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Identification of a Tissue-Specific, C/EBPβ-Dependent Pathway of Differentiation for Murine Peritoneal Macrophages

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Macrophages and dendritic cells (DC) are distributed throughout the body and play important roles in pathogen detection and tissue homeostasis. In tissues, resident macrophages exhibit distinct phenotypes and activities, yet the transcriptional pathways that specify tissue-specific macrophages are largely unknown. We investigated the functions and origins of two peritoneal macrophage populations in mice: small and large peritoneal macrophages (SPM and LPM, respectively). SPM and LPM differ in their ability to phagocytose apoptotic cells, as well as in the production of cytokines in response to LPS. In steady-state conditions, SPM are replenished by bone marrow precursors following radiation injury. Transcription factor analysis revealed that SPM and LPM express abundant CCAAT/enhancer binding protein (C/EBP)β. Cebpb−/− mice exhibit elevated numbers of SPM-like cells but lack functional LPM. Alveolar macrophages are also missing in Cebpb−/− mice, although macrophage populations in the spleen, kidney, skin, mesenteric lymph nodes, and liver are normal. Adoptive transfer of SPM into Cebpb−/− results in SPM differentiation into LPM, yet donor SPM do not generate LPM after transfer into C/EBPβ-sufficient mice, suggesting that endogenous LPM inhibit differentiation by SPM. We conclude that C/EBPβ plays an intrinsic, tissue-restricted role in the generation of resident macrophages.


Animal tissues contain macrophages and dendritic cells (DC) that constitute a network of cells dubbed the mononuclear phagocyte system. Among tissues, resident phagocytes exhibit substantial phenotypic heterogeneity, likely reflecting tissue-specific function. Macrophage diversity is thought to result from environmental cues in tissues that induce distinct differentiation programs in macrophages or their precursors. However, the differentiation pathways that generate tissue-specific macrophages are largely unknown. Moreover, recent studies have challenged the long-standing hypothesis that tissue-resident macrophages arise from circulating monocytes (1), instead indicating that some macrophage compartments are established by fetal precursors and maintained independently of hematopoiesis (4, 5).

Macrophages of the murine peritoneal cavity are among the best-studied tissue macrophage compartments. Peritoneal macrophages play important roles in clearing apoptotic cells (6) and coordinating inflammatory responses (7–9). A recent study (10) demonstrated that mouse peritoneal macrophages can be divided into two subsets: large peritoneal macrophages and small peritoneal macrophages (LPM and SPM, respectively). LPM make up the majority of peritoneal macrophages and express high levels of F4/80, CD11b, and CD93 but lack MHC class II (MHCII); SPM, in contrast, express lower levels of F4/80, CD11b, and CD93 but high levels of MHCII (10). Both SPM and LPM phagocytose bacteria and produce NO (10), but little is known about their origins and independent functions.

In this study, we investigated the functions and origins of SPM and LPM; SPM and LPM diverged in their ability to phagocytose apoptotic cells and exhibited differences in cytokine production after LPS exposure. Transcription factor analysis revealed C/EBPβ expression by both SPM and LPM; however, in Cebpb−/− mice, the peritoneal cavity lacked LPM, whereas elevated numbers of SPM-like cells were present. We identify SPM as precursors of LPM and show that C/EBPβ acts intrinsically in the differentiation or survival of LPM. Finally, adoptively transferred SPM efficiently generated
LPN in Cebpb<sup>−/−</sup> mice, but they did not differentiate in normal mice in the presence of endogenous LPN. We interpret this suppression of differentiation as evidence of a demand-driven process for the replenishment of peritoneal macrophages from hematopoietic precursors.

