Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages

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Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages

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Distinguishing dendritic cells (DCs) from other cells of the mononuclear phagocyte system is complicated by the shared expression of cell surface markers such as CD11c. In this study, we identified Zbtb46 (BTBD4) as a transcription factor selectively expressed by classical DCs (cDCs) and their committed progenitors but not by plasmacytoid DCs (pDCs), monocytes, macrophages, or other lymphoid or myeloid lineages. Using homologous recombination, we replaced the first coding exon of Zbtb46 with GFP to inactivate the locus while allowing detection of Zbtb46 expression. GFP expression in Zbtb46fl/fl/− mice recapitulated the cDC-specific expression of the native locus, being restricted to cDC precursors (pre-cDCs) and lymphoid organ– and tissue-resident cDCs. GFP+ pre-cDCs had restricted developmental potential, generating cDCs but not pDCs, monocytes, or macrophages. Outside the immune system, Zbtb46 was expressed in committed erythroid progenitors and endothelial cell populations. Zbtb46 overexpression in bone marrow progenitor cells inhibited granulocyte potential and promoted cDC development, and although cDCs developed in Zbtb46−/− mice, they maintained expression of granulocyte colony-stimulating factor and leukemia inhibitory factor receptors, which are normally down-regulated in cDCs. Thus, Zbtb46 may help enforce cDC identity by restricting responsiveness to non-DC growth factors and may serve as a useful marker to identify rare cDC progenitors and distinguish between cDCs and other mononuclear phagocyte lineages.

Abbreviations used: cDC, classical DC; CDP, common DC progenitor; CPU-E, CFU-erythroid; CMP, common myeloid progenitor; DTR, diphtheria toxin receptor; GMP, granulocyte macrophage precursor; MLN, mesenteric LN; mRNA, messenger RNA; pDC, plasmacytoid DC; RPM, red pulp macrophage; SLN, skin-draining LN; Tip-DC, TNF/iNOS-producing DC.
express fluorescent reporters, Cre recombinase, or the diphtheria toxin receptor (DTR) for the purpose of allowing tracking of DCs or to allow the inducible depletion of DCs for functional studies in vivo (Jung et al., 2002; Lindquist et al., 2004; Caton et al., 2007). However, the interpretation of studies using these reagents has been confounded by the expression of CD11c by other lineages (Probst et al., 2005; Murphy, 2011). For example, in CD11c-DTR mice, the depletion of DCs by administration of diphtheria toxin A also leads to depletion of other cell types, including tissue-resident, marginal zone, and metallophilic macrophages, NK cells, and NKT cells, as well as some CD11c+ B and T cells (van Rijt et al., 2005; Bennett and Clausen, 2007; Hume, 2011). Other loci, including Lym-1, Gsf1r, Cx3cr1, and CD11b, have been modified to express fluorescent proteins, but these are also expressed not only in DCs but in additional myeloid populations as well (Hume, 2011). No single marker has been identified to uniquely distinguish cDCs from other myeloid or lymphoid lineages, prompting the suggestion that identification of bona-fide cDCs may require the simultaneous analysis of surface phenotype, cellular derivation, function, and anatomical location (Geissmann et al., 2010).

DCs are derived from BM myeloid progenitors that undergo progressive lineage restriction. The common myeloid progenitor (CMP) generates the common DC progenitor (CDP), which has lost the ability to give rise to granulocytes and macrophages (derived from the downstream granulocyte macrophage precursor [GMP]) but still retains both pDC and cDC potential (Onai et al., 2007; Naik et al., 2007). The CDP appears to be the immediate precursor of both pre-pDCs (Cisse et al., 2008) and pre-cDCs (Naik et al., 2006; Liu et al., 2009), which are restricted to their respective lineage but not yet fully mature. Terminal cDC development occurs in peripheral lymphoid and nonlymphoid organs after Flt3L (fms-like tyrosine kinase 3 ligand)-dependent expansion (Waskow et al., 2008).

We previously suggested that accurately defining the cDC lineage might benefit from methods based on lineage-specific transcription factors induced during these early stages of commitment (Satpathy et al., 2011). Several transcription factors have already been found to regulate DC development (Satpathy et al., 2011) but are not suitable for the purpose of specifically marking the lineage in vivo. For example, If8−/− mice lack pDC as well as CD8α+ cDC lineages but also have additional defects in other immune cell types (Schiavoni et al., 2002; Wang and Morse, 2009). Likewise, the transcription factor Batf3, which is required for the development of the CD8α+ cDC lineage, is expressed in cDCs but also in other myeloid cells (Heng and Painter, 2008) and differentiated Tδ1 cells (Hildner et al., 2008).

In this study, we identify a member of the BTB-ZF (Broad complex, Tramtrack, Bric-à-brac, and Zinc finger) family (Kelly and Daniel, 2006) for which no studies have been reported. To examine its expression pattern and function in vivo, we designed a targeting strategy to inactivate the locus while also allowing for detection of its expression. We inserted a GFP reporter cassette into exon 2 beginning at the first coding methionine of Zbtb46, thus removing the native translational start site and completely eliminating exon 2, which encodes the Zbtb46 BTB domain (Fig. 2A). Two ES cell clones harboring the correctly targeted Zbtb46 allele were obtained (Fig. 2B) and used to generate germline-targeted mice on the pure C57BL/6 genetic background (Fig. 2C). Both of these correctly targeted clones were successfully transmitted through the germline, and progeny derived from each clone exhibited the same phenotype and patterns of GFP expression. Data in this study are expressed not only in DCs but in additional myeloid populations, including closely related myeloid lineages. Furthermore, Zbtb46 expression identifies the earliest committed precursor of cDCs. Zbtb46 is not required for cDC development in vivo but regulates silencing of G-CSF and leukemia inhibitory factor receptors that normally occur during cDC differentiation.

