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Absence of the Transcriptional Repressor Blimp-1 in Hematopoietic Lineages Reveals Its Role in Dendritic Cell Homeostatic Development and Function

Yueh-Hsuan Chan,*† Ming-Feng Chiang,*† Yueh-Chiao Tsai,† Shin-Tang Su,*† Ming-Hsu Chen,‡ Mau-Sheng Hou,‡ and Kuo-I Lin*†

Dendritic cells (DCs) are important for the initiation and regulation of immune responses. In this study, we demonstrate that DC homeostatic development in peripheral lymphoid organs is negatively regulated by the transcriptional repressor, Blimp-1, which is critical for regulation of plasma cell differentiation and T cell homeostasis and function. Deletion of Prdm1, the gene encoding Blimp-1, in mouse hematopoietic lineages resulted in an increase in the steady-state number of conventional DCs (cDCs). Specifically, Prdm1 deletion increased immediate CD8⁺ cDC precursors in peripheral lymphoid organs, causing selective expansion of the CD8⁺ cDC population. Upon stimulus-induced maturation, Blimp-1 was up-regulated in bone marrow-derived DCs via the p38 MAPK and NF-κB pathways. Notably, Blimp-1-deficient DCs matured poorly upon stimulation in vitro and in vivo. Blimp-1 binds to the proinflammatory cytokine/chemokine genes, Il-6 and Ccl2, and negatively regulates their expression. Collectively, our findings reveal two new roles for Blimp-1: negative regulation of a select subset of cDCs during homeostatic development, and enhancement of DC maturation. *The Journal of Immunology, 2009, 183: 7039–7046.

Dendritic cells (DCs) are a type of APCs that are present in virtually all tissues. They are crucial for initiation and regulation of immune responses against pathogens and are important for maintaining steady-state self-tolerance. The maturation of DCs is initiated through pattern recognition receptors, such as TLRs (1), upon Ag conjugation. By capturing, processing, and presenting Ags, DCs move into local draining lymph nodes as such, resulting in the initiation of T cell activation and adaptive immune responses (2).

In mouse spleen and lymph nodes, DCs are heterogeneously distributed but they are generally subdivided into two major groups: conventional DCs (cDCs) and plasmacytoid DCs (3). cDCs can be further divided into CD4⁺CD8⁻, CD4⁺CD8⁺, and CD4⁻CD8⁺ subsets (4, 5). The developmental pathways that regulate differentiation of DCs from hematopoietic stem cells were recently deciphered. In mouse bone marrow, cDCs and plasmacytoid DCs are derived from a common proliferative DC-restricted progenitor that expresses fms-like tyrosine kinase-3 (Flt3) and M-CSF receptor (M-CSFR) (6–8). A significant number of steady-state cDCs in the spleen are generated from immediate splenic cDC precursors (pre-cDCs), which may be derived from bone marrow DC progenitors through blood circulation (9). The expression of surface CD24 on splenic pre-cDCs segregates the developmental lineages of CD8⁻ and CD8⁺ DCs (3, 9).

Many transcription factors and cytokines have been implicated in regulation of cDC development. For example, CD8⁻ cDC generation is defective in mice lacking IFN-regulatory factor (IRF)-2 (10) and IRF-4 (11), and the number of CD8⁺ cDCs is reduced in mice lacking IRF-8 (12). Flt3 ligand (13) and STAT3 (14) are critical for multiple subsets of DC development. Thus, accumulating evidence suggests there is an important interplay between these positive regulators in the general development of cDCs or development of specific DC subsets.

B lymphocyte-induced maturation protein-1 (Blimp-1), also called PRDI-BF1, was discovered as a transcriptional repressor of the IFNβ promoter (15). Blimp-1 orchestrates plasma cell differentiation by regulating cascades of gene expression (16–18), controls T cell homeostasis and tolerance (19, 20), and triggers myeloid lineage differentiation (21). Blimp-1 expression can be activated in mature B cells in many ways, including activation by TLR ligands (22–24). We wonder whether Blimp-1 is involved in DC function as DC maturation is often mediated by TLR activation. Thus, an animal system in which the Blimp-1 gene, Prdm1, was conditionally deleted in the pan-hematopoietic lineage was generated. In addition to its known roles in B and T cell lineages, we found that Blimp-1 negatively regulates homeostatic development of the CD8⁻ cDC subset by maintaining the numbers of immediate cDC precursors that acquire a CD8⁻ cDC fate in spleen. We also found that Blimp-1 is critical for DC maturation in response to several stimuli. Together, we have identified two novel roles for Blimp-1 in DC: homeostatic development and maturation in peripheral lymphoid tissues.
Materials and Methods