**Materials and Methods**

**Mice**

C57BL/6 and congenic lysozyme M (LysM<sup>Creet</sup> and C3crl<sup>Creert</sup>) mice came from The Jackson Laboratory. Cebpb<sup>−/−</sup> mice on 129/Sv and C57BL/6 backgrounds were kindly provided by Dr. P. Johnson (National Cancer Institute, Frederick, MD); 129/Sv × C57BL/6.F1 Cebpb<sup>−/−</sup> mice and control subjects were generated from hemizygous parents (14). 129/Sv and control subjects were generated from hemizygous parents (14). 129/Sv mice came from The Jackson Laboratory.

**Characterization of peritoneal macrophages and DC**

Peritoneal cells were harvested by lavage with 7 ml cold PBS. Blood was harvested with a heparinized 27G needle from the inferior vena cava. Mice were perfused with PBS and lung, liver, mesenteric lymph nodes, spleen, kidneys, and ears were harvested. Organs were manually dissociated and digested for 1 h at 37°C with 2 mg/ml collagenase A (Roche Applied Science). Nonspecific IgM (NS IgM) (Southern Biotech) or rabbit IgG isotype control (Santa Cruz Biotechnology); primary Abs were detected with PE-conjugated anti-rabbit protein, cells were fixed and permeabilized (eBioscience fixation/permeabilization buffers) before incubation with anti-C/EBPβ or rabbit IgG isotype control (Santa Biotechnology); primary Abs were detected with PE-conjugated anti-rabbit IgG (Southern Biotechnology).

Quantitative RT-PCR

Peritoneal B cells, DC, SPM, and LPN were purified by FACS. RNA was harvested using a Nanoprep RNA kit (Agilent Technologies), and cDNA was generated with SuperScript III reagents (Invitrogen) and oligo-dT primers. Gene transcription was determined using SsoFast EvaGreen reagents (Bio-Rad) and the following primers: Gapdh, forward 5′-AAC TTT GTC ATT GTG GAA GGG-3′ and reverse 5′-ACA TAG TGG GGG TAG GAA CA-3′; Actb, forward 5′-AGC CAT GTA CGT AGC CAT CC-3′ and reverse 5′-CTC TCA GCT GTG GTG GTA AA-3′; and Cebpb, forward 5′-AAG CTG AGC GAC GAG TAC AAG A-3′ and reverse 5′-GTC AGC TCC AGC ACC TTG TG-3′. Cebpb gene expression was calculated in the peritoneal DC compartment were set as 1; expression levels in other compartments were normalized to this value.

Cytokine induction by LPS

Peritoneal DC, SPM, and LPN were isolated by FACS then suspended at 10<sup>5</sup> cells/ml in RPMI 1640 medium containing 10% FBS, 10 mM HEPES, 55 μM 2-ME, and penicillin/streptomycin. Cells were treated with or without 1 μg/ml LPS (0127:B8; Sigma-Aldrich) overnight at 37°C/5% CO<sub>2</sub>; supernatants were harvested at 8°C.

Phagocytosis of apoptotic cells

Phagocytic activity was assayed as previously described (18). Briefly, syngeneic thymocytes (10<sup>6</sup> cells/ml) were cultured with 0.1 μM dexamethasone overnight to induce apoptosis. The frequency of Annexin V<sup>+</sup> cells was determined by light scattering and apoptosis. Cell death was monitored using an Annexin V-apoptosis kit (BD Biosciences). Cells were fixed and stained with an Alexa Fluor 647-labeled anti-CD40 ligand and an Alexa Fluor 488-labeled anti-mouse IgM. For intracellular detection of F4/80, cells were fixed/permeabilized using Cytofix/Cytopermeabilization buffer (BD Biosciences) and stained with a PE-labeled anti-F4/80 Abs. After staining, cells were acquired on a FACSAria (BD Biosciences).

Average expression levels of Gapdh and Cebpb in the peritoneal DC compartment were set as 1; expression levels in other compartments were normalized to this value.