**RESULTS**

**Zbtb46 is selectively expressed in cDCs and committed cDC progenitors**

To identify transcription factors expressed in early cDC progenitors but not in other myeloid precursors, we purified CMPs, GMPs, CDPs, and BM and splenic pre-cDCs (Fig. 1A) and performed gene expression analysis (Fig. 1B). Transcription factors were compared for their induction in splenic pre-cDCs compared with BM CDPs and for their lineage specificity in cDCs compared with a broad panel of hematopoietic and nonhematopoietic cells (Fig. 1B; Lattin et al., 2008). This method identified previously studied cDC genes, Batf3 and Id2, as well as the novel factor Zbtb46. Zbtb46 was induced nearly 20-fold in splenic pre-cDCs relative to CDPs and was highly cDC specific across the tissue panel (Fig. 1B).

Zbtb46 was expressed in both the CD8α− and CD4+ subsets of cDCs but at very low levels in pDCs, monocytes, granulocytes, and T and B cells (Fig. 1C). Zbtb46 is conserved as BTBD4 in humans, where it was also specifically expressed in both major subsets of cDCs but not pDCs, monocytes, granulocytes, or T, B, or NK cells (Fig. 1D). In mouse, Zbtb46 was first expressed in the BM pre-cDC and increased during differentiation into mature cDC subsets (Fig. 1E). In contrast, If8 was expressed in the earlier CMP and GMP stages, highly induced in the CDP, and maintained in the pre-cDC and subsequent CD8α− cDC and pDC. Expression of Batf3 was low in BM progenitors and was induced beginning in the later splenic pre-cDC stage and maintained in mature cDCs (Fig. 1E; Edelson et al., 2010).

**Zbtb46-driven GFP expression identifies cDCs in lymphoid organs**

Zbtb46 encodes a 601-aa member of the BTB-ZF family of transcriptional repressors (Kelly and Daniel, 2006) for which no studies have been reported. To examine its expression pattern and function in vivo, we designed a targeting strategy to inactivate the locus while also allowing for detection of its expression. We inserted a GFP reporter cassette into exon 2 beginning at the first coding methionine of Zbtb46, thus removing the native translational start site and completely eliminating exon 2, which encodes the Zbtb46 BTB domain (Fig. 2A). Two ES cell clones harboring the correctly targeted Zbtb46 allele were obtained (Fig. 2B) and used to generate germline-targeted mice on the pure C57BL/6 genetic background (Fig. 2C). Both of these correctly targeted clones were successfully transmitted through the germline, and progeny derived from each clone exhibited the same phenotype and patterns of GFP expression. Data in this study are expressed not only in DCs but in additional myeloid populations, including closely related myeloid lineages. Furthermore, Zbtb46 expression identifies the earliest committed precursor of cDCs. Zbtb46 is not required for cDC development in vivo but regulates silencing of G-CSF and leukemia inhibitory factor receptors that normally occur during cDC differentiation.
Figure 1. **Zbtb46** is expressed in BM pre-cDCs and cDCs in both mouse and human. (A) BM cells from WT mice were stained for expression of the indicated markers. Two-color histograms are shown for live cells pregated as indicated above the diagram. Numbers represent the percentage of cells within the indicated gate. Data are representative of five independent experiments with at least two mice each. (B) Sorted populations in A were analyzed by gene expression microarray as described in Materials and methods. Gene expression levels were determined for a list of all transcription factors using dChip software (Li and Wong, 2001a,b) for the splenic pre-cDC, BM CDP, CD8α+ and CD4+ cDCs, and a broad panel of tissues (Lattin et al., 2008). For each transcription factor, the horizontal axis indicates the ratio of gene expression in the pre-cDC compared with the CDP, and the vertical axis indicates the ratio of the mean expression in CD8α+ and CD4+ DCs compared with the mean expression in the tissue panel excluding DCs. Each dot indicates an individual probe set. (C) Shown is the normalized expression value of **Zbtb46** mRNA determined by quantitative RT-PCR for the indicated cell populations. Data represent three independently sorted replicates from three mice. (D) Shown is the expression value of **BTBD4** mRNA in the indicated cells derived from Lindstedt et al. (2005) and Du et al. (2006). (E) Shown are the expression values for mouse **Zbtb46**, **IrF8**, and **Batf3** mRNA transcripts in the indicated cell populations determined by expression microarray. Microarray analysis in B and E represents either two or three independently obtained arrays from progenitors sorted from three to five pooled WT mice. cDC arrays in E are derived from Robbins et al. (2008). (C–E) Bars represent the mean ± SEM.
absence of Zbtb46 (Fig. 3 A). In spleen, GFP+ cells were overwhelmingly CD11c+ MHCII+ and comprised both CD172α+ CD24− and CD172α− CD24+ populations (Fig. 3 B), indicating Zbtb46 expression in both major subsets of cDCs. In LNs, GFP was not only expressed by cells with high levels of CD11c, but also by migratory cDCs that expressed reduced levels of CD11c. Here, CD11c+ cells expressed intermediate levels of MHCII, representing lymphoid-resident DCs, whereas CD11c− cells expressed high levels of MHCII, representing migratory DCs (Fig. 3 B; Shortman and Naik, 2007).

normal Mendelian frequencies from heterozygous breeding pairs. Zbtb46 transcripts were reduced in DCs isolated from Zbtb46+ mice to background levels, indicating that this targeting strategy interrupted normal gene transcription as intended (Fig. 2 D).

We first analyzed Zbtb46 expression in lymphoid organs, including spleen, skin-draining LNs (SLNs), mesenteric LNs (MLNs), and thymus (Fig. 3 A). In CD45+ cells analyzed by FACS, GFP-expressing cells were present at equivalent frequencies in both Zbtb46+ and Zbtb46+ mice, indicating that no major populations of cells failed to develop in the absence of Zbtb46 (Fig. 3 A). In spleen, GFP+ cells were overwhelmingly CD11c+ MHCII+ and comprised both CD172α+ CD24− and CD172α− CD24+ populations (Fig. 3 B), indicating Zbtb46 expression in both major subsets of cDCs. In LNs, GFP was not only expressed by cells with high levels of CD11c, but also by migratory cDCs that expressed reduced levels of CD11c. Here, CD11c+ cells expressed intermediate levels of MHCII, representing lymphoid-resident DCs, whereas CD11c− cells expressed high levels of MHCII, representing migratory DCs (Fig. 3 B; Shortman and Naik, 2007). Again, GFP+ resident DCs in SLNs and MLNs contained both...
CD172+CD24− and CD172−CD24+ populations (Fig. 3 B), indicating that Zbtb46 is expressed in all cDC subsets in peripheral lymphoid organs as well.

