric analysis of spleens of WT mice (CD45.1) that received an adoptive transfer of polyclonal CFSE-labeled CD4$^+$ or CD8$^+$ T cells isolated from CD11c:DTA mice or littermate controls (CD45.2) for the presence of graft-derived cells (CD45.2) and their proliferation status. Note absence of CD11c:DTA T cell proliferation in recipients. Results are representative example of two independent experiments with two mice per group.

assays and compared hematopoietic precursor frequencies of CD11c:DTA mice and littermate controls. The analysis revealed a significant increase of cfu counts in the blood, the spleen, and the BM, although the elevation in the first two organs was more pronounced (Figure 5A). In accordance with this finding, a 2 hr BrdU pulse resulted in an increased label of myeloid cells in the spleens of CD11c:DTA mice as compared to littermate controls, whereas the percentage of proliferating myeloid BM cells was hardly affected (Figure 5B). Extramedullary hematopoesis was also confirmed by a histological examination of spleens of 3-month-old CD11c:DTA mice (Figure S8). Moreover, flow-cytometric analysis of 3-month-old CD11c:DTA spleens revealed a significant increase of the lineage marker-negative (Lin^-) Sca-1^+ c-Kit^+ LSK cell subset representing hematopoetic stem cells (Kondo et al., 2003) (Figure 5C). However, even with progressing age, CD11c:DTA mice did not spontaneously progress into a transplantable leukemia (data not shown). Rather, cDC-less mice develop a nonmalignant chronic, nonfatal myeloproliferative disorder (MPD).

The Myeloid-Proliferative Disorder Is Triggered by the Absence of Functional cDCs

The observed MPD could be a response to ongoing cDC apoptosis in the CD11c:DTA mice. Alternatively, it could result from activation of a hitherto-unknown mechanism sensing the absence of peripheral cDCs and triggering myeloid regeneration. To distinguish between these options, we generated mixed BM chimeras through reconstitution of lethally irradiated WT recipient mice with an equal mixture of CD11c:DTA (CD45.2) and WT (CD45.1) BM. For controls, we included mice reconstituted with CD11c:DTA or WT BM only. [CD11c:DTA > WT] chimeras, which constitutively lacked cDCs (Figure 6A), displayed markedly elevated frequencies of monocytes and $CD11b^+$ myeloid cells in blood and spleen, when compared to [WT > WT] chimeras (Figures 6B and 6C). In contrast, in the mixed [CD11c:DTA-WT > WT] BM chimeras, which retained $CD45.1^+$ cDC of WT donor origin (Figure 6A), blood monocyte and myeloid cell counts were similar to the [WT > WT] controls (Figure 6C). The direct linkage between the MPD development and the absence of cDCs is further corroborated by the fact that additionally, extended conditional cDC ablation—as achieved through repetitive DTx treatment of [CD11c-DTR > WT] chimeras (Zaft et al., 2005)—resulted in the significant elevation of myeloid cells in blood and spleen (Figure 6D**)**. Collectively, these data establish that the chronic myeloproliferation observed in CD11c:DTA mice is a response to the lack of cDCs, suggesting the existence of a feedback mechanism that ensures appropriate myelogenesis in homeostasis.

Mice deficient for the interferon (IFN) regulatory factor (IRF)-8 (also known as interferon consensus sequence binding protein [ICSBP]) display a severe myeloid hyper-proliferation (Holtschke et al., 1996). Interestingly, *Irf8*$^{-/-}$ mice also exhibit a DC deficiency comprising $CD11b^-$ $CD8\alpha^+$ cDCs, as well as LCs and PDCs (Aliberti et al., 2003; Schiavoni et al., 2002). The MPD in mice lacking IRF-8 is thought to result from the tumor-suppressive activity of the transcription factor (Hao and Ren, 2000), rather than from its role in cDC development. In the light of our present findings, we decided to readdress this issue and test the possibility that the MPD of *Irf-8*$^{-/-}$ mice could be triggered by a DC-restricted deficiency. To this end, we generated irradiation chimeras with BM obtained from *Irf8*$^{-/-}$ mice (Holtschke et al., 1996), CD11c:DTA mice, and WT mice. Mice reconstituted with *Irf8*$^{-/-}$ BM developed MPD similar to mice that received CD11c:DTA BM, as indicated by the prominent accumulation of splenic $CD11b^+$ cells 8 weeks after reconstitution (Figure 6E). Mixed [*Irf8*$^{-/-}$-WT > WT] chimeras displayed normal numbers of $CD11b^+$ cells in their blood and spleens. In contrast, mixed [*Irf8*$^{-/-}$-CD11c:DTA > WT] chimeras displayed myelohyperproliferation. The MPD reported for *Irf8*$^{-/-}$ mice can thus be explained as a result of a specific defect in the DC compartment of these mice, such as the absence of $CD8\alpha^+$ cDCs or an additional functional DC impairment (Mattei et al., 2006). Together with the results obtained from the CD11c:DTA mice, this finding highlights the existence of a feedback mechanism sensing peripheral cDC numbers in the steady state.