Histology and immunofluorescence

Tissues were frozen in OCT compound. Sections (5 μm) were fixed in methanol/acetic (1:1) and blocked with rat IgG and Fc block. Sequential sections were stained with FITC MOMA-1, PE F4/80, and biotin B220 or Alexa Fluor488 SIGN-R1, PE TCRβ, and biotin B220. Biotin mAb were detected with streptavidin–Alexa Fluor 350, and Alexa Fluor 488. Images were acquired at room temperature using a Zeiss Axiovert 200M microscope with 10× objective lenses (0.30 numerical aperture), Zeiss AxioCam MRm camera, and AxioVision software. Composite micrographs were generated in Adobe Photoshop using B220 labeling to align images from sequential sections.

Quantification of cytokines in culture supernatants and sera

Soluble cytokines were quantified in the Duke Human Vaccine Institute Immune Reconstitution and Biomarker Shared Resource Facility. Supernatants from untreated and LPS-treated DC, SPM, and LPN were analyzed. Sera were prepared from Cebpb<sup>+/−</sup> and Cebpb<sup>−/−</sup> mice. Cytokine concentrations in samples were determined using a Bio-Plex Pro Group I 23-Plex kit (Bio-Rad).

Adoptive transfer of peritoneal macrophages

Peritoneal cells of 129/Sv × C57BL/6.eGFP<sup>+</sup>F1 mice were stained with fluorochrome–Ab conjugates, fixed/permeabilized, treated with DNase, and stained with FITC anti-BrdU. Frequencies of BrdU<sup>+</sup> SPM and LPN were determined by flow cytometry using control cell populations from untreated mice as background controls.

Adaptive reconstitution

C57BL/6 mice were sublethally irradiated (600 rad) and reconstituted with 10<sup>7</sup> BM cells from congenic C57BL/6.CD45.1 mice. Peritoneal cells of chimeric mice were assessed 4–5 wk after reconstitution; donor-derived cells were distinguished by CD45.1 expression.

Statistics

Significance in paired data was determined by the Student t test. Significance in multiple comparisons was determined by ANOVA with the Tukey post hoc test (JMP Pro).

**Results**

Characterization of peritoneal macrophages and DC

Peritoneal lavage cells from naive C57BL/6 mice were analyzed by flow cytometry, and IgM<sup>SSC<hi>lo</hi></sup> B cells were excluded for analysis.
of myeloid cells (Fig. 1A). We identified a population of IgM<sup>+</sup>CD11c<sup>+</sup>SSC<sup>low</sup> cells that was uniformly MHCII<sup>+</sup>CD93<sup>+</sup> and exhibited a broad distribution of CD115, the M-CSF receptor (Fig. 1A). Most (75–90%) IgM<sup>+</sup>CD11c<sup>+</sup>SSC<sup>low</sup> cells were CD11b<sup>+</sup> (Fig. 1A), consistent with the myeloid DC phenotype of lymphoid tissues, whereas the remaining CD11b<sup>+</sup> cells expressed CD205, similar to lymphoid DC (19) (Fig. 1A). Hereafter, we refer to IgM<sup>+</sup>CD11c<sup>+</sup>SSC<sup>low</sup> cells collectively as DC.

Within the IgM<sup>+</sup>CD11c<sup>+</sup>SSC<sup>low</sup> cell population, we observed two CD11b<sup>+</sup> populations with high side-scatter properties that could be distinguished by intermediate and high levels of CD11b (Fig. 1A). CD11b<sup>int</sup>SSC<sup>hi</sup> cells were CD115<sup>low</sup>Siglec F<sup>+</sup> and identified as eosinophils by histological analysis (data not shown). The CD11b<sup>hi</sup>SSC<sup>hi</sup> compartment was subdivided by F4/80 staining; the F4/80<sup>low</sup> cells, accounting for <5% of CD11b<sup>hi</sup>SSC<sup>hi</sup> cells, exhibited greater light scattering than did DC and were CD115<sup>+</sup>MHCII<sup>hi</sup>CD93<sup>+</sup>CD205<sup>low</sup> (Fig. 1A), consistent with the SPM phenotype (10). The F4/80<sup>hi</sup> cells exhