Zinc finger transcription factor zDC is a negative regulator required to prevent activation of classical dendritic cells in the steady state

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Classical dendritic cells (cDCs) process and present antigens to T cells. Under steady-state conditions, antigen presentation by cDCs induces tolerance. In contrast, during infection or inflammation, cDCs become activated, express higher levels of cell surface MHC molecules, and induce strong adaptive immune responses. We recently identified a cDC-restricted zinc finger transcription factor, zDC (also known as Zbtb46 or Btbd4), that is not expressed by other immune cell populations, including plasmacytoid DCs, monocytes, or macrophages. We define the zDC consensus DNA binding motif and the genes regulated by zDC using chromatin immunoprecipitation and deep sequencing. By deleting zDC from the mouse genome, we show that zDC is primarily a negative regulator of cDC gene expression. zDC deficiency alters the cDC subset composition in the spleen in favor of CD8+ DCs, up-regulates activation pathways in steady-state cDCs, including elevated MHC II expression, and enhances cDC production of vascular endothelial growth factor leading to increased vascularization of skin-draining lymph nodes. Consistent with these observations, zDC protein expression is rapidly down-regulated after TLR stimulation. Thus, zDC is a TLR-responsive, cDC-specific transcriptional repressor that is in part responsible for preventing cDC maturation in the steady state.
can present antigen to MHC I– or MHC II–restricted T cells, CD4+/CD11b+ cDCs are better suited for antigen presentation on MHC II to induce CD4+ T cell responses, and CD8+/CD103+ cDCs are specialized for cross-presentation on MHC I for CD8+ T cell responses (Dudziak et al., 2007; Kamphorst et al., 2010; Hashimoto et al., 2011).

zDC is an evolutionarily conserved broad complex, trans- track, bric-a-brac and zinc finger protein (BTB-ZF) transcription factor which is specifically expressed by cDCs in humans and mice (Meredith et al., 2012; Satpathy et al., 2012). To understand the role of zDC in cDC development and homeostasis, we identified zDC's target genes by ChIP-seq and produced zDC-deficient mice. We show that zDC associates with >1,000 gene promoters and acts as a transcriptional repressor of many of these target genes. Furthermore, cDC gene expression is profoundly altered in the absence of zDC, resulting in aberrant partial induction of activation and maturation pathways in the steady state.

RESULTS

zDC target genes
zDC is a member of the BTB-ZF transcription factor family and is therefore likely to function as a transcription factor. To identify genes regulated by zDC, we performed chromatin immunoprecipitation sequencing (ChIP-seq) on steady-state splenic cDCs. Genome-wide, zDC was enriched in a region 100 base pairs upstream of transcription start sites (TSSs), which would optimally position it to interact with transcriptional machinery (Fig. 1 A). For example, zDC binds upstream of the first exon of all four classical MHC II genes expressed by C57BL/6 mice (Fig. 1 B). Furthermore, zDC can also associate with elements downstream of the final exon (Fig. 1 B), suggesting that it may bind to enhancers and other regulatory elements outside of promoters.

zDC contains two C2H2 zinc finger domains, each of which should bind a unique trinucleotide sequence (Bulyk et al., 2001), and therefore zDC would be predicted to recognize a six-nucleotide sequence motif. To identify zDC's DNA binding motif, we used MEME software to identify a consensus motif in the zDC ChIP-seq library (Bailey et al., 2009). MEME software identified a 10-nucleotide sequence featuring a prominent 6-nucleotide TGACGT core motif (Fig. 1 C). To test the binding of zDC to this predicted sequence, we performed gel shift assays between recombinant zDC and double-stranded DNA probes containing the predicted binding motif or control probe with a scrambled version of the motif. We found that zDC bound to the probe containing the predicted 6-nucleotide motif but not the scrambled control (Fig. 1 D). Consistent with this finding, our ChIP-seq library identified 1,309 genes occupied by zDC (Fig. 1 E), >99% of which contained the TGACGT consensus motif within 1 kilobase of the TSS (P < 0.01). zDC binding sites did not consistently colocalize within 1 kilobase of other specific cis-regulatory elements defined in the Open Regulatory Annotation (ORegAnno) database.