Absence of Functional cDCs Triggers a Flt3-Ligand-Associated Feedback Loop Resulting in Myeloproliferation

Our data suggest a critical link between the size control of myeloid compartment and the presence of functional peripheral cDCs. In search for a soluble factor that could mediate this feedback loop, we analyzed the sera of CD11c:DTA mice suffering from MPD and healthy littermate controls for the elevation of growth factors that have been implied in myeloid or DC differentiation, e.g., M-CSF (Wiktor-Jedrzejczak et al., 1990), GM-CSF (Vremec et al., 1997), and Flt3 ligand (McKenna et al., 2000). In addition, we tested the serum of the mice for TNFα, which recently has been associated with MPD development (Walkley et al., 2007). Interestingly of the factor tested we found only the serum concentrations of Flt3 ligand consistently and markedly increased in the CD11c:DTA mice as compared to age-matched littermate controls (Figure 7A). Moreover, the Flt3-ligand serum elevation was observed also in young CD11c:DTA mice (<5 weeks of age) and thus considerably preceded overt development of a disorder in the animals. This finding supports a scenario in which Flt3L causes MPD, as shown in other settings (Brasel et al., 1996). The Fms-like tyrosine kinase 3 (Flt3) receptor is expressed by immediate cDC precursors (Naik et al., 2007; Onai et al., 2007) and peripheral cDCs (Tussiwand et al., 2005), and its corresponding ligand has been identified as a critical factor in the control of DC development (Maraskovsky et al., 1997; McKenna et al., 2000) and peripheral cDC maintenance (Waskow et al., 2008). Notably, Flt3 is also expressed in hematopoietic stem or progenitor cells.

To obtain further evidence for a link between Flt3 ligand and the chronic myeloproliferation, we tested the serum titers of the growth factor in the set of BM chimeras that was described

(F) Flow-cytometric analysis of thymi and spleens of CD11c:DTA mice and LM controls indicating percentages of $Foxp3^+$ Treg cells out $CD4^+$ T cells. n = 3 for each group; results are representative of three independent experiments.
(G) In vitro suppression assay with $CD25^+$ $CD4^+$ Treg cells isolated from CD11c:DTA mice and littermate controls and WT $CD25^-$ $CD4^+$ T responder cells (Tresp). Cells are stimulated with splenic DCs and soluble anti-CD3. The bar graph shows mean of triplicates ± SD.