**Mice and cell lines**

*Pdml*<sup>105</sup> mice, as described (18), were bred with *Tie2-Cre* mice, B6, Tg(Tek-cre)12Flv/J (The Jackson Laboratory). Sex-matched *Pdml*<sup>105</sup> Tie2-Cre<sup>+</sup> (conditional knockout, CKO) and littermate control (Ctrl) mice (2- to 4-mo-old) were used for all experiments in this study. All mice were maintained under specified pathogen free conditions in the animal facility of the Institute of Cellular and Organismic Biology at Academia Sinica. The Institute Animal Care and Utilization Committee of Academia Sinica approved all experiments. Mice were genotyped by genomic PCR using primers described in supplemental Table I. C57BL/6 and BALB/c mice were purchased from the Laboratory Animal Center in Taiwan and used at 6–10 wk of age. For BrdU labeling in vivo, 1 µg BrdU (BD Biosciences) was injected into Ctrl or CKO mice by i.p. at 1 day before sacrifice for flow cytometric analysis of the percentage of BrdU-positive cells in splenic cDC subsets. JAWS II cells purchased from American Type Culture Collection were grown in MEMα medium (Invitrogen) supplemented with 20% FBS (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin, 4 mM L-glutamine, 1 mM sodium pyruvate, and 5 ng/ml murine GM-CSF (eBioscience).

**Preparation and culture of cells from lymphoid organs**

For analysis of cell populations, lymphoid organs were harvested using 0.02 mg/ml Liberase Blendzyme II (Roche Applied Science) for 20 min at room temperature. Single cell suspensions were washed with RPMI 1640 (Invitrogen) containing 50 µg/ml Dnase I (Roche Applied Science) and 3% FCS (HyClone). RBCs were removed using RBC lysis buffer (Sigma-Aldrich). Spleen Cdl<sup>1</sup> cells were isolated by positive selection using an autoMACS separator (Miltenyi Biotec) and stimulated with 1 µg/ml LPS for indicated days. Spleen CD4<sup>+</sup> T cells were isolated from total spleenocytes cultured with 5 µg/ml anti-CD3 (eBioscience) and 10 ng/ml IL-2 (R&D Systems) for 3 days. The immediate spleen DC precursors (pre-cDCs) were defined as previously described (9). In brief, by density using Nycodenz (Axis-Shield), and cells of density between 1.076 and 1.084 were collected. B, T, and NK cells were excluded by staining with specific Abs to CD3, CD19, and CD49b. The lymphocyte-excluded medium-density fraction was further analyzed by the following parameters: low side scatter and expression of CD11c, CD45RA, CD43, SIRP-α, and CD24.

**Bone marrow-derived DC (BMDC) cultures**

GM-CSF culture of BMDCs was performed essentially as described (25). Cells (2 × 10<sup>5</sup> cells/ml) were cultured in RPMI 1640 supplemented with 20 ng/ml murine GM-CSF (eBioscience), 10% FBS (BenchMark), 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin. After 8 days in culture, nonadherent cells were harvested as immature BMDCs. The re-arranging cells were replated at a density of 1 × 10<sup>6</sup> cells/ml for stimulation with 10 ng/ml (or other indicated doses) LPS, 0.5 µg/ml poly I:C, 50 ng/ml CpG oligodeoxynucleotide 1826 (InvivoGen), or 10 ng/ml TNF-α (PeproTech) for 20 h. In some experiments, immature BMDCs harvested on day 8 were treated for 1 h with the NF-κB inhibitor BAY 11–0782 (Calbiochem), or the p38 MAPK inhibitor SB203580 (Calbiochem), followed by LPS stimulation for 20 h. FliL-cultured BMDCs were prepared as described (26). Cells were seeded at 1 × 10<sup>6</sup> cells/ml and cultured in RPMI 1640 supplemented with 50 ng/ml murine Flt3L (R & D Systems), 10% FBS (BenchMark), 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were left untreated or stimulated with 0.5 µg/ml poly I:C or 0.3 µM CpG D19 (GGTGCATCGATGCAGGGGGG phosphorothioate-modified at sites underlined) on day 8 and harvested on day 9.