Notably, GFP expression was not detected in any other myeloid or lymphoid lineages (Fig. 3 C). In particular, pDCs were devoid of GFP expression in spleen, SLNs, and thymus, as were monocytes, granulocytes, and NK cells. Red pulp macrophages (RPMs) showed a low level of autofluorescence that did not represent authentic GFP expression, as similar levels of autofluorescence were observed in RPMs of Zbtb46+/+ (WT) mice (not depicted) and anti-GFP staining was not detected in RPMs by immunofluorescence microscopy (see generated in BM cultures treated with GM-CSF (Fig. 4 A; Sallusto and Lanzavecchia, 1994). Adherent cells either from GM-CSF–treated cultures, which represent macrophages and granulocytes, or from M-CSF–treated cultures, which represent macrophages, did not express Zbtb46 (Fig. 4 A). Purified monocytes cultured with GM-CSF induced the expression of Zbtb46 after 24 h (Fig. 4 B) and induced high levels of Zbtb46 after 4 d in culture (Fig. 4 C). Notably, after 4 d of treatment with GM-CSF alone, Zbtb46 was induced in ~75% of cells, whereas with the addition of both GM-CSF and IL-4, they became uniformly MHCIi high and >95% GFP positive, indicating that IL-4 may enhance DC differentiation.

Interestingly, Zbtb46 was also expressed in nonadherent CD11c+cDCs

Fig. 3. Zbtb46 specifically identifies cDCs in the mouse. Cells from Zbtb46+/+, Zbtb46+/−, and Zbtb46−/− were harvested from the spleen, SLNs, MLNs, and thymus as indicated and stained for CD11c, MHCIi, CD172, CD24, and CD45. (A) Shown are two-color histograms for the indicated markers after gating for CD45 expression. Numbers represent the percentage of cells in the indicated gate. (B) Shown are two-color histograms for the indicated markers derived from a GFP+ gate as indicated in A from Zbtb46−/− mice. (C) Shown are single-color histograms for GFP expression of cells from Zbtb46−/− mice (WT) or cells from Zbtb46+/− mice stained to identify cDCs (MHCIi+CD11c+), resident cDCs (MHCIi+CD11c+), migratory cDCs (MHCIi+CD11c−), pDCs (CD317+B220+), RPMs (F4/80+ autofluorescent), monocytes (CD11b+CD11c+MHCIi+Ly6G−), granulocytes (Ly6G+CD11b+), CD8+ T cells (CD4+CD11c+), B cells (B220+MHCIi−), NK cells (NKp46+), DP thymocytes (CD4+CD8−Lin−), or DN thymocytes (CD4−CD8−Lin−). (D) BM cells from Zbtb46−/− or Zbtb46−/− mice were cultured with 100 ng/ml Flt3L for 6–9 d as indicated, harvested, and stained for CD11c, MHCIi, and CD317. Gates identifying pDCs, cDCs, and precursors are indicated in two-color histograms for CD11c and MHCIi expression on the left. Shown on the right are two-color histograms for CD11c and GFP expression for pDCs, cDCs, and precursor cells. All data in this figure are representative of three to five independent experiments using at least two mice per group.

CD172+CD24− and CD172−CD24+ populations (Fig. 3 B), indicating that Zbtb46 is expressed in all cDC subsets in peripheral lymphoid organs as well.
Figure 4. GM-CSF-derived cDCs express Zbtb46<sup>gfp</sup>. (A) BM cells from C57BL/6 mice were treated with 20 ng/ml GM-CSF or M-CSF, and cultures were harvested on day 7 and divided into adherent and nonadherent fractions. Nonadherent CD11c<sup>+</sup> cDCs from the GM-CSF–treated cultures were purified by positive selection, and microarray analysis was performed on cDCs (GM-CSF CD11c<sup>+</sup>) and both adherent populations (Adh) for the indicated cultures. Shown is the expression value of the Zbtb46 probe set. (B) BM monocytes were purified by negative selection using antibodies for Ly6G, B220, CD4, CD8a, DX5, CD11c, Ter119, and CD117, resulting in a fraction of cells that were >90% pure for expression of CD11b and Ly6C. Cells were cultured with 20 ng/ml GM-CSF for the indicated number of hours and harvested, and microarray analysis was performed. Shown is the expression value of the Zbtb46 probe set. Data in A and B are from one experimental replicate obtained from cells pooled from three mice. (C) Monocytes were purified from BM by cell sorting as CD11b<sup>-</sup>Ly6C<sup>-</sup>Ly6G<sup>-</sup>CD11c<sup>-</sup>MHCII<sup>-</sup>Siglec-H<sup>-</sup> cells and cultured in 20 ng/ml GM-CSF with or without 20 ng/ml IL-4 as indicated and analyzed after 4 d for expression of Zbtb46<sup>gfp</sup> and MHCII. Data are representative of one experiment in which each sample was performed in triplicate. Numbers represent the percentage of cells in the indicated gate.

Zbtb46<sup>gfp</sup> expression distinguishes cDCs from macrophages in peripheral tissues

A particular problem arises in using CD11c to distinguish cDCs from macrophages in peripheral nonlymphoid tissues such as lung (GeurtsvanKessel and Lambrecht, 2008), intestine (Manicassamy et al., 2010; Murphy, 2011), or kidney (Kriger et al., 2004). In the lung, cDCs and macrophages both express CD11c but differ in expression of CD103 and CD11b, with cDCs comprising CD103<sup>+</sup>CD11b<sup>+</sup>, CD103<sup>-</sup>CD11b<sup>+</sup>, and CD103<sup>-</sup>CD11b<sup>-</sup> populations and macrophages residing within a CD103<sup>-</sup>CD11b<sup>-</sup> autoimmune fluorescent gate (Edelson et al., 2010). Consistent with this, we found that Zbtb46<sup>gfp</sup> was uniformly expressed in CD103<sup>-</sup>CD11b<sup>-</sup> and CD103<sup>-</sup>CD11b<sup>+</sup> populations of cDCs and not expressed in CD11c<sup>+</sup>CD103<sup>-</sup>CD11b<sup>-</sup> macrophages (Fig. 5 A). Notably, the CD11c<sup>+</sup>CD103<sup>-</sup>CD11b<sup>+</sup> cells were heterogeneous for GFP expression, and although considered to represent cDCs (Edelson et al., 2010), our result suggests that this heterogeneous population likely includes GFP<sup>-</sup> macrophages that resemble cDCs in marker expression but not transcriptional profile. This agrees with the suggestion of a recent study that peripheral CD11c<sup>+</sup>CD103<sup>-</sup>CD11b<sup>+</sup> cells contain macrophages as they show partial dependence on M-CSF (Ginhoux et al., 2009). Furthermore, Zbtb46 expression determined by microarray was high in both the CD103<sup>-</sup>CD11b<sup>-</sup> and CD24<sup>+</sup> CD11b<sup>+</sup> cDC populations and low in lung macrophages (Fig. 5 B). The CD24<sup>+</sup>CD11b<sup>-</sup> lung cDCs expressed Zbtb46 at an intermediate level, consistent with the notion of heterogeneity. Accordingly, analysis of lung tissue sections by immunofluorescence microscopy showed that only a subset of CD11b<sup>+</sup> cells (Fig. 5 C) or CD11c<sup>+</sup> cells (not depicted) were positive for expression of Zbtb46<sup>gfp</sup>. These GFP-positive cells were largely found to surround large vessels and conducting airways.

Another peripheral tissue in which the distinction of cDCs and macrophages is problematic is the lamina propria of the intestinal tract (Bogunovic et al., 2009; Varol et al., 2009), where both macrophages and DCs can express CD11c (Manicassamy et al., 2010; Murphy, 2011). In the lamina propria, we found that the majority of CD11c<sup>+</sup> cells lacked expression of MHCII; these classically have been suggested to represent macrophages (Pabst and Bernhardt, 2010). In agreement, we found that none of these CD11c<sup>+</sup> MHCII<sup>-</sup> cells expressed Zbtb46<sup>gfp</sup> (Fig. 5 D). Within CD11c<sup>+</sup>MHCII<sup>+</sup> cells, Zbtb46<sup>gfp</sup> was uniformly expressed in the CD11b<sup>-</sup> (CD103<sup>+</sup>) subset (Fig. 5 D), previously shown to be derived from CDPs (Bogunovic et al., 2009; Varol et al., 2009). However, among the CD11b<sup>+</sup> subset of CD11c<sup>+</sup>MHCII<sup>+</sup> cells, only half expressed Zbtb46<sup>gfp</sup> (Fig. 5 D). Again, this is consistent with previous suggestions that some CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>-</sup> cells in the lamina propria arise from monocytes and represent MHCII<sup>+</sup> macrophages (Bogunovic et al., 2009; Varol et al., 2009). Consistently, Zbtb46 expression by microarray analysis was high in both of the CD103<sup>-</sup> cDC subsets but low in lamina propria macrophages (Fig. 5 E).

In the kidney, Zbtb46 expression can similarly distinguish between cDCs and macrophages as in lamina propria and lung (Fig. 5 F). CD11c<sup>+</sup> cells in the kidney have also been recognized to contain both cDCs and macrophages.
which are CD11c+MHCII+CD11b- (Ginhoux et al., 2009), were uniformly positive for GFP expression. Finally, the CD11c+MHCII+CD11b+ cells, which have been suggested to represent CD11b+ cDCs, were heterogeneous for GFP expression, suggesting that some of these cells may represent an MHCII+ macrophage population as well.

Figure 5. *Zbtb46* distinguishes cDCs from macrophages in peripheral tissues. (A) Cells were harvested from perfused lungs of *Zbtb46*+/+ mice and analyzed for expression of CD11c, MHCII, CD45, CD103, CD11b, GFP, and autofluorescence (centered on 700 nm). Shown (right) are two-color histograms for GFP expression and autofluorescence for the indicated populations. Numbers represent the percentage of cells in the indicated gate. Data are representative of three independent experiments with three mice per group. (B) Shown are the mean expression values of *Zbtb46* (±SEM) determined by microarray for the indicated cell populations derived from the ImmGen Database (Heng and Painter, 2008). Data are assembled from two to four replicate arrays. (C) Lung sections from *Zbtb46*+/+ BM chimeras were stained for CD11b and anti-GFP. Data are representative of five independent experiments. Bars, 200 µm. (D) Cells were harvested from small intestinal lamina propria of *Zbtb46*+/+ mice and analyzed for expression of CD11c, MHCII, CD45, CD103, CD11b, and GFP. Shown (right) are two-color histograms for CD11b and GFP expression for the indicated populations. Data are representative of three independent experiments with two mice each. (E) Shown are the mean expression values of *Zbtb46* (±SEM) in the indicated cells derived from Heng and Painter (2008). Data are assembled from four to seven replicate arrays. (F) Cells harvested from the kidney from *Zbtb46*+/+ mice were stained as in D. Data are representative of three independent experiments with two mice each.
**Zbtb46** expression indicates the identity of unclassified myeloid cell types

*Zbtb46* expression also clarifies the identity of several recently described myeloid cell types that have characteristics of both cDCs and other myeloid lineages. The recently described CX3CR1<sup>+</sup>CD8α<sup>+</sup> splenic population was initially thought to represent a pDC-related lineage based on its E2-2 expression and immunoglobulin gene rearrangements but also resembles cDCs by its high expression of CD11c and MHCII (Bar-On et al., 2010). LPS-induced CD206<sup>+</sup> DCs arise from monocytes during inflammation, and so may represent macrophages or DCs but were interpreted as DCs based on their **Zbtb46** expression indicates the identity of unclassified myeloid cell types

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that this subset of CD169⁺CD11c⁺⁺ cells represents cDCs. Finally, TNF/iNOS-producing DCs (Tip-DCs), which are of monocyte origin and are induced after infection with *Listeria monocytogenes* (Serbina et al., 2003), were negative for expression of *Zbtb46* **gfp** (Fig. 6 A). This finding may indicate that Tip-DCs are more highly related to a form of activated monocyte than to DCs, as recently suggested (Geissmann et al., 2010). Accordingly, these cells expressed Ly6C and CD317 similar to activated monocytes (Fig. 6 E). Of note, during *L. monocytogenes* infection or LPS-induced inflammation, *Zbtb46* **gfp** expression remained restricted to cDCs and loss of *Zbtb46* did not affect the induction or maturation of cDCs (not depicted).

**Zbtb46** **gfp** is expressed in definitive erythroid precursors and endothelial cells

Above we observed that a small fraction of *Zbtb46**gfp**-expressing cells were negative for CD11c and MHCI expression in lymphoid organs (Fig. 3 B). To determine the identity of these GFP-expressing cells, we first examined BM for indications of GFP expression in progenitor populations (Fig. 8 A). First, we noted that there were no changes in the frequency of progenitor populations between *Zbtb46**+/**+/**+/+** control and *Zbtb46**gfp/gfp** mice (Fig. 8 A). Within these identified progenitor populations, GFP was not detectable in mature erythrocytes (Fig. 8 B). However, unlike the persistent expression of GFP in mature DCs, GFP was not detectable in mature erythrocytes (Fig. 8 B).
Figure 8. Zbtb46 is expressed in definitive erythroid precursors and endothelial cells. (A) BM cells from Zbtb46+/+ (WT) or Zbtb46gfp/gfp [KO] mice were stained for expression of lineage markers B220, NKp461, and CD3 (Lin), Sca-1, CD11b/32, CD41, CD105, CD117, CD135, and CD150 and analyzed using a FACSia. Shown are two-color histograms for the indicated markers on cells previously gated for expression as indicated above the diagram. Numbers represent the percentage of cells within the indicated gate. The gates used to identify MkP, GMP, CMP, CFU-E, pre–CFU-E, pre-MegE, and pre-GM
Consistently, among these populations, Zbtb46 messenger RNA (mRNA) was only observed in committed erythroid progenitors at ~50% of the level of cDCs by microarray analysis (Fig. 8 C). To determine whether expression of Zbtb46 in erythroid progenitors impacted gene expression, we purified pre-MegE, pre-CFU-E, and CFU-E populations from Zbtb46+/+, Zbtb46+/−, and Zbtb46−/− mice and performed microarray analysis. Notably, there was very little change in gene expression caused by the loss of Zbtb46 in these progenitors (Fig. S1 A).

We next examined Zbtb46 expression in the spleen using immunofluorescence (Fig. 8 D, left). As expected, GFP was expressed in a subset of cells coexpressing CD11c, consistent with its expression by cDCs as determined by FACS. However, in addition, we observed that GFP was also expressed by cells associated with the vasculature of the splenic marginal zone and red pulp (Fig. 8 D), suggesting expression by endothelium. To test this, we reexamined splenic stromal cells by FACS for GFP expression using markers to identify endothelium and distinguish between these cells and DCs (Fig. 8 E). In spleen, the majority of GFP-expressing cells expressed CD11c, but 6% were negative for CD11c and were positive for CD31 expression. Of these cells, 24% expressed MADCAM-1 and Flk1, markers of endothelial cells associated with the marginal sinus (Zindl et al., 2009). Because endothelial cells in adult mice are not of hematopoietic origin, we generated chimeras by transferring Zbtb46+/+ BM into lethally irradiated WT mice. In these chimeras, Zbtb46 expression remained specific but was no longer found in CD11c−CD31+ endothelial cells either by FACS or by immunofluorescence (Fig. 8, D [right] and E). The use of radiation chimeras, either Zbtb46+/− or Zbtb46−/− into WT or WT into Zbtb46−/−, could be used to isolate the GFP expression for functional studies of either DCs or endothelium, respectively. In summary, outside of its selective expression in cDCs within immune lineages, Zbtb46 is also expressed transiently by erythroid progenitors and constitutively by endothelial cells.

**Zbtb46** identifies cDC-committed progenitors in BM and peripheral lymphoid organs

The lack of truly cDC-specific markers has confounded the purification of committed cDC progenitors away from cells capable of pDC development. The most successful purifications of a committed pre-cDC relied on CD11c and CD135, but capable of pDC development. The most successful purifications of committed cDC progenitors away from cells outside of its selective expression in cDCs within immune lineages, Zbtb46 is also expressed transiently by erythroid progenitors and constitutively by endothelial cells.

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**Zbtb46** identifies cDC-committed progenitors in BM and peripheral lymphoid organs

The lack of truly cDC-specific markers has confounded the purification of committed cDC progenitors away from cells capable of pDC development. The most successful purifications of pre-cDCs relied on CD11c and CD135, but capable of pDC development. The most successful purifications of committed cDC progenitors away from cells outside of its selective expression in cDCs within immune lineages, Zbtb46 is also expressed transiently by erythroid progenitors and constitutively by endothelial cells.
Figure 9. Zbtb46 expression identifies committed pre-cDCs in BM and peripheral lymphoid tissues. (A) BM from Zbtb46+/+ (WT) and Zbtb46<sup>gfp/gfp</sup> (KO) mice was stained for expression of Siglec-H, CD317, B220, CD11c, and MHCII. Shown are two-color histograms for Siglec-H and GFP expression for cells previously gated for positive expression of B220, CD317, and Siglec-H (pDC) or for positive expression of CD11c and MHCII (cDC) as indicated above the diagram. Numbers represent the percentage of cells within the indicated gate. Data are representative of three independent experiments with three mice each. (B) BM cells from Zbtb46+/+ (WT) and Zbtb46<sup>gfp/gfp</sup> (KO) mice were stained for expression of B220 and NKp46 (Lin), Sca-1, CD105, MHCII, CD117, CD135, CD11c, and Siglec-H. Shown are two-color histograms for expression of the indicated markers for cells previously gated as indicated above the diagram. Data are representative of five independent experiments with two mice each. (C) Splenocytes, SLNs, and MLNs from Zbtb46+/+ (WT) and Zbtb46<sup>gfp/gfp</sup> (KO) mice were analyzed for expression of CD11c, MHCII, B220, Siglec-H, and GFP. Shown are histograms depicting Siglec-H and GFP expression for cells previously gated as indicated above the diagram. Data are representative of two independent experiments with one or two mice each. (D) BM cells from Zbtb46<sup>gfp/gfp</sup> mice were sorted into populations of Lin−Sca-1−CD105−CD117−CD135−MHCII− (CDP, left), Lin−Sca-1−CD105−MHCII−CD117−CD135−Siglec-H−GFP− (Siglec-H−GFP−; middle), or Lin−Sca-1−CD105−MHCII−CD117−CD135−Siglec-H+ and GFP+ (Siglec-H+GFP+; right) as indicated above the diagram. Cells were cultured in 100 ng/ml Flt3L for 4 d and analyzed by FACS for expression of Siglec-H, GFP, and MHCII. Data are representative of three independent experiments with two to three mice each.
whether the divergence of pDCs and cDCs takes place at the Siglec-H$^+$/Zbtb46$^-$ stage of CDP development.

**Zbtb46 represses alternate myeloid potential in BM progenitor cells**

Because Zbtb46 expression first became apparent at the precDC stage, we wondered whether it might act in regulating lineage restriction of cDCs. We expressed Zbtb46 by retrovirus in BM cells and cultured them with growth factors as described in Materials and methods. On day 5, cells were harvested and analyzed for expression of Gr1, MHCII, and GFP. Shown are two-color histograms for the retroviral constructs indicated above the diagram for the indicated markers for cells gated on positive expression of GFP (GFP$^+$). Numbers represent the percentage of cells in the indicated gates. Data are representative of three independent experiments using at least two mice per group.

(B) Replicates from two experiments (each experiment is uniquely colored) described in A are shown. Numbers represent the percentage of cells on day 5 that are present within the gates for cDCs or granulocytes (Gr1$^+$) as indicated below the bars. Bars represent the mean ± SEM. (C) BM from WT or BXH2 recombinant inbred mice (Tailor et al., 2008) was infected with GFP-RV, a retrovirus encoding full-length mouse Irf8 (Irf8-RV) or Zbtb46-RV as described in A and analyzed on day 5 for expression of CD11c and MHCII. Similar diminishment of cDC development was seen with Irf8$^-/-$ BM, which was similarly restored by Zbtb46-RV (not depicted). Data are representative of two independent experiments with two mice per group.

(D) GFP-RV vector (Empty-RV) or retroviruses expressing full-length Zbtb46 (Zbtb46-RV) or a truncated Zbtb46 in which the region of cDNA encoding the C-terminal zinc finger domains (amino acids 404–601) were removed (dZF-Zbtb46-RV) and were used to infect BM progenitors as described in A, and cells were transferred into sublethally irradiated CD45.1 recipients. After 14 d, splenocytes were harvested from recipient mice, and CD45.2$^+$GFP$^+$ cells were analyzed for expression of CD11c, B220, and MHCII. Shown are the percentage of cells within the B220$^-$CD11c$^+$ gate for cells previously gated as CD45.2$^+$GFP$^+$ for the indicated retroviral construct. Data are representative of three independent experiments with one mouse per group.

(E) Sorted steady-state splenic CD172$^+$CD4$^+$ cDCs from Zbtb46$^+$+/+ (WT) and Zbtb46$^+$gfp/gfp (KO) mice were purified, and gene expression microarrays were performed. Genes that are increased in the WT sample relative to the Zbtb46$^+$gfp/gfp (KO) sample (left) are listed with the associated fold induction. Genes that are increased in Zbtb46$^+$gfp/gfp (KO) mice relative to WT are listed with their fold induction (right).

(F) Fold changes of the indicated genes derived from E are shown for their expression in WT compared with Zbtb46$^+$gfp/gfp (KO) mice.
when Zbtb46 was expressed in BM progenitors from Irf8 mutant mice (either Irf8-deficient [not depicted] or BXH2 recombinant inbred mice, harboring a point mutation in the Irf8 gene [Fig. 10 C; Tailor et al., 2008]), which normally do not support the development of cDCs. The effects of Zbtb46 are specific because deletion of its zinc finger domains abrogated the enhancement of cDC development (Fig. 10 D).

Because Zbtb46 belongs to a family of transcriptional repressors, some of which are known to participate in lineage fate decisions within the immune system (Wildt et al., 2007), we analyzed gene expression microarrays from WT or Zbtb46^gfp/− CD4^+ splenic cDCs to identify genes that may be induced or repressed by the loss of Zbtb46 (Fig. 10 E and Fig. S1 B). In these steady-state DCs, it appeared that Zbtb46 regulated relatively few target genes. Notably, among the few genes altered were the receptors for G-CSF (Csf3R) and leukemia inhibitory factor (Fig. 10 F), which were increased in their relative expression in Zbtb46-deficient CD4^+ cDCs. These results suggest that Zbtb46 may enforce cDC lineage restriction by extinguishing the expression of alternate myeloid growth factor receptors.

DISCUSSION

The precise assignment of cellular identity can be hampered by the fact that many lineages share expression of common surface markers. This is particularly true for cDCs, which have overlapping expression of various markers in common with pDCs, monocytes, and macrophages. The hypothesis that transcription factors define distinct lineages prompted our efforts to generate a Zbtb46-knockin reporter to help distinguish between these closely related myeloid subsets. We find that within the immune system, Zbtb46 expression specifically identifies the cDC lineage, both in lymphoid and nonlymphoid tissues. Because Zbtb46 is also expressed in endothelial cells and transiently during erythroid differentiation, studies such as intravital microscopy or lineage ablation using the Zbtb46 locus may benefit from the use of BM chimeras to eliminate effects of expression by non-DCs. In examining Zbtb46-deficient mice, it seems that cDCs, vasculature, and erythrocytes each develop without major abnormalities, suggesting that potential defects will reside in induced responses or that there is compensation by an unidentified factor.