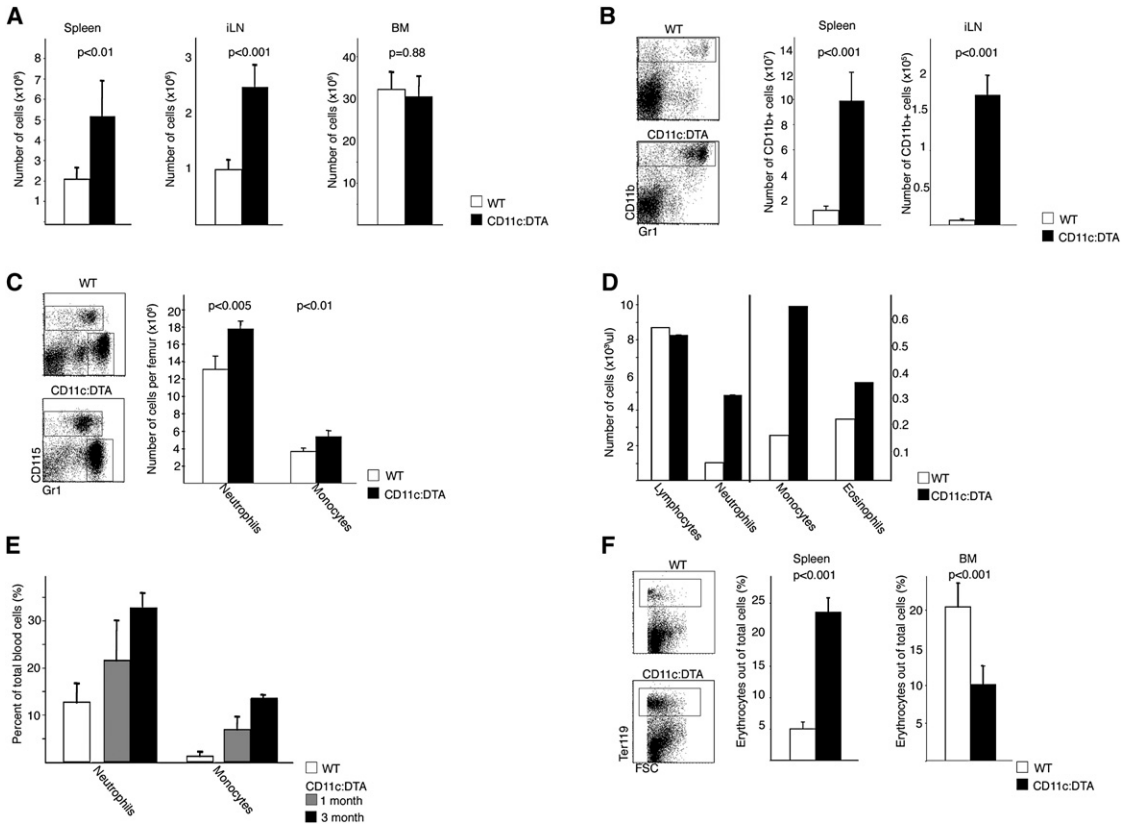

Figure 4. CD11c:DTA Mice Develop a Myeloid-Proliferative Disorder

(A) Bar graph showing total cell numbers of spleen, LN, and BM cells of 3-month-old CD11c:DTA mice and LM controls ± SD (n = 5).

(B) Myeloid cell number (identified as $CD11b^+$ cells) in the spleen and inguinal LNs of 3-month-old CD11c:DTA mice and LM controls (n = 5 for each group). Bar graphs represent mean ± SD (n = 4).

(C) Flow-cytometry analysis of BM neutrophils ($Gr1^{hi}$ $CD115^-$) and monocytes ($CD115^+$) of 3-month-old CD11c:DTA mice and LM controls. Bar graphs summarize means of cell numbers ± SD (n = 4).

(D) Bar graph representing data obtained from blood cell count ± SD (n = 2) of 3-month-old CD11c:DTA mice and LM controls.

(E) Comparison of neutrophil and monocyte percentages (±SD, n = 3) in the blood of 3-month-old versus 1-month-old CD11c:DTA mice and LM controls.

(F) Flow-cytometric analysis of BM and spleen of 3-month-old CD11c:DTA mice and LM controls for $Ter119^+$ erythrocytes. Bar graph shows means ± SD (n = 6).

above and that we generated with CD11c:DTA and *Irf8*$^{-/-}$ BM. The Flt3-ligand elevation was restricted to [*Irf8*$^{-/-}$ > WT] and [*Irf8*$^{-/-}$-CD11c:DTA > WT] chimeras (Figure 7B) and thus correlated with MPD development (Figure 6E). Flt3-ligand-driven expansion of myeloid precursor cells thus could provide a causative explanation for the observed myeloid expansion. Taken together, our results show a critical role of cDCs in the control of steady-state hematopoiesis, revealing a feedback loop that links peripheral cDCs to myelogenesis through soluble growth factors, such as Flt3 ligand.

DISCUSSION

Here, we report the use of a binary transgenic system to generate mice that constitutively lack $CD11c^{hi}$ DCs. cDC-less mice were born at normal Mendelian frequencies and displayed no developmental abnormalities. Confirming the critical role of cDCs as APCs in the efficient activation of naive T lymphocytes, T cell responses in CD11c:DTA mice were severely impaired. However, surprisingly, cDCs were largely dispensable for T cell homeostasis and repertoire shaping including thymic negative selection for autoreactivity. Moreover, T cells that developed in CD11c:DTA mice, i.e., in absence of cDCs, did not show overt functional impairments. With time, however, CD11c:DTA mice developed a progressive MPD as a direct result of the absence of cDCs.

On the basis of shared progenitors and differentiation markers, DCs have been grouped together with monocytes and macrophages into the mononuclear phagocyte system (van Furth and Cohn, 1968; Gordon and Taylor, 2005), although the exact categorization and relation of the subpopulations remains controversial. Two properties that distinguish DCs from related cell types such as macrophages are their unrivaled capacity to stimulate naive T cells (Steinman and Witmer, 1978) and their unique migration propensity. Murine cDCs can furthermore also be genetically defined in transgenic animals on the basis of the activity of the CD11c promoter (Brocker, 1999; Caton et al., 2007; Jung et al., 2002; Lindquist et al., 2004), although this definition is not absolute (Sapoznikov and Jung, 2008).

Homeostasis of naive $CD4^+$ and $CD8^+$ T cells is critically dependent on MHC expression and the presence of distinct cytokines (Boyman et al., 2007; Dummer et al., 2001; Gruber and Brocker, 2005). Moreover, among the various APCs, DCs have

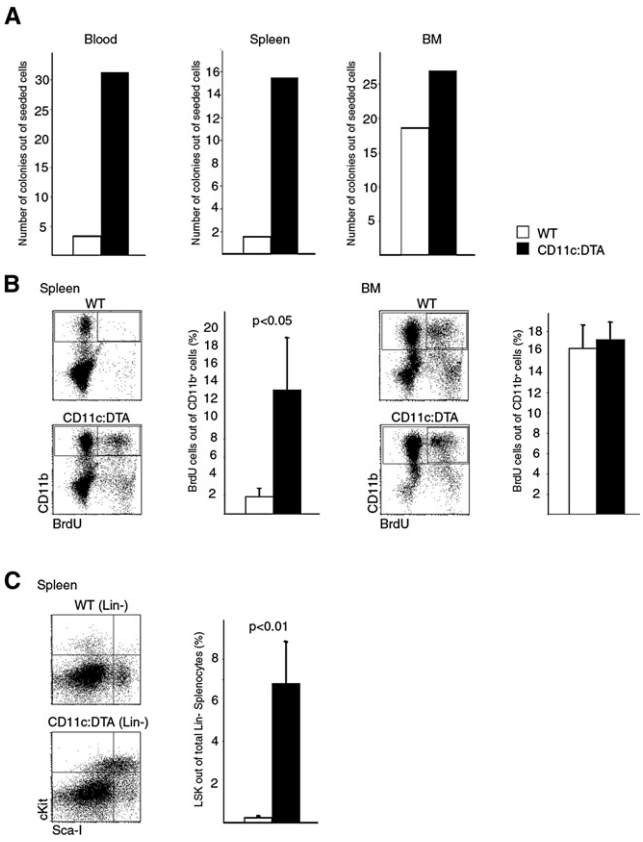

Figure 5. Enhancement of Peripheral Hematopoiesis in CD11c:DTA Mice

(A) Number of colonies per 2×10^5 seeded blood cells (left), 1.5×10^4 seeded BM cells (middle), and 16.7×10^5 splenocytes (right) of (black bar) CD11c:DTA mice and (white bar) LM control. Bar graphs represent duplicates ± SD. Results are representative of three independent experiments.

(B) Percentages of $BrdU^+$ cells out of myeloid ($CD11b^+$) cells in the (left) spleen and (right) BM of CD11c:DTA mice and LM controls 2 hr after BrdU pulse. Bar graphs represent mean ± SD (n = 3).

(C) Flow-cytometric analysis of hematopoietic stem cells (defined as Lin^- $Sca\text{-}1^+$ and $c\text{-}kit^+$ cells) (LSK cells) in spleens of CD11c:DTA mice and LM controls. Bar graphs represent LSK mean percentages ± SD (n = 4) out of splenic cells.

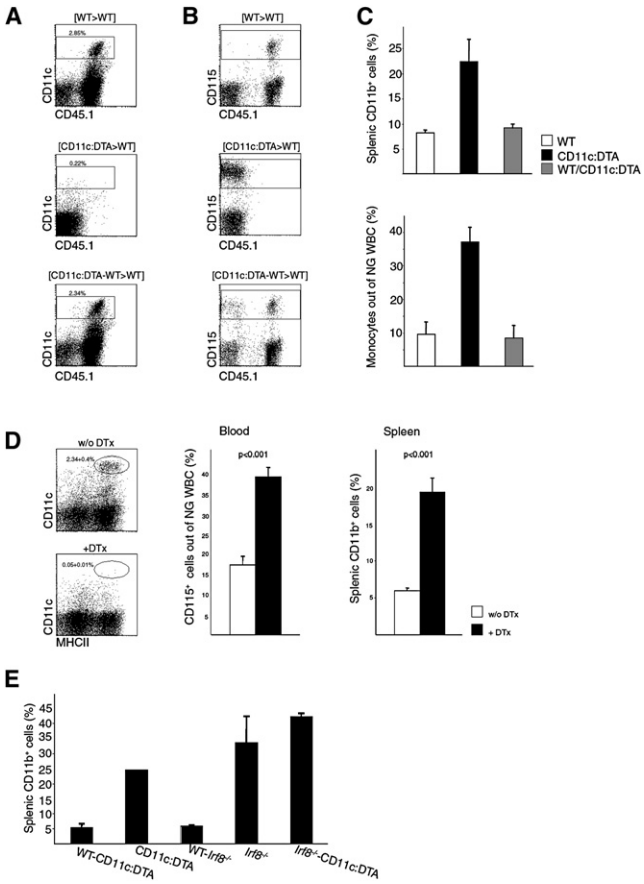

Figure 6. Absence of Functional cDCs Results in Myeloproliferation

(A) Flow-cytometric analysis of splenic DCs of [WT > WT], [CD11c:DTA > WT] and [CD11c:DTA-WT > WT] chimeras. DCs are gated as $CD11c^{hi}$ cells. Donor BM is indicated above the dot blots.

(B) Flow-cytometric analysis of percentage of blood monocytes ($CD115^+$) out of total nongranular white blood cells (ngWBC) in the three chimeras. Splenic myloid cells defined as $CD11b^+$ cells were analyzed in the same way. Donor BM is indicated above the dot blots.

(C) Bar graphs summarizing mean ± SD percentages (n = 3) of $CD115^+$ monocytes in the blood (out of total ngWBC) and $CD11b^+$ cells in the spleen of indicated chimeras. Results are representative example of three independent experiments.

(D) Myeloproliferative disorder after persistent conditional cDC ablation. Flow-cytometric analysis of splenic and blood cells of [CD11c-DTR > WT] chimeras treated for 2 weeks with diphtheria toxin (DTx) (every second day). Controls were left untreated. FACS analysis representing percentages of $CD11c^{hi}$ splenic DCs in DTx-treated and untreated mice is shown. The bar graph represents mean percentages ± SD (n = 3) of blood monocytes and splenic myeloid cells in (black bar) DTx-treated CD11c-DTR mice versus (white bar) nonreated mice.

(E) Flow-cytometric analysis of BM chimeras generated with CD11c:DTA BM and $Irf8^{-/-}$ BM. Bar diagrams summarize mean percentages ± SD (n = 3) of CD11b-positive cell out of total splenocytes in the indicated BM chimeras.

been proposed to play a unique role in the maintenance of the steady-state T cell repertoire (Brocker et al., 1997; Gruber and Brocker, 2005; Zaft et al., 2005). Surprisingly however, CD11c:DTA mice displayed a largely unimpaired $CD4^+$ and $CD8^+$ T cell compartment, with respect to naive T cell numbers, subset ratios, and Vβ TCR representation. Although this finding does not generally negate a role of DCs in thymic selection, it establishes that in contrast to the current notion, steady-state T cell survival and T cell homeostasis can be maintained largely without cDCs.

Exclusive expression of MHC class II on DCs was shown to promote thymic negative selection of $CD4^+$ T cells (Brocker et al., 1997). Moreover, although antigen expression and presentation by mTEC was reported to suffice for the deletion of autoreactive $CD8^+$ T cells (Gallegos and Bevan, 2006), the cellular requirement for $CD4^+$ T cell tolerization in the thymus has remained under debate. Data from chimeras in which paternal BM was transferred into lethally irradiated F1 offspring suggested that BM-derived cells are dispensable for thymic negative selection (Gao et al., 1990; Sprent et al., 1992), although a more recent study reported the requirement of DCs (Gallegos and Bevan, 2004) for $CD4^+$ T cell tolerization. Here, we showed that thymic DCs were dispensable for the establishment of deletional tolerance, including the clearance of the T cell repertoire from endogenous MMTV super antigen-reactive T cells, polyclonal

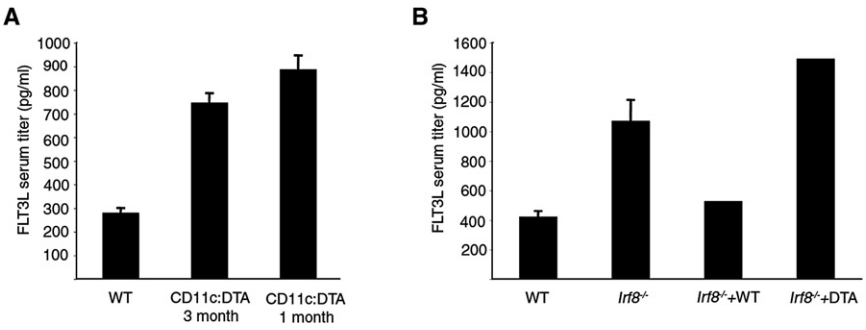

Figure 7. Absence of cDC Results in Increase of Serum Titers of Flt3 Ligand

(A) Serum analysis of CD11c:DTA mice and littermate controls for Flt3 ligand by ELISA. The bar graph shows means of triplicates ± SD (n = 3).

(B) Serum Flt3-ligand titers as analyzed by ELISA of lethally irradiated mice reconstituted with WT, *Irf8*$^{-/-}$, *Irf8*$^{-/-}$-WT, or *Irf8*$^{-/-}$-CD11c:DTA BM. The bar graph shows means of triplicates ± SD (n = 3).

autoreactive CD4$^+$ and CD8$^+$ T cells, as well as CD4$^+$ T cells responsive to a model self-antigen (OVA).

DCs are believed to play a critical role in the maintenance of peripheral tolerance by their induction and/or stimulation of Treg cells. Human TSLP-activated DCs within the thymic medulla were reported to stimulate developing T cells to differentiate into Treg cells (Watanabe et al., 2005). Furthermore, in vitro studies support a unique role of murine DCs in the generation of CD4$^+$Foxp3$^+$ Treg cells from peripheral naive T cells (Coombes et al., 2007; Yamazaki et al., 2007). DCs are most effective in triggering Treg in vitro proliferation (Fehervari and Sakaguchi, 2004) and regulating Treg homeostasis in vivo (Cong et al., 2005). Additionally, in vivo targeting of antigens to immature DCs was shown to result in Treg cell induction (Mahnke et al., 2003). Surprisingly however, CD11c:DTA mice harbored normal numbers of functional CD4$^+$Foxp3$^+$ Treg cells in the thymus and periphery. This establishes that DCs are dispensable for homeostasis and generation of natural Treg cells, supporting the notion that other cells might perform this task as well, including thymic mTECs (Aschenbrenner et al., 2007). Collectively, our results do not support an essential role of cDCs in T cell homeostasis, survival, and negative selection, as well as in the generation of natural Treg cells, that cannot be compensated by other cells.

Mice that constitutively lack cDCs developed a MPD. Until recently MPD were considered to be hematopoietic cell intrinsic, a notion supported by the involvement of Bcr-Abl in human CML (Van Etten and Shannon, 2004) and various murine MPD models (Passegue et al., 2004; Wernig et al., 2006). In contrast, the MPD developed by CD11c:DTA mice is not cell autonomous but arises as a response of the organism to the lack of cDCs. This notion is supported by the results obtained from mixed BM chimeras generated with WT and CD11c:DTA BM, as well as the studies involving persistent conditional cDC ablation. As such, CD11c:DTA mice resemble reported models of microenvironment-induced, stroma-regulated MPD (Rupec et al., 2005; Walkley et al., 2007). Moreover, we provide evidence that the MPD in mice deficient for the transcription factor IRF-8 (Holtschke et al., 1996) may also develop as a consequence of their impaired cDC compartment. Taken together, these results argue that the absence of cDCs triggers a feedback loop resulting in systemic myeloid expansion.

Interestingly, serum analysis of CD11c:DTA mice and mice that were reconstituted with *Irf8*$^{-/-}$ BM revealed a marked elevation of the growth factor Flt3 ligand, which probably caused constitutive activation of Flt3 receptor expressed on hematopoietic stem or progenitor cells. Importantly, activating Flt3 mutations are frequently involved in human myeloid leukemia (Stirewalt and Radich, 2003); moreover, constitutive activity of endogenous Flt3 through an activating knockin mutation was recently shown to cause MPD (Lee et al., 2007; Li et al., 2008). Finally, experimentally induced elevation of Flt3-ligand concentrations causes a disorder similar to the one observed in CD11c:DTA mice (Brasel et al., 1996). Thus, elevated serum levels of Flt3 ligand provide a potential explanation for the MPD observed in CD11c:DTA mice. A unique role of Flt3 ligand in the feedback loop is supported by the fact that we failed to detect an elevation of serum titers of other candidate growth factors and cytokines, such as M-CSF, GM-CSF, and TNFα.

Several mutually nonexclusive scenarios may explain the increased Flt3-ligand levels in the absence of DCs. First, cDCs (along with the less numerous PDCs) are the only mature peripheral hematopoietic cells expressing Flt3 (Karsunky et al., 2003) and therefore might serve as a major constitutive "ligand sink." Alternatively, cDCs might provide a secreted or membrane-bound signal that regulates Flt3-ligand production by stromal cells or lymphocytes. In the absence of such DC-mediated feedback, Flt3-ligand production would be increased, ultimately resulting in MPD. Although these possibilities remain to be tested, our current findings describe a critical and unexpected role of DCs in the feedback regulation of steady-state hematopoiesis. Future elucidation of the molecular mechanism linking the cDC loss to the myeloproliferative disorder might provide critical insight in the etiology of MPDs and help develop novel strategies for therapeutic interventions of chronic nonmalignant myeloid disorders.

EXPERIMENTAL PROCEDURES

Mice

The following mice were used in this study: 8- to-12 week-old C57BL/6 mice, BALB/c mice, CD11c-DTR transgenic mice (B6.FVB-Tg Itgax-DTR/GFP 57Lan/J) carrying a transgene encoding a human DTR-GFP fusion protein under the control of the murine CD11c promoter (Jung et al., 2002); *CD11c*-Cre mice (Caton et al., 2007); R26-DTA mice (backcrossed for ten generations onto C57BL/6) (Brockschnieder et al., 2006); OT-I (C57BL/6) TCR transgenic mice harboring OVA-specific CD8$^+$ T cells (Hogquist et al., 1994); OT II (C57BL/6) TCR transgenic mice harboring OVA-specific CD4$^+$ T cells (Barnden et al., 1998); RIPmOVA transgenic mice expressing a membrane-bound form of OVA (residues 139–385) under control of the rat insulin promoter (RIP) (Kurts et al., 1996); MHC class I-deficient *B2m*$^{-/-}$ mice (Koller et al., 1990); *Irf8*$^{-/-}$ mice (Holtschke et al., 1996); and B6.Cg-Igh^{a}Thy1^{a}Gpi1^a/J mice (kindly provided by H.-W. Mittruecker, Berlin). R26-DTA mice were crossed with *CD11c*-Cre transgenic mice for generating *CD11c*-Cre:DTA mice. Mixed [CD11c-DTR > WT], [WT > WT], [DTA > WT], [50% DTA-50% WT > WT], [*Irf8*$^{-/-}$ > WT], [50% DTA-50% *Irf8*$^{-/-}$ > WT], [DTA OTII > RIPmOVA], [DTA OTII > WT], [OTII > RIPmOVA], and [OTII > WT] BM chimeras were generated as reported (Zaft et al., 2005). For conditional DC ablation, [CD11c-DTR > WT]

BM chimeras were inoculated intraperitoneally every second day for 2 weeks with 16 ng diphtheria toxin (DTx) per g body weight. For BrdU labeling, mice were i.p. injected with 1 mg/ml of BrdU (Sigma) and analyzed for incorporation 2 hr later by FACS analysis. All animals were maintained under specific pathogen-free (SPF) conditions and handled according to protocols approved by the Weizmann Institute Animal Care Committee as per international guidelines.

Flow-Cytometry Analysis

Staining reagents used in this study included the PE-coupled antibodies anti-MHC II, mPDCA-1 (Milteny Biotec), CD4, CD8, Vα2, Vβ3, Vβ5, Vβ6, Vβ8.1, Vβ8.3, Vβ11, Vβ12, CD11b, CD115, Ter119, and Sca1; the biotinylated antibodies anti-CD45.1, CD4, CD3, and Thy1.2; the APC-coupled antibodies anti-SA, CD11c, CD45.1, CD4, Foxp3, CD19, Gr1(Ly6C/G), and CD117 (c-Kit); the PerCP-coupled antibodies anti-CD8, CD11b; and the FITC-coupled antibodies anti-Brdu (BD PharMingen), CD8, CD4, Gr1, B220, NK1.1, and CD11b. Unless indicated otherwise, the reagents were obtained from eBioscience or Biolegend. The cells were analyzed on a FACS Calibur cytometer (Becton Dickinson) with CellQuest software (Becton Dickinson).

Mixed-Leukocyte Reactions

Stimulator cells were isolated from spleens and thymi of CD11c:DTA mice, CD11c-DTR transgenic mice, and littermates controls as indicated. Responder T cells were enriched from spleens of BALB/c mice by positive selection with anti-CD4 microbeads (Miltenyi Biotec). A total of 5×10^5 splenocytes or thymocytes were cultured with 10^5 responder $CD4^+$ T cells (BALB/c). Cultures were pulsed after 72 hr with 1μCi of $[H^3]$ thymidine incorporation was measured 16 hr later.

Histology and Immunohistochemistry

Tissues were fixed in 4% para-formaldehyde for 24 hr, embedded in paraffin, sectioned serially (4 μm), and stained with hematoxyline and eosin (Sigma). Photographic documentation was performed with and E800 microscope equipped with a digital camera (DXM 1200, NIKON, Japan). For immunohistochemistry, freshly removed organs were immersed in Hank's balanced-salt solution and were "snap-frozen" in liquid nitrogen. Tissue sections that were 5 μm in thickness were air-dried, fixed for 10 min with acetone, and stored at −70°C. Cryosections were blocked for 30 min with 1 g Fc-blocking antibody 2.4G2 per sample, washed in PBS, and incubated for 1 hr at 4°C with the appropriate fluorescent antibodies. Where needed, streptavidin-tetramethyl rhodamine isothiocyanate was added in a second step. After sections were washed with PBS, and they were mounted with fluorescence mounting solution (Dako).

Analysis of In Vivo T Cell Proliferation

TCR transgenic T cells were isolated from spleens and LNs of respective mice, enriched by MACS cell sorting with anti-CD8 or anti-CD4 antibodies according to the manufacturer's protocol (Miltenyi Biotec GmbH), and labeled with carboxy fluorescein succinimidyl Ester (CFSE, C-1157; Invitrogen) (Lyons, 2000). CFSE-labeled T cells (1 to 2×10^6 cells/mouse) were injected into the tail veins of the recipient mice. Cells were analyzed 4 days later for CFSE dilution with FACS.