**Abs and flow cytometry**

Single cell suspensions were stained with appropriate Abs as described in supplemental Table II. For intracellular cytokine staining, T cells were washed and stimulated with 50 ng/ml PMA and 1 µg/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A (Biolegend) for 6 h. After fixation and permeabilization, cells were stained with anti-IFN-γ-APC (Biolegend) and anti-IL-4-PE (Biolegend). All flow cytometric analysis was performed using FACSCanto or FACSCalibur and results were analyzed by FACSDiva software (BD Biosciences) or FlowJo software (Tree Star). In some experiments, CD11c<sup>hi</sup>CD4<sup>+</sup>CD8<sup>+</sup>, CD11c<sup>lo</sup>CD4<sup>+</sup>CD8<sup>+</sup>, or CD11c<sup>lo</sup>CD4<sup>+</sup>CD8<sup>+</sup> cells from purified splenic CD11c<sup>+</sup> cells were sorted by FACSaria (BD Biosciences).

**Western blotting and immunofluorescence staining**

Western blotting with an anti-Blimp-1 mAb was performed as described (27). For detection of cytoplasmic IκBα degradation by rabbit anti-IκBα Ab (Santa Cruz Biotechnology) or nuclear translocation of p50 subunit by rabbit anti-p50 Ab (Santa Cruz Biotechnology), cytoplasmic and nuclear extracts were prepared as described (22). Goat anti-lamin B Ab was purchased from Santa Cruz Biotechnology. For NF-κB p65 subunit immunofluorescence staining, in brief, BMDCs were left unstimulated or stimulated with 10 ng/ml LPS for 2 h and prepared by cytospin at 500 rpm for 5 min. The cells were then fixed by 4% paraformaldehyde, permeabilized with 0.1% triton X-100, and blocked with 1% BSA, followed by staining with rabbit anti-p65 Ab (Santa Cruz Biotechnology) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (Molecular Probes). The nuclei were counterstained by 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). The fluorescent images were examined under a fluorescence microscope (Leica DM6000B).

**Mixed lymphocyte reaction**

Allogeneic T cell proliferation was measured using 10<sup>5</sup> splenic CD3<sup>+</sup>CD4<sup>+</sup> T cells from BALB/c as responders and various numbers of BMDCs (1000, 3000, or 10000 cells) irradiated with 50 Gy as stimulators. After 4 days in culture, cells were pulsed with 1 µCi [H]thymidine for 16 h. [H]Thymidine incorporation was quantified with a microplate-scintillation counter (TopCount NXT, Packard). Cell proliferation was measured in cpm.

**In vivo DC maturation**

Ctrl and CKO mice were subjected to i.p. injection of LPS (3 µg/g body weight) solubilized in PBS. Control groups were injected with PBS alone. Ten hours after injection, mice were sacrificed. Single cell suspensions were prepared from mesenteric lymph nodes, stained, and subjected to flow cytometric analysis.

**Lentiviral vector preparation and transduction**

pFGW, pFUW-GFPBlimp1, pFUWCtrl, and pFUWGBlimp1-3952I have been described (27, 28). Generation of pseudotyped lentivirus was performed essentially as described (27, 29). Targeted BMDCs and JAWS II cells were infected at a multiplicity of infection of 5 in the presence of 8 µg/ml polybrene (Sigma-Aldrich).

**Cytometric bead array (CBA), multiplex bead array assays, and ELISA**

The Mouse Inflammation CBA Kit (BD Pharmingen) was used in accordance with the manufacturer’s protocol to measure select cytokines and chemokines (IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70) in supernatants from BMDC cultures stimulated with 1 µg/ml LPS. Cytokines and chemokines in sera were determined by the MILLIPLEX Mouse Cytokine/ Chemokine kit (Millipore) according to the manufacturer’s protocol and analyzed by Luminex 200. IgM production in culture supernatants was determined by ELISA essentially as described (22).

**RNA isolation and reverse transcription-quantitative PCR (RT-QPCR)**

These procedures have been described (22). The gene specific Taqman primer and probe sets, including *Pdml* (assay ID: Mm 00777741_sH) were purchased from Applied Biosystems. The primer specific setters information was described in supplemental Table I.