zDC-targeted genes represent a highly diverse group of genes, the largest number of which is implicated in regulation of gene expression, including transcription factors, RNA processing factors, and chromatin regulators (Fig. 1 E and Table S1). A second large group of zDC-associated genes regulate protein metabolism, including kinases, peptidases, ubiquitin ligases, and phosphatases. Although zDC target genes are spread throughout the genome, they are most abundant on chromosome 17 in the region of the mouse MHC locus (Fig. 1 F). In fact, zDC is associated with all the MHC II genes expressed in C57BL/6 mice (Fig. 1 B). We conclude that zDC binds a variety of genes in cDCs, including MHC II antigens.

Members of the BTB-ZF family are typically transcriptional repressors but have also been shown to function as transcriptional activators (Collins et al., 2001; Kelly and Daniel 2006; Beaulieu and Sant’Angelo 2011). To begin to define the role of zDC in regulating gene expression in vivo, we compared the expression of zDC target gene mRNAs in fully differentiated cDCs and monocytes by gene array. Monocytes were selected for comparison because they are closely related to cDCs but lack zDC expression (Meredith et al., 2012; Satpathy et al., 2012). Consistent with the ability of BTB-ZF transcription factors to function as suppressors or activators, we found a broad range of differential gene expression of zDC target genes in cDC and monocytes (Fig. 1 G). 564 zDC-associated gene probes were expressed at two- to fourfold higher levels in monocytes compared with cDCs (57% of ChIP target gene probes differentially expressed between cDCs and monocytes; Fig. 1 G). This pattern suggests that zDC suppresses the expression of these genes in differentiated cDCs. Conversely, a smaller group of genes (381 probes; 40% of differentially expressed ChIP gene probes) was up-regulated in cDCs compared with monocytes, representing targets that may be activated by zDC binding. Therefore, like other BTB-ZF family members, zDC represses the expression of many of its target genes but can also activate gene expression.

zDC is not required for cDC development
To examine the function of zDC in vivo, we produced a conditional knockout of zDC by flanking its second exon, which encodes the translational start site and the BTB protein-binding domain, with loxP sites (Fig. 2 A, zDClox/lox mice). These mice were generated and maintained on the C57BL/6 background. zDClox/lox mice were crossed with EIIA-Cre mice to produce mice that carry a zDC null mutation (zDC−/−). Despite the absence of zDC protein in mutant mice (Fig. 2 B), early cDC development was unaffected. We found normal numbers of macrophage and DC progenitors, common DC progenitors, and pre-cDCs in the bone marrow (Fig. 2 C). Furthermore, the total number of cDCs in the spleen and skin-draining LNs were normal in zDC−/− mice compared with littermate controls (Fig. 2 D and E). However, the number of CD11c+MHCII+ migratory DCs (mDCs), which arrive from
Figure 1.  zDC target genes. (A) Genome-wide location of zDC-associated sequences relative to the closest gene’s TSS. The number of genes at various distances from the TSS is shown on the y axis. (B) zDC ChIP reads relative to MHC II genes. Histograms show zDC density, and boxes below each histogram show the exon structure of the MHC genes at that position. A 1-kb scale bar is included for each locus. (C) Consensus motif for zDC ChIP sequences. (D) Gel shift assay with predicted zDC motif and mutant probe with scrambled motif. Two concentrations of zDC protein are included, as well as glutathione S-transferase (GST) control protein. The gel is representative of three independent experiments with comparable results. (E) Pie chart shows gene families represented among zDC ChIP target genes. Gene ontologies for target genes were determined by PANTHER Classification (pantherdb.org). Numbers in parentheses indicate the number of genes included in the indicated group. (F) Genome-wide distribution of zDC target genes. The mouse genome was divided into 10-Mbp segments and the number of zDC target genes within each segment is shown. Alternating red and black portions and matching numbers indicate chromosome number. (G) Fold difference expression of zDC target gene probes by cDCs relative to monocytes. Red bars indicate probes expressed at higher levels in cDCs, and blue for those in monocytes. Probes expressed equally by cDCs and monocytes (i.e., less than twofold difference) are not shown.
mice showed approximately equal numbers of CD4+ and CD8+ cDCs (Fig. 2 F). zDC−/− mice likewise demonstrated CD4+ cDC reduction and CD8+ cDC expansion, and subset composition in zDC−/+ and zDC−/− mice did not significantly differ (P = 0.830 and 0.136 for CD4+ and CD8+ cDC numbers, respectively). CD4−CD8− double-negative cDCs were not significantly affected by zDC deficiency.

We conclude that zDC is not required for early cDC development, but zDC deficiency alters the proportions of CD4+ and CD8+ cDCs in the spleen and results in a small increase of mDC numbers in the skin-draining LNs.

**zDC regulation of gene expression**

To determine the effects of zDC deficiency on cDC gene expression genome-wide, we compared gene array expression

the skin via CCR7-dependent migration after maturation (Alvarez et al., 2008), were slightly increased in the skin-draining LNs of zDC+/− and zDC−/− mice (Fig. 2 E). Furthermore, although zDC−/− mice contained the same number of total cDCs as wild-type littermates, zDC−/− mice contained fewer splenic CD4+ cDCs and reciprocally greater numbers of CD8+ cDCs (Fig. 2 F). Whereas CD4+ cDCs were threefold more abundant than CD8+ cDCs in wild-type littermate controls, zDC-deficient

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**zDC regulation of gene expression**

To determine the effects of zDC deficiency on cDC gene expression genome-wide, we compared gene array expression
data from wild-type and zDC-deficient splenic CD8+ and CD4+ cDCs. Overall, 2,653 genes were up-regulated, whereas 1,195 genes were down-regulated in CD4+ zDC−/− cDCs (Fig. 3 A). Similarly, 2,333 genes were up-regulated and 1,078 genes down-regulated in CD8+ zDC−/− cDCs. These effects on gene expression in zDC−/− cDCs were highly correlated between CD4+ and CD8+ cDCs (Fig. 3 B; \( m = 0.78 \pm 0.0023; r^2 = 0.73 \)). To validate our microarray dataset, we compared the expression of a subset of genes in wild-type and zDC-deficient cDCs by quantitative PCR (Q-PCR; Fig. 3 C). Therefore, zDC deficiency profoundly alters the transcriptional profiles of CD4+ and CD8+ cDCs, and these changes are shared in both subsets of cDCs.

To better appreciate the function of zDC at its target genes, we compared the expression of zDC-bound genes identified by ChIP-seq with gene set enrichment analysis (GSEA; Subramanian et al., 2005). Overall, zDC target genes were up-regulated in zDC-deficient cDCs (Fig. 3 D; \( P < 0.001 \)). Furthermore, when we compared the expression of zDC target genes up-regulated in wild-type cDCs or wild-type monocytes (defined in Fig. 1 G) to those that differed between wild-type and zDC-deficient cDCs, monocyte-up-regulated target genes were uniformly up-regulated by zDC-deficient cDCs (Fig. 3 E). Conversely, cDC-up-regulated target genes were more randomly deregulated (Fig. 3 E). This up-regulation of zDC-bound genes in the knockout, particularly among those genes typically expressed higher by monocytes, is consistent with the idea that zDC acts as a transcriptional repressor at many of its targets, similar to other members of the BTB-ZF family (Collins et al., 2001; Kelly and Daniel 2006; Beaulieu and Sant’Angelo 2011).

**zDC deficiency results in partial activation in the steady state**

cDCs express numerous pathogen recognition molecules and signaling receptors which allow them to sense pathogen- and self-derived activation signals. Signaling through these receptors induces cDC maturation, including the up-regulation of MHC II and co-stimulatory markers, which enables cDCs to stimulate adaptive immune responses (Steinman 2007). GSEA software showed that multiple activation and inflammatory pathways are up-regulated in zDC-deficient cDCs, including gene sets up-regulated in response to LPS (Foster et al., 2007) and by treatment with TNF/IL-1β (Lindstedt et al., 2002; Fig. 4 A; \( P < 0.005 \)). Furthermore, in addition to the transcriptional up-regulation of these gene sets, zDC−/− cDCs expressed up to twofold higher MHC II levels by flow cytometry compared with wild-type littermates (Fig. 4 B). Therefore, steady-state zDC-deficient cDCs up-regulate the expression of gene pathways that are normally only expressed after activation.