Our data indicate that Zbtb46 expression at the stage of the pre-DC is a marker for cells committed to the cDC lineage that have lost potential for pDCs and other myeloid cells. This is a useful distinction because it has not been precisely clear at which point potential for pDC development is extinguished (Liu and Nussenzweig, 2010; Belz and Nutt, 2012). By combining Zbtb46^gfp expression with analysis of other markers, we have determined that although Siglec-H is specific for pDCs in peripheral organs, BM progenitors expressing Siglec-H actually retain potential for cDC development. This result might explain discrepancies between two recent studies that attempted to deplete pDCs based either on Siglec-H−DTR (Takagi et al., 2011) or BDCA2-DTR (Swiecki et al., 2010) because the former would likely cause the loss of some cDC populations not depleted by the latter.

Besides cDCs in lymphoid and peripheral tissues, several unconventional myeloid subsets have been described whose precise origin and identity have been unclear. In these cell types, examination of Zbtb46 expression seems to aid in their classification into either cDC or macrophage lineages. For example, Tip-DCs were originally described as a type of induced inflammatory DC lineage (Serbina et al., 2003) but recently were suggested to more closely resemble human M1 macrophages (Geissmann et al., 2010). The lack of Zbtb46 expression in Tip-DCs may support this latter interpretation. In addition, subcapsular macrophages expressing CD169 were considered to represent an F4/80-negative macrophage lineage (Junt et al., 2007; Phan et al., 2009). Our results suggest that this population is heterogeneous with respect to Zbtb46 expression and could indicate that these cells may be more closely related to cDCs than previously thought.

Although current results indicate that Zbtb46 is dispensable for development of the recognized types of cDCs, our analysis has not yet examined the functional properties of these cells in settings of immune challenge. We may conclude that specification and commitment in vivo appears normal, but we have uncovered some evidence for subtle changes in gene expression in CD4^+ cDCs. These findings will require further studies to clarify the functional consequences of such changes. Importantly, heterozygous Zbtb46^gfp/− mice or BM chimeras should benefit studies in which the identification of cDCs or committed pre-cDCs from monocytes and macrophages is necessary.

MATERIALS AND METHODS

Mice. Zbtb46^gfp mice were maintained on the pure 129SveEv background by mating germline progeny from the EDJ22 ESC line with mice purchased from Taconic. Experiments were performed with sex-matched littermate mice at 6–10 wk of age. Irf8^−/− mice were obtained from the European Mutant Mouse Archive and maintained on the C57BL/6 background. BxH2/Tij mice were purchased from the Jackson Laboratory and maintained on the C57BL/6 background. Mice were bred and maintained in our specific pathogen-free animal facility according to institutional guidelines and with protocols approved by the Animal Studies Committee of Washington University in St. Louis.

Generation of the Zbtb46^gfp targeting construct. The Zbtb46^gfp targeting construct was assembled with the Gateway recombination cloning system (Invitrogen). To construct pENTR-lox-GFP.Neo, a 1,017-bp PCR product containing cGFP followed by the SV40-pA terminator was amplified from gDNA of CX3CR1^gfp mice (Hung et al., 2000). Using primers pENTR-lox-GFP-N, 5′-AAATGTCGACCGTCCACCATGTT-3′, and pENTR-lox-GFP-N1, 5′-ATCTCGAGATTAAGCCGATTACATTTACGCTTAAAGAC-3′, and cloned into pGEM-T Easy (Promega). The resulting plasmid was digested using SalI and XhoI, and the 1,004-bp eGFP-pA cassette was cloned into the Sall site of the PGK-neo (phosphoglycerate kinase promoter-neomycin phosphotransferase) gene cassette of the pLNTK targeting vector (Gorman et al., 1996) to generate pLNTKGFP (8,313 bp). Finally, the 2,825-bp eGFP-PGK–neor cassette from pLNTK-EGFP was released using SalI and XhoI and ligated bluntly into the 2,554-bp backbone of NotI-digested pENTR-lox-Puro (Iiizumi et al., 2006). To construct pENTR-Zbtb46-SHA, the 5′ homology arm was amplified by PCR from 129SveEv-EDJ22 ESC genomic DNA using the following oligonucleotides,
Staining was performed at 4°C in the presence of Fc Block (clone 2.4G2; BD) in FACS buffer (DPBS + 0.5% BSA and 2 mM EDTA). The following antibodies were purchased from BD: APC anti-F4/80 (P84). These were purchased from eBioscience: PE anti-NKp46 (29A1.4), PE-Cy7 anti-F4/80 (M1/70), APC eFluor780 anti-CD11c (N418), PE anti-CD103 (2E7), APC anti-CD31/B220 (eBio297), eFluor450 anti-MHCII (I-A/I-E; M5/114.15.2), APC anti-CD45-R (104), PerCP-Cy5.5 anti-CD16/32 (93), PE anti-CD41 (eBioMrVre30), APC anti-FKl1 (Avs12a1), APC anti-CD150 (mShad150), APC-eFluor780 anti-CD11c (B2B), V500 anti-B202 (RA3-6B2), eFluor450 anti-CD105 (M7/18), PE anti-CD135 (A2F10), biotin anti-MADCAM-1 (MECA-367), APC anti-F4/80 (BM8), Alexa Fluor 700 anti-Sca-1 (D7), PerCP-eFluor710 anti-siglec-H (eBio44C), eFluor450 anti-CD31 (390), eFluor450 anti-Ter119 (Ter119), and PerCPCy5.5 anti-CD14 (S2A-8). These reagents were purchased from Miltenyi Biotec; PE anti-CD205/DEC205 (NLDC-145) and APC anti-CD205/DEC205 (NLDC-145). APC anti-CD206 (MRSD3) was purchased from BioLegend. In general, all antibodies were used at a 1:200 dilution. Anti-DEC205 was used at a 1:20 dilution. Cells were analyzed on FACSCan II or FACSria II flow cytometers (BD), and data were analyzed with FlowJo software (Tree Star). For immunofluorescence experiments, the following antibodies were purchased from Invitrogen: rabbit polyclonal anti-GFP, Alexa Fluor 488 anti-rabbit IgG (A11034), streptavidin Alexa Fluor 555, and biotin anti-F4/80 (BM8); from BioLegend: Alexa Fluor 647 anti-CD11c (N418); from AbD Serotec: APC anti-CD169 (D6T12); and from ebioscience: biotin anti-B220 (RA3-6B2).