Viral Infection

Mice were injected with indicated pfu of MHV A59 (i.p.) and with 200 pfu of LCMV WE (i.v.) and sacrificed at the indicated time points. Organs were stored at −70°C until further analysis. For measuring the amount of the liver enzyme alanine 2-oxoglutarate-aminotransferase (ALT), which is indicative of liver damage, blood was incubated at RT to coagulate and centrifuged, and serum was used for ALT measurements with a Hitachi 747 auto-analyzer (Tokio, Japan). MHV titers were determined by standard plaque assay on day 8 after infection with L929 cells. LCMV titers in the spleens were determined 4 days after i.v. infection in an LCMV infectious focus assay. MHC class I (H-2Db) monomers complexed with GP33 were produced as described (Krebs et al., 2005) and tetramerized by addition of streptavidin-PE (Molecular Probes). At the indicated time points after immunization, animals were bled and single-cell suspensions were prepared from spleens and lymph nodes. Aliquots of 5×10^5 cells or three drops of blood were stained with 50 ml of a solution containing tetrameric class I-peptide complexes at 37°C for 10 min and then stained with anti-CD8-FITC (BD PharMingen) at 4°C for 20 min. The cells were analyzed by flow-cytometry gating on viable leukocytes.

Analysis of IFN-γ Production

Specific ex vivo production of IFN-γ was determined by intracellular cytokine staining. Organs were removed at the indicated time points after infection. For intracellular cytokine staining, single-cell suspensions of 1×10^6 splenocytes were incubated for 5 hr at 37°C in 96-well round-bottom plates in 200 ml culture medium containing 25 U/ml IL-2 and 5 mg/ml Brefeldin A (Sigma). Cells were stimulated with phorbol-myristate acetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) (both purchased from Sigma, Buchs, Switzerland) as a positive control or left untreated as a negative control. For analysis of peptide-specific responses, cells were stimulated with 1 μM of GP33 peptide or 100 μM of MHV S598 peptide. The percentage of $CD8^+$ T cells producing IFN-γ was determined with a FACSCalibur flow cytometer. Both S598 (RCQIFANI) and GP33 (KAVYNFATC) peptides were purchased from Neosystem.

Analysis of In Vitro T Cell Proliferation

$CD4^+$ and $CD8^+$ T cells were isolated from spleens and LNs of respective mice, enriched by MACS cell sorting with bead-coupled anti-CD4 or anti-CD8 antibodies according to the manufacturer's protocol (Miltenyi Biotec GmbH). A total of 10^5 cells were plated on 96-well round-bottom plates coated with anti-CD3 antibody in different concentrations (10.1, 0.5, and 0.1 μg/ml) (BD PharMingen). Cultures were pulsed after 72 hr with 1μCi of $[H^3]$ thymidine, incorporation was measured 16 hr later.

Treg Cell-Suppression Assay

$CD25^-$ and $CD25^+$ $CD4^+$ T cells were isolated from WT or CD11c:DTA mice by magnetic depletion according to the manufacturer's protocol (Miltenyi Biotec GmbH). $CD4^+CD25^-$ WT responder T cells (10^5) were cultured in 96-well round-bottom plates in a total volume of 0.2 ml in the presence or absence of freshly isolated $CD4^+CD25^+$ Treg cells (10^5). A total of 2×10^4 splenic $CD11c^+$ DCs/well isolated by MACS (Miltenyi Biotec GmbH) were used as accessory cells, and anti-CD3 mAb (145-2C11; 2 μg/ml) was used as stimulus. Cultures were pulsed after 96 hr with 1μCi of $[H^3]$ thymidine; incorporation was measured 16 hr later.

Colony-Forming-Unit Assay

Semisolid cultures were performed as previously described (Petit et al., 2005). In brief, murine splenocytes (5×10^5 cells/ plate), peripheral blood cells (2×10^5 cells/plate), and BM cells (1.5×10^4 cells/plate) were plated in 0.9% methylcellulose (Sigma), 30% FCS (Biological Industries), 50 ng/ml SCF, 5 ng/ml IL-3, 5 ng/ml GM-CSF (Kirin), and 2 u/ ml Erythropoietin (Orto Bio Tech). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and scored 7 days later according to morphologic criteria.

Detection of Serum Titers of Growth Factors and Cytokines

Sera of CD11c:DTA mice and age matched littermate controls were tested by ELISA for Flt3L and M-CSF (catalog #DY416; R&D Systems), as well as GM-CSF and TNFα (catalog #555167, 555268; Becton Dickinson) according to the manufacturer's protocol. The detection levels as determined by analysis of the respective recombinant standards diluted in PBS-BSA or 20% serum/ 80% PBS-BSA were ∼15 pg/ml; 22 pg/ml (GM-CSF); 10 pg/ml; 14 pg/ml (M-CSF); and 15 pg/ml, 20 pg/ml (TNFα). Note that dilution of the standard in serum lowered the sensitivity of the commercial ELISA kits.

Statistical Analysis

All statistics were generated with a Student's t test. All error bars in diagrams and numbers following a plus-minus sign are standard deviations (SD).

SUPPLEMENTAL DATA

Supplemental Data include eight figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00503-7.

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