Because zDC-deficient steady-state cDCs appear to phenocopy some aspects of cDC activation, we asked whether zDC expression is normally modulated during cDC activation by measuring zDC protein after TLR stimulation. As early as 3 h after poly I:C injection, zDC protein could not be detected in splenic cDCs by Western blotting (Fig. 4 C). In addition to poly I:C, other TLR agonists including LPS and CpG also rapidly down-regulated zDC protein (unpublished data). This loss of zDC protein expression is not the result of a loss of zDC transcription because zDC transcripts are still expressed by splenic cDCs 3 h after poly I:C injection (Fig. 4 D) as well as by lung cDCs in poly I:C–stimulated mice and gut cDCs in *Salmonella*–infected mice (immgen.org). We conclude that zDC protein is down-regulated after TLR stimulation, most likely via posttranslational regulation. The down-regulation of zDC protein after TLR stimulation is consistent with the up-regulation of MHC II expression during cDC maturation (Cella et al., 1997; Landmann et al., 2001).
The choice between the induction of tolerance or adaptive immune responses is in part determined by the activation state of cDCs (Steinman and Nussenzweig 2002). To examine the consequences of zDC deficiency during antigen presentation to T cells, we tested zDC−/− cDCs for the ability to induce OVA-specific OT-I T cell tolerance (Hawiger et al., 2001; Dudziak et al., 2007). Specifically, wild-type and zDC−/− mice received 1–2 × 10⁶ CFSE-labeled OT-I T cells and were treated with 3 µg DEC-205 antibody expressed as a fusion protein with OVA antigen (DEC-OVA). 2 wk after DEC-OVA targeting, OT-I cells were examined by flow cytometry. Whereas wild-type littermates deleted the transferred OT-I T cells, zDC-deficient mice retained CFSEloCD62lo OT-I T cells that had divided and developed phenotypic

Figure 4. zDC-deficient cDC gene expression. (A) GSEA plots for inflammatory response after LPS exposure (Foster et al., 2007) and DC maturation (Lindstedt et al., 2002) gene sets significantly up-regulated in zDC−/− compared with wild-type cDCs (P < 0.001). (B) Histograms depicting surface MHC II expression by splenic CD11c+CD8α+ and CD11c+CD4+ cDCs. Results are representative of four experiments. (C) Western blot for zDC expression by CD11c-enriched splenic cDCs from naive wild-type mice, wild-type mice 3 h after i.v. injection with 30 µg poly I:C, and naive zDC−/− mice. The histone H4 blot is included as a loading control. (D) zDC transcript expression by splenic CD8α+ and CD8α− cDCs from naive wild-type mice and wild-type mice 3 h after i.v. injection with 30 µg poly I:C. zDC levels normalized to Gapdh. Each point represents a single mouse from three independent experiments. (E) Surviving CD8+CD45.1+Vα2+ OT-I T cells measured by flow cytometry in the spleens of wild-type and zDC−/− mice 2 wk after treatment with 3 µg DEC-OVA. The graph on the right shows results from three experiments with each point representing a single mouse. Horizontal bars show means. (F) Histogram showing CFSE labeling on CD8+CD45.1+Vα2+ OT-I T cells described in E. The gray shaded curve represents cells collected from PBS controls, the blue line from DEC-OVA–treated wild-type mice, and the red line from DEC-OVA–treated zDC−/− mice. (G) CD62L expression on surviving OT-I cells described in E. The graph on right shows results pooled from three experiments with each point representing a single mouse.
features of effector cells (Fig. 4, E–G). Conversely, naïve OT-I T cells from wild-type mice that did not receive DEC-OVA treatment, as well as the few remaining OT-I T cells from DEC-OVA–treated wild-type mice, maintained a naïve phenotype indicated by high expression of CD62L (Fig. 4 G). Therefore, OVA presentation to OT-I cells by steady-state zDC−/− cDCs does not result in complete deletion of OT-I T cells.