Progenitor isolation and cell culture. BM was harvested from femur, tibia, humerus, and pelvis. Bones were fragmented by mortar and pestle, and debris was removed by gradient centrifugation using Histopaque 1119 (Sigma-Aldrich). Cells were passed through a 70-µm strainer, and red blood cells were lysed with ACK lysis buffer. Cells were counted on a Vi-CELL analyzer, and 5 × 10^6 to 10^7 were used for analysis. Gates were defined using FSC (sidewalls) and FSC-A versus FSC-H. For monocyte cultures, BM monocytes were identified as Ly6C lo and IL-7R α and IL-7R α (Lin−CD45hiCD11c−MHCII−Siglec-H−). Cells were sorted into PBS supplemented with 0.5% BSA and 2 mM EDTA. Cell purities of at least 95% were confirmed by postsort analysis. For Flt3L culture experiments, whole BM (2 × 10^7 cells/ml in 4 ml cIMDM for 6 or 9 d) or sorted cells (1 × 10^7 cells/ml in 200 µl cIMDM for 4 d) were cultured at 37°C in 100 ng/ml Flt3L (PeproTech). For GM-CSF and M-CSF culture experiments, whole BM (10^7 cells/ml in 4 ml cIMDM) was cultured at 37°C in 20 ng/ml GM-CSF or M-CSF (PeproTech). For monocyte cultures, BM monocytes were identified by Ly6C°C hi, D11bLy6G−CD11c−MHCII−Siglec-H−, and 5 × 10^6 cells were sorted and cultured in 20 ng/ml GM-CSF with or without 20 ng/ml IL-4 for 4 d (PeproTech).

Expression microarray analysis. Total RNA was isolated from cells using the RNAqueous-Micro kit (Invitrogen). For Mouse Genome 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybriddized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, gene expression was modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, gene expression was modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, gene expression was modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, gene expression was modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, gene expression was modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, gene expression was modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, gene expression was modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a). For Mouse Gene 430 2.0 arrays, RNA was amplified, labeled, fragmented, and hybridized using the 3′IVT Express kit (Affymetrix). Data were normalized within experiments, and expression values were modeled using DNA-Chip analyzer (dChip) (http://www.dChip.org; Li and Wong, 2001a).
**Immunofluorescence microscopy.** Organs were fixed in 2% paraformaldehyde for 6 h followed by incubation in 30% sucrose/H2O overnight, embedded in OCT compound, and 7-µm cryosections were produced. After two washes in DPBS, sections were blocked in CAS Block (Invitrogen) containing 0.2% Triton X-100 for 10 min at room temperature. Sections were stained with primary and secondary reagents diluted in CAS Block containing 0.2% Triton X-100 and mounted with ProLong Gold Antifade reagent containing DAPI (Invitrogen). Four-color epifluorescence microscopy was performed using an AxioCam MRc microscope equipped with an AX10 camera (Carl Zeiss). Monochrome images were acquired with AxioVision software (Carl Zeiss) using either 10× or 40× objectives and exported into ImageJ software (National Institutes of Health) for subsequent color balancing and overlaying. Confocal microscopy was performed using an LSM 510 META confocal laser-scanning microscope and analyzed with LSM Image Examiner software (Carl Zeiss). Images were taken using a water immersion C-Apochromat 40× objective, and z-stack images were acquired at 1-µm intervals.

**BM chimeras.** BM cells from Zbtb46+/− were collected as described in Progenitor isolation and culture, and 2 × 10^6 total BM cells were transplanted into subrenal i.v. injection into WT 129SvEv recipients that had received 1,200 rads of whole body irradiation. Chimeras were analyzed at 6–8 wk after transplant. Sublethal irradiation was accomplished with 600 rads of whole body irradiation.

**LPS and L. monocytogenes.** Mice were infected with 10^4 live L. monocytogenes (WT strain EGD). L. monocytogenes was stored as glycerol stocks at −80°C and diluted into pyrogen-free saline for i.v. injection into mice, and analysis was performed 2 d later. 5 μg LPS E. coli 055:B5 (Sigma-Aldrich) diluted in 200 μl pyrogen-free saline was injected i.v. to induce CD206+ DCs, and analysis was performed 18 h later in SLNs. Intracellular staining for CD206 was performed after cells were fixed in 4% paraformaldehyde in DPBS and permeabilized in 0.1% saponin/DPBS.

**Retrovirus infection.** The Zbtb46 full-length cDNA was cloned from splenic cDNA using RT-PCR with the following primers: Zbtb46isoform1 forward, 5′-ATTACCGAGCTAGGAGATCCAGGCAAAGTCT-3′, and Zbtb46isoform1 reverse, 5′-AAAAGGTACCCTAGCATCGATCATCATGAACACCGAGAAGAATA-3′. The resulting 1.8-kB PCR fragment was digested with BamHI and XhoI and cloned into the BglII and XhoI sites of the GFP-RV (Ranganath et al., 1998). Retroviral vectors were transfected into Phoenix E cells as described previously (Sedy et al., 2005), and viral supernatants were collected 2 d later. BM cells were infected with viral supernatants in the presence of 2 μg/ml polybrene by spin infection at 2,500 rpm for 30 min. Cells were cultured in the presence of 10 ng/ml of each of the factors: Flt3L, stem cell factor (SCF), GM-CSF, and EPO (PeproTech). Cells were harvested on day 5 and analyzed by FACs.

**Statistical analysis.** Differences between groups were analyzed by an unpaired, two-tailed Student’s t test, with p-values ≤0.05 considered significant ( Prism; GraphPad Software).

**Online supplemental material.** Fig. S1 shows gene expression analysis of erythroid precursors and CD4+ DCs from WT and Zbtb46 KO mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20120030/DC1.

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