**Chromatin immunoprecipitation (ChIP) assay**

Blimp-1 binding in vivo was detected essentially as reported (28, 30) using rabbit antiserum against Blimp-1 as described (31). In brief, 2–3 × 10<sup>6</sup> Ctrl or CKO BMDCs stimulated with LPS for 24 h were used per ChIP experiment. The immunoprecipitated DNA was analyzed by real-time PCR with SYBR green method on an ABI prism 7300 system. The primer sequences for ChIP assay were listed in the supplemental Table I.

**DNA pull-down assay and EMSA**

The DNA pull-down assay was performed essentially as described (32). In brief, biotinylated wild-type or mutated oligonucleotides coupled with...
streptavidin-conjugated Dynabeads (Invitrogen) were incubated with 150 μg preincubated extract prepared from LPS–stimulated Ctrl and CKO BMDCs. After 1 h incubation at room temperature, the complexes were collected with a magnetic apparatus. Coprecipitated proteins were eluted and fractionated by 8% SDS-PAGE, followed by Western blotting analysis. In the competition assay, a 200-fold excess of unlabeled oligonucleotides was added. EMSA was performed using 2.5 μg nuclear extract prepared from 293T cells transfected with Blimp-1 expression vector and the LightShift chemiluminescent EMSA kit (Pierce) as described (22). For the supershift assay, 1 μg of mouse anti-Blimp-1 Ab or control Ab (Sigma-Aldrich) was added to the described reactions and incubated on ice for 30 min. The oligonucleotides used in this study were described in supplemental Table III.

Luciferase assay
Genomic fragments from +69 to +223 of murine Il-6, located in intron 1, and from −600 to +64 of murine Ccl2 were amplified by PCR from genomic DNA of primary mouse BMDCs derived from C57BL/6 mice and cloned into Smal/BglII-digested pGL3 promoter vector (Promega) and KpnI/BglII-digested pGL3 basic vector (Promega), respectively. Detailed primer information for luciferase reporter constructions was described in supplemental Table I. JAWS II cells were cotransfected with 0.2–1 μg luciferase reporter construct as mentioned above and various amounts (for IL-6 reporter assays: 50, 150, and 450 ng; for Ccl2 reporter assays: 30, 100, and 300 ng) of Blimp-1 expression vector (pCMV-Blimp1) or control vector (pCMV-5b) using TransIT-TKO reagent (Mirus) (33). Luciferase activity measurement and fold repression calculation was performed as previously described (30).

Statistical analysis
Statistical significance was measured using the two-tailed paired Student’s t test. Data represent means ± SD or means ± SEM as indicated.

Results
Blimp-1 negatively regulates CD8+ cDC development
Prdm1f/f mice harboring Prdm1 alleles containing two LoxP sites flanking exons 5 and 8 were generated previously (18) and have been used to demonstrate the role of Blimp-1 in B cell differentiation and T cell homeostasis in vivo (18, 19). To further understand the role of Blimp-1 in the immune system, such as in DC lineage development and function, we deleted Prdm1 in the hematopoietic system by crossed Prdm1f/f mice with expressing the cre-recombinase (Cre) transgene under the control of the Tie2 regulatory element, Tie2-Cre (34). A three primer-based PCR cre ing the Tie2 regulatory element, Tie2-Cre (34). A three primer-based PCR

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The Journal of Immunology 7041

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mediated by NF-
Stimulus-induced Blimp-1 expression during DC maturation is
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represent means ± SD for one representative experiment of three. E. Flow
cytometric analysis of splenic CD4+ T cells from Ctrl and CKO mice.
Numbers in the quadrants indicate the percentage of naive
(CD62LhighCD44low) and effector (CD62LlowCD44high) T cells.
F. Total splenocytes from Ctrl and CKO mice were cultured with PMA plus iono-
mycin in the presence of brefeldin A for 6 h for intracellular cytokine
staining and subsequent flow cytometric analysis. Numbers in the quad-
Drants indicate the percentage of IFN-γ and IL-4-producing cells.

from Ctrl and CKO mice (supplemental Fig. 2B). Collectively, these results suggest that the increased number of steady-state
CD8+ cDCs in CKO spleen likely results from increased generation
of immediate CD8− cDC precursors.

Stimulus-induced Blimp-1 expression during DC maturation is
mediated by NF-κB and p38 MAPK pathways

DCs can be generated by addition of GM-CSF in the bone marrow
progenitors in culture (37). We next measured the expression of
Blimp-1 in bone marrow cultures supplemented with GM-CSF and
evaluated whether its expression changed following stimulus-in-
duced maturation. The Blimp-1 mRNA levels in sorted CD11c+ cells obtained from BMDCs were almost equivalent in cells cul-
tured with GM-CSF for 4 and 8 days (Fig. 4A), whereas Blimp-1
was greatly induced in BMDCs cultured with LPS for 24 h (Fig. 4A).
Similarly, maturation of GM-CSF-cultured BMDCs by
TNF-α, CpG, or poly I:C induced Blimp-1 mRNA and protein
expression (Fig. 4, B and C). Using pharmacological inhibitors, we
examined the signaling pathway(s) required for Blimp-1 induction
in BMDCs. Induction of Blimp-1 by LPS was inhibited in a dose-
dependent manner by the NF-κB inhibitor BAY 11-7082, and by
the p38 MAPK inhibitor SB203580 (Fig. 4D), but the JNK inhib-
itor SP600125, the ERK inhibitor PD98059, and the PI3K inhibitor
LY294002 had no effect (data not shown). Thus, during BMDC
maturation, Blimp-1 is induced by TLR ligands through the p38
MAPK and NF-

Blimp-1 negatively regulates CD8+ DC homeostasis. A. Percentage of peripheral lymph node CD3+ T cells (n = 9), B220+ B cells
(n = 9), and F4/80+ macrophages (n = 10) in Ctrl and CKO mice. B, Percentage (left) and total number (middle) of splenic CD11chigh cDCs
(n = 12), and percentage of peripheral lymph node CD11c+ cDCs (n = 8, right) in Ctrl and CKO mice. C. Percentage of CD8− and CD8+ cDCs
in the CD11c+ gate in Ctrl and CKO spleens (n = 12). D. Total number of splenic CD11c+CD8− and CD11c+CD8+ cDCs in Ctrl and CKO mice
(n = 12). E. Percentage of splenic CD11c+CD4+CD8− and CD11c+CD4−CD8+ cDCs in the CD11c+CD8− gate from Ctrl and
CKO mice (n = 12). F. Total number of splenic CD11c+CD4+CD8−
and CD11c+CD4−CD8+ cells in Ctrl and CKO mice (n = 12). Data
represent means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001 vs Ctrl.

We next examined whether BMDCs cultured with GM-CSF re-
quired Blimp-1. We verified impaired Blimp-1 induction by LPS
treatment of CKO BMDCs cultured with GM-CSF (Fig. 5A). The
maturation status of DCs, which was analyzed by the expression of
a panel of surface markers including CD25, CD40, CD86, and
MHCI-I/A/E), did not differ significantly between Ctrl and CKO
GM-CSF-cultured BMDCs after 8 days of culture (Fig. 5B), sug-
uggesting that Blimp-1 is not essential for DC development under
these conditions. Following stimulation with a variety of TLR li-
gands or by TNF-α for 24 h, induction of CD825, CD40, CD86, and
MHCI-I/A/E) was partially impaired in CKO GM-CSF-cultured BMDCs
(Fig. 5B). Likewise, up-regulation of CD86 and MHCI in BMDCs
cultured with GM-CSF derived from C57BL/6 mice was blocked

![FIGURE 1. The generation of CKO mice. A, PCR analysis of genomic DNA isolated from tissues (peripheral lymph node (LN), spleen (Sp), thymus (Thy), bone marrow (BM), and brain), from cells (splenic CD3+ T cells (T), B220+ B cells (B), and CD11c+ DCs (DC)) of Prdm1f/+Tie2-Cre-/- and Prdm1f/+Tie2-Cre+/+ mice. B, Western blotting of Blimp-1 in Ctrl and
CKO B cells treated with LPS for 3 days. a-tubulin was as a loading
control. C. Flow cytometric analysis of plasma cells generated from Ctrl and
CKO splenic B cells stimulated with LPS for 3 days. One representa-
tive experiment of three is shown. D, IgM levels in supernatants har-
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![FIGURE 2. Blimp-1 negatively regulates CD8+ DC homeostasis. A. Percentage of peripheral lymph node CD3+ T cells (n = 9), B220+ B cells
(n = 9), and F4/80+ macrophages (n = 10) in Ctrl and CKO mice. B. Percentage (left) and total number (middle) of splenic CD11c+ cDCs
(n = 12), and percentage of peripheral lymph node CD11c+ cDCs (n = 8, right) in Ctrl and CKO mice. C. Percentage of CD8− and CD8+ cDCs
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Blimp-1 is required during DC maturation