**Increased lymphangiogenesis in zDC−/− mice**

zDC-deficient mice have enlarged skin-draining LNs with prominent blood vessels, which bear proportional expansion of B and T lymphocytes and a small relative increase of mDCs (Fig. 2 E; and Fig. 5, A–D). The degree of LN enlargement in zDC-deficient mice is similar to the amount of cDC-dependent LN hypertrophy that has been reported in response to TLR stimulation (Herman et al., 1972; Webster et al., 2006). TLR-stimulated cDCs mediate LN enlargement and angiogenesis by producing vascular endothelial growth factor (VEGF; Webster et al., 2006; Tzeng et al., 2010; Wendland et al., 2011). To determine if enhanced VEGF production by zDC-deficient cDCs contributes to LN expansion, we measured its expression in zDC−/− cDCs. Gene array analysis suggested Vegfc is up-regulated in zDC−/− cDCs (Fig. 5 E), and this up-regulation was confirmed by Q-PCR (Fig. 5 F). Consistent with elevated Vegfc mRNA, popliteal LN from zDC−/− mice determined by flow cytometry. Results are pooled from four experiments. SEM is shown with error bars. (E) Log2-scaled heat maps for three Vegfc gene array probes showing Vegfc expression by splenic CD4+ and CD8+ cDCs collected from zDC+/+ and zDC−/− mice. (F) Q-PCR for Vegfc expression by splenic CD4+ cDCs normalized to Gapdh expression. (G) Western blot for VEGF protein from whole popliteal LN extracts collected from zDC+/+→zDC+/+ (+/+), zDC+/+→zDC−/− (−/+), and zDC−/−→zDC+/+ bone marrow chimeras. β-Actin is included as a loading control. The graph on the right shows VEGF band quantification normalized to β-actin band intensity with ImageJ software from three experiments. Horizontal bars in C, F, and G show means.

**DISCUSSION**

zDC is a member of the BTB-ZF family. This family contains dozens of members, many of which control immune cell development and function (Collins et al., 2001; Kelly and Daniel 2006; Beaulieu and Sant’Angelo 2011). BTB-ZF transcription factors typically regulate gene expression by binding to specific DNA sequences via their DNA-binding zinc finger domains and recruiting cofactors that mediate chromatin remodeling.
and transcriptional silencing or activation (Kelly and Daniel 2006; Beaulieu and Sant’Angelo 2011).

In the immune system, BTB-ZF transcription factors, including Bcl6, PLZF, ThPOK, PLZP, MARZ, BAZF, LRF, and Miz, function as transcriptional repressors that control lineage commitment decisions and cellular activation (Collins et al., 2001; Kelly and Daniel 2006; Beaulieu and Sant’Angelo 2011). Bcl6 is a typical BTB-ZF transcriptional repressor that is required for germinal center B cell and follicular helper T cell development (Dent et al., 1997; Ye et al., 1997; Nurieva et al., 2009; Yu et al., 2009). Its effect on B cell differentiation is mediated in part through repression of Blimp-1, which is required for plasma cell development (Shaffer et al., 2000; Tunyaplin et al., 2004). Similarly, ThPOK is required for CD4+ T cell lineage commitment and iNKT function (He et al., 2005; Engel et al., 2010). ThPOK-deficient thymocytes bearing TCRs that would normally differentiate into CD4+ T cells or iNKT cells are instead diverted toward CD8+ T cell commitment. This redirection is a result of the loss of ThPOK-mediated repression of CD8+ T cell determinants, including Runx3 (Beaulieu and Sant’Angelo 2011). In contrast, PLZP primarily modulates T cell proliferation and cytokine secretion after TCR stimulation (Piazza et al., 2004). PLZP-deficient CD4+ T cells become hyperproliferative after TCR signaling and produce excessive IL-4, IL-5, and IL-13. Likewise, stimulated PLZP-deficient CD8+ T cells also hyperproliferate and produce more IFN-γ. Less is known about what roles BTB-PZ transcription factors play in the development and regulation of myeloid lineage cells.

zDC is unique among members of the BTB-ZF family as it is highly restricted to cDCs (Meredith et al., 2012; Satpathy et al., 2012). Like other BTB-ZF factors, it acts primarily as a transcriptional repressor as evidenced by the up-regulation of zDC bound genes in zDC−/− cDCs. zDC directly and/or indirectly represses the expression of genes normally induced during cDC maturation, including MHC II. GSEA analyses identified gene pathways associated with TLR signaling and immune stimulation are up-regulated in zDC-deficient cDCs. Consistent with the idea that the absence of zDC expression in zDC−/− mice causes cDC activation, zDC protein levels rapidly decrease in response to TLR stimulation in wild-type cDCs. Thus, normal cDC maturation involves activation of positive regulators like NF-κB (Rescigno et al., 1998) but also the extinction of zDC, which actively helps maintain cDCs in the quiescent steady state.

Although zDC motifs are located in the X2 box of MHC promoters, this position is also occupied by the MHC activator Creb1, suggesting that these two regulators with opposite effects on gene expression might compete for MHC promoter binding (Krawczyk et al., 2008; Handunnetthi et al., 2010). Moreover, zDC−/− cDCs express higher Creb1 transcript levels than wild-type controls (Fig. 3C), and ChIP-seq demonstrated that zDC binds the Creb1 locus. Therefore, zDC may directly repress Creb1 expression by occupying its promoter, thus lowering Creb1 expression and allowing zDC to outcompete Creb1 for occupation of MHC II promoters in steady-state cDCs. According to this model, zDC degradation during TLR stimulation would alleviate the competition for MHC binding, permitting Creb1 to freely associate with MHC promoters. This scenario is consistent with the kinetics of zDC protein down-regulation within 3 h of TLR stimulation and the short burst of MHC transcription that occurs during the first 3–4 h after cDC maturation (Cella et al., 1997; Landmann et al., 2001). Further work is required to test whether zDC and Creb1 compete for MHC promoters and whether this competition also occurs at other zDC target genes.

Steady-state cDCs are in part responsible for maintaining immune tolerance to self- and innocuous antigens by instructing self-reactive T cells to undergo deletion, anergy, or differentiation into regulatory T cells (Hawiger et al., 2001, 2004; Kretschmer et al., 2005). In contrast, antigen presentation by steady-state zDC−/− cDCs to OT I cells resulted in an intermediate phenotype wherein many of the responding cells survive and acquire an effector T cell phenotype (CD62Llo). However, this break in peripheral tolerance is insufficiently penetrant to produce autoimmunity because we have not noticed any autoimmune disease (weight loss, proteinuria, or glycosuria) in zDC−/− mice aged for up to 4 mo. Similar effects were also found in a second independently produced zDC knockout strain lacking exons 2 and 3.

zDC-deficient mice develop hyperplastic skin-draining LNs. This effect is consistent with increased VEGF production by zDC−/− cDCs. This growth factor is normally produced by activated cDCs after TLR stimulation and is required for the vascularization and growth of LNs in response to inflammation (Webster et al., 2006; Tzeng et al., 2010; Wendland et al., 2011). Because VEGF is not a direct target of zDC in our ChIP-seq library, the up-regulation of VEGF is likely a downstream effect of zDC deficiency, for example, the up-regulation of cDC maturation pathways.

zDC is haploinsufficient, as indicated by altered cDC subset composition and elevated MHC II expression in zDC−/+ heterozygotes. This observation may be relevant to human disease because human zDC expression is cDC-restricted and a genome-wide association study identified single nucleotide polymorphisms (SNPs) within the human zDC gene that are associated with pediatric-onset inflammatory bowel disease (IBD; Kugathasan et al., 2008). Further work is necessary to address the effects of these SNPs on zDC function and to evaluate zDC’s role in IBD and other human inflammatory diseases.

In conclusion, we have defined the genes regulated by the zinc finger zDC and its DNA recognition element. zDC is a repressor of a transcriptional network that is extinguished upon immune stimulation by TLR ligands. This network is in part responsible for maintaining cDC quiescence in the steady state.

**MATERIALS AND METHODS**

**Antibodies and other reagents.** The following reagents were from BD or eBioscience: anti-CD16-CD32 (2.4G2), anti-I-A/I-E (M5/114.15.2), anti-CD45R (RA3-6B2), anti-CD115 (AFS98), anti-Fli3 (A2F10), anti-CD3 (145-2C11), anti-CD4 (L3T4), anti-CD8 (53–6.7), anti-CD19 (1D3), anti-NK1.1 (PK136), anti-Ter119 (TER-119), anti-Scia-1 (D7), anti-CD11b.
HRP (Jackson ImmunoResearch Laboratories, Inc.), anti–rabbit HRP (Jackson protein G Sepharose; GE Healthcare), rabbit anti-Histone H4 (Abcam), RIPA buffer and sonication for 15 min on the high setting of a Diagenode Western blots.

for 4 h at 65°C. ChIP DNA was then isolated by phenol/chloroform extraction fer), and protein was digested with 1 mg/ml Proteinase K and 0.3% SDS in TE NaDOC[,] once with TE buffer + 0.2% Triton X-100, and once with TE buf-

were washed rigorously (twice with RIPA buffer, twice with RIPA buffer

linking (fixed with 1% formaldehyde in PBS for 10 min at 37°C, and quenched with 125 mM glycine) and genomic DNA was sheared to 200–1,000-bp

were cross-linked (sonicated for 15 min at 4°C in RIPA buffer [50 mM Tris-HCl, with 125 mM glycine) and genomic DNA was sheared to 200–1,000-bp

confirmed to be >95% pure by flow cytometry. Protein and DNA were cross-

microbeads were from Miltenyi Biotec. Other reagents included PBS, HBSS, FBS, ACK lysis buffer, and EDTA (Gibco) and Collagenase d (Roche).

ChIP-seq. 30 × 10^6 MACS-purified cDC (CD3, CD19, NK1.1, and Gr1, CD14R-depleted, followed by CD11c enrichment) and B cells (CD3, CD1c-depleted, followed by CD19 enrichment) were isolated from FhL-

jected and naive C57BL/6 mice, respectively. Both populations were

were washed rigorously (twice with RIPA buffer, twice with RIPA buffer

fragments (sonicated for 15 min at 4°C in RIPA buffer [50 mM Tris-HCl,

ions (P84), anti-CD117 (2B8), anti–PDCA-1 (eBio927), anti-Ly6C (Sa2-8), anti-CD169 (MOMA-1), anti-F4/80 (BM8), anti-CD11c (N418), anti-CD172a (P64), anti-CD117 (2B8), anti–PDCA-1 (eBio927), anti-Ly6C (HK1.4), anti-Ly6G (IA8), and anti–IFN-γ (XMG1.2).

Pharm Lyse lysing buffer, Cytoperm/Cytofix solution, and Perm/Wash buffer were purchased from BD. Anti-biotin, anti-CD11c, and anti-CD19 microbeads were from Miltenyi Biotec. Other reagents included PBS, HBSS, FBS, ACK lysis buffer, and EDTA (Gibco) and Collagenase d (Roche).

Western blots. Whole cell or LN lysates were prepared by resuspension in RIPA buffer and sonication for 15 min on the high setting of a Diagenode Bioruptor. Antibodies for Western blots were purchased from the following: Armenian hamster anti-zDC (produced by hybridomas and purified with protein G Sepharose; GE Healthcare), rabbit anti-Histone H4 (Abcam), rabbit anti-VIEGF (Abcam), mouse anti-β-actin (Merck), anti–Armenian hamster HRP (Jackson ImmunoResearch Laboratories, Inc.), anti-rabbit HRP (Jackson ImmunoResearch Laboratories, Inc.), and anti-mouse HRP (Jackson ImmunoResearch Laboratories, Inc.).

Protein purification and gel shift assay. Recombinant zDC was produced as fusion protein to GST in BL21 competent cells (Promega) transformed with pGEX-6p-1 vector (GE Healthcare) containing the mouse zDC cDNA sequence. Glutathione Sepharose beads and PreScission Protease (GE Healthcare) were used to purify zDC without the GST tag using the manufacturer's protocols. GST control protein was purified from cells transformed with pGEX-6p-1 vector (GE Healthcare) were used to purify zDC without the GST tag using the manufacturer's protocols. GST control protein was purified from cells transformed with empty vector.