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(Fig. 5B). Likewise, up-regulation of CD86 and MHCI in BMDCs
cultured with GM-CSF derived from C57BL/6 mice was blocked
by lentiviral vectors expressing short hairpin RNAs against Blimp-1 (Fig. 5C), suggesting that impaired maturation of CKO BMDCs is a specific effect. We excluded the possibility that defective BMDC maturation in CKO mice was caused by changes in the levels of various TLR mRNAs (supplemental Fig. 3A), the kinetics of IkB degradation, or nuclear translocation of the NF-κB subunits p50 and p65 that is mediated by TLR4 receptor signaling (supplemental Fig. 3, B and C).

Using a MLR assay, we demonstrated that LPS, poly I:C, CpG, and TNF-α stimulation of BMDCs from CKO mice resulted in a partially reduced ability to induce alloegenic T cell proliferation, when compared with Ctrl BMDCs (Fig. 5D). The significance of Blimp-1 in DC maturation was further strengthened by in vivo LPS injection (Fig. 5E). The percentage of Lin- c-kit+Flt3+ progenitors in the Lin- gate and Lin- c-kit+Flt3+IL-7Rα- M-CSFR- subset of DC progenitors (gate 3, right contour plots) are indicated in successive gates. One representative experiment of five is shown. B. Percentage of Lin- c-kit+Flt3+ (left, n = 6) in the Lin- gate and Lin- c-kit+Flt3+IL-7Rα- M-CSFR- (right, n = 5) progenitors in the Lin- c-kit+Flt3+ gate in Ctrl and CKO bone marrow cells. C. Percentage of CD8+ T cells that correspond to CD8+ T cells, naive (CD62LhighCD44low) CD4+ T cells, effector (CD62LlowCD44high) T cells, and T cells + anti-CD5+IL-2 stimulation for 3 days. Results in E and F were calculated relative to nonstimulated B cells. Data represent means ± SEM. *p < 0.05 vs Ctrl.

by lentiviral vectors expressing short hairpin RNAs against Blimp-1, this increase occurred at the transcriptional level (supplemental Fig. 4A). IL-6 and MCP-1 mRNA levels were also increased in LPS- and poly I:C-stimulated splenic CD11c+ DCs cultured from CKO mice (supplemental Fig. 4B). We thus hypothesized that Il-6 and Ccl2 expressed in the DC lineage are subject to Blimp-1 regulation. Il-6 contains three potential Blimp-1 binding sites: one in intron 1 and two in intron 3. The Ccl2 promoter region contains one potential Blimp-1 binding site, as revealed based on known Blimp-1 consensus sites (31) (Fig. 6B). A ChIP assay using a Blimp-1-specific Ab showed that endogenous Blimp-1 bound to the Il-6 intron 1 and Ccl2 promoter sites in Ctrl BMDCs stimulated with LPS, and that Blimp-1 did not bind significantly to either site in LPS-stimulated CKO BMDCs (Fig. 6C). In support of this finding, DNA pull-down assay demonstrated that Blimp-1 in nuclear extracts harvested from LPS-stimulated Ctrl BMDCs could bind to biotinylated oligonucleotides containing wild-type but not mutated Il-6 or Ccl2 sites (Fig. 6D), and that competition assays using unlabeled oligonucleotides containing a known Blimp-1 binding site in the c-myc promoter (36) largely reduced the binding (Fig. 6D). In contrast, nuclear proteins isolated from LPS-stimulated CKO BMDCs bound much less to wild-type Il-6 or Ccl2 biotinylated oligonucleotides (Fig. 6D). Accordingly, EMSA showed that oligonucleotides containing these sites were shifted by nuclear protein extracts containing exogenously expressed Blimp-1 (supplemental Fig. 5, A and B). The shifted complex was disrupted by addition of excess unlabeled probe corresponding to the c-myc site (supplemental Fig. 5, A and B) and was supershifted by a Blimp-1-specific mAb (supplemental

II-6 and Ccl2 were suppressed directly by Blimp-1

We also measured cytokine/chemokine production to assess the function of GM-CSF-primed BMDCs from Ctrl and CKO mice (Fig. 6A). After LPS stimulation for 1 day, CKO cultures produced more IL-6 and MCP-1 (encoded by Ccl2) than Ctrl cultures, as determined by CBA (Fig. 6A), and this increase occurred at the transcriptional level (supplemental Fig. 4A). IL-6 and MCP-1 mRNA levels were also increased in LPS- and poly I:C-stimulated splenic CD11c+ DCs cultured from CKO mice (supplemental Fig. 4B). We thus hypothesized that Il-6 and Ccl2 expressed in the DC lineage are subject to Blimp-1 regulation. Il-6 contains three potential Blimp-1 binding sites: one in intron 1 and two in intron 3. The Ccl2 promoter region contains one potential Blimp-1 binding site, as revealed based on known Blimp-1 consensus sites (31) (Fig. 6B). A ChIP assay using a Blimp-1-specific Ab showed that endogenous Blimp-1 bound to the Il-6 intron 1 and Ccl2 promoter sites in Ctrl BMDCs stimulated with LPS, and that Blimp-1 did not bind significantly to either site in LPS-stimulated CKO BMDCs (Fig. 6C). In support of this finding, DNA pull-down assay demonstrated that Blimp-1 in nuclear extracts harvested from LPS-stimulated Ctrl BMDCs could bind to biotinylated oligonucleotides containing wild-type but not mutated Il-6 or Ccl2 sites (Fig. 6D), and that competition assays using unlabeled oligonucleotides containing a known Blimp-1 binding site in the c-myc promoter (36) largely reduced the binding (Fig. 6D). In contrast, nuclear proteins isolated from LPS-stimulated CKO BMDCs bound much less to wild-type Il-6 or Ccl2 biotinylated oligonucleotides (Fig. 6D). Accordingly, EMSA showed that oligonucleotides containing these sites were shifted by nuclear protein extracts containing exogenously expressed Blimp-1 (supplemental Fig. 5, A and B). The shifted complex was disrupted by addition of excess unlabeled probe corresponding to the c-myc site (supplemental Fig. 5, A and B) and was supershifted by a Blimp-1-specific mAb (supplemental
Fig. 5, A and B). By contrast, mutated oligonucleotides or control isotype Ab did not affect the mobility of the shifted complex (supplemental Fig. 5, A and B). Furthermore, ectopic Blimp-1 expression was sufficient to suppress the expression of endogenous IL-6 and MCP-1 in JAWS II cells, a murine immature DC line (33) (Fig. 6E). Finally, we used a construct consisting of a minimal basal promoter fused with an intron 1 fragment of Il-6 containing the Blimp-1 binding site and the luciferase gene, and a similar construct containing the Ccl2 promoter fragment. We observed dose-dependent Blimp-1 suppression of luciferase expression with both constructs (Fig. 6F). Taken together, these data indicate that Il-6 and Ccl2 are direct targets for Blimp-1 and that Blimp-1 negatively regulates these genes in DCs.

Discussion
A role for Blimp-1 in B cell and T cell lineages has been reported (18–20). In this study, we found increased DC populations in mice lacking Blimp-1. Specifically, the number of CD8<sup>+</sup> cDCs was affected in CKO mice. Higher Blimp-1 expression is found in CD8<sup>+</sup> cDC subpopulations. Our results suggest that Blimp-1 may function in CD8<sup>+</sup> cDC homeostasis by providing a balance that prevents excessive expansion of CD8<sup>+</sup> cDCs by certain positive regulators. Blimp-1 also regulates DC maturation, and therefore we have identified a novel role for Blimp-1 in the DC lineage that is functionally relevant to innate immunity.

Similar to mice lacking Prdm1 specifically in the B or T cell lineage (18–20), in vitro plasma cell differentiation is defective and T cell homeostasis is dysregulated in CKO mice. One striking phenotype found in T cell-specific deletion of Prdm1 in mice is severe colitis and body weight loss (19). Autoimmune disease symptoms were not observed in CKO mice up to 5 mo of age (data not shown), although these mice displayed increased steady-state effector T cell populations. These discrepancies may have resulted from the use of a different lineage-specific Cre in the different systems. In particular, DC function was altered in our animals,
which may have impaired the initiation of the inflammatory response. In contrast, we think that the altered levels of CD8\(^+\) cDCs in this study are probably not attributable to an indirect effect resulting from changes in cytokine levels from T and B cell lineages, for the following reasons. First, Ctrl and CKO mice do not differ significantly with respect to steady-state serum cytokine/chemokine levels as determined by multiplex bead array assays (supplemental Fig. 6). Second, cDC homeostasis is unaffected by deletion of Prdm1 in the mouse B cell lineage (data not shown). 3) T cell-specific Blimp-1 transgenic mice have normal distribution and numbers of splenic CD11c\(^{hi}\) cDCs when compared with control littermates (Y.-H. Chan and K.-I Lin, unpublished observation). In addition, the mice used in our study also harbored a Prdm1 deletion in endothelial cells, but Blimp-1 expression in endothelial cells is not functionally relevant during embryonic development (40). The numbers and frequencies of T cells, B cells, and precDCs were normal or minimally affected in CKO spleen or peripheral lymphoid organs, suggesting that Blimp-1 deficiency in blood vessel endothelial cells does not interfere with the trafficking of newly generated hematopoietic cells into peripheral lymphoid tissues.

In this study, we demonstrated the expression of Blimp-1 transcripts in steady state murine splenic cDCs and BMDCs cultured with GM-CSF, and that Blimp-1 is induced following stimuli-induced maturation of BMDCs. What are the signals/molecules involved in regulating Blimp-1 expression in DC lineages during either steady state or maturation? We determined that various TLR ligands induce Blimp-1 expression in BMDCs, consistent with previous findings that conjugation of TLR4, TLR2, or TLR9 results in Blimp-1 activation in the B cell lineage (22–24). Blimp-1 induction through TLR signaling is dependent on p38 MAPK and NF-κB signaling pathways. Essential roles for these two pathways in DC maturation have been described in vitro (41, 42). These findings are concordant with our finding that Blimp-1 is critical for BMDC maturation because it acts downstream of p38 MAPK and NF-κB. Blimp-1 transcripts in B cells are also regulated by AP-1 and STAT3 (43, 44). A JNK inhibitor did not influence Blimp-1 expression during BMDC maturation (data not shown), thus it is unlikely that AP-1 is involved in regulation of Blimp-1 expression in the DC lineage. The role of STAT3 in Blimp-1 induction during BMDC maturation remains to be determined. Whether p38 MAPK and NF-κB pathways regulate steady state Blimp-1 expression in splenic CD8\(^+\) cDCs also awaits further investigation. Because CD8\(^+\) cDCs are found largely in the marginal zone of spleen or the subcapsular layer of peripheral lymph nodes (45, 46), the microenvironment of these areas may provide signals for steady state Blimp-1 expression.

Blimp-1 negatively regulates homeostatic development of cDC subsets in vivo, and it also positively regulates DC maturation. CKO GM-CSF-supplemented BMDCs are less able to up-regulate MHCII in response to stimulation, which may explain why stimulation of BMDCs from CKO mice did not induce efficient alloreactive T cell proliferation. Accordingly, the induction of CD25, CD40, CD86, and MHCII surface markers was reduced in Flt3L-cultured CKO BMDCs following either poly I:C or CpG stimulation (supplemental Fig. 7), suggesting that Blimp-1 may regulate genes generally important for response to DC maturation. Further identification of Blimp-1 targets in DC lineages may improve our understanding of the underlying causes of defective CKO BMDC maturation. Blimp-1-mediated suppression of IL-6 in the DC lineage may provide a clue. IL-6 knockout mice have a greater number of mature DCs in vivo and pretreatment with IL-6 suppresses BMDC maturation (47), suggesting that defective DC maturation in CKO mice or cultures may result from enhanced IL-6 production, at least in part.

In conclusion, we have established two new roles for the transcriptional repressor Blimp-1 in the immune system: (1) maintenance of CD8\(^+\) cDC homeostasis and (2) promotion of DC maturation or function. Our studies provide insights into the mechanisms governing maintenance of homeostatic cDC development and also provide a molecular basis for designing agents to modulate immune tolerance or immunogenic responses.
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Disclosures

The authors have no financial conflict of interest.

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