Double-stranded DNA probes containing the MEME-predicted motif (5′-ACCGGTGATGACGTCAGGAGCCGCAA-3′) and mutated motif (5′-ACCGCTTCATTAAAGGGAGCCGCAA-3′) were labeled with P32-dATP by T4 polynucleotide kinase (NEB). Binding reactions between labeled probes and recombinant zDC or GST control were performed in assay buffer (2 mM TrisCl, pH 7.9, 10 mM KCl, 0.02 mM EDTA, 2% glycerol, 0.25 mg/ml BSA, 1 mM DTT, 0.1% NP-40, and 1 mM PMSE) at 30°C for 30 min, run on 5% polyacrylamide gel, and exposed to photographic film overnight at ~80°C.

zDC conditional knockout mouse. We targeted exon 2 of the zDC locus with a pCON-DTA targeting vector containing a 1.7-kb short arm within intron 1 (640 bp upstream of exon 2) and 5.9-kb long arm within intron 2 (520 bp downstream of exon 2) to insert loxp sites flanking exon 2. zDC conditional knockout mice were generated by homologous recombination in C57BL/6 albino embryonic stem cells at The Rockefeller University Gene Targeting Resource Center and maintained on the C57BL/6 background by setting up littermate crosses. C57BL/6 mice were purchased from The Jackson Laboratory, and CD45.1° OT-I were bred and maintained at The Rockefeller University. All mice were housed in The Rockefeller University Comparative Bioscience Center under specific pathogen-free conditions, and all experiments were performed in accordance with National Institutes of Health guidelines and approved by The Rockefeller University Animal Care and Use Committee.

Microarray. Total RNA extraction and hybridization on MOE-430 2.0 arrays (Affymetrix) were performed at Memorial Sloan-Kettering Cancer Center (New York, NY). Microarray data were analyzed using GeneSpring 10.0 software (Agilent Technologies). Triplicates of each population were collected and averaged in GeneSpring. Splenic cDCs were collected from wild-type (GEO accession: GSE6259; Dudzak et al., 2007) and zDC°/° (GEO accession: GSE37995) mice. Monocytes (GEO accession: GSE37566; Meredith et al., 2012) were collected from wild-type bone marrow.

zDC down-regulation with poly I:C and other TLR agonists. 30 µg poly I:C (Invitrogen), 50 µg LPS serotype 0111:B4 (Sigma-Aldrich), or 20 µg 1826 CpG plus 15% DOTAP (Roche) in PBS was injected i.v. in C57/BL6 mice. 6 h after injection, mice were euthanized and spleen DCs were enriched with CD11c beads (Miltenyi Biotec) for analysis by Western blotting.

OT-I deletion. OT-I T cells were isolated from CD45.1° OT-I mice using CD8° T cell negative selection (Miltenyi Biotec) and were labeled with 2 µM CFSE (Molecular Probes). Each mouse received 1–2 × 10^6 CFSE-labeled OT-I T cells i.v. and 3 µg DEC-OVA i.p. or footpad s.c. 12–18 h after OT-I transfer. 2 wk after DEC-OVA treatment, the amount of CD8° CD45.1°Vε2° OT-I cells in the spleen were quantified by flow cytometry.

Quantitative real-time PCR. Total RNA was isolated from 20,000-100,000 FACS-sorted C57BL/6 and zDC°/° cDCs with TRizol (Invitrogen), from which cDNA libraries were reverse-transcribed using Superscript II (Invitrogen) and random primers. Mouse 14gc cDNA (forward: 5′-TGTGTCACGG-TGATAGGC-3′; reverse: 5′-TGGCATGCATTGAGTCTTTC-3′) was normalized to Gapdh cDNA (forward: 5′-TGAACGAGGCTATCGAGG-3′; reverse: 5′-CGAAGGTGAAGTGAGGG-3′). All quantitative PCR reactions were performed with Brilliant SYBR Green (Agilent Technologies) on an Mx3005P system.

Online supplemental material. Table S1 shows zDC target genes. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20121003/DC1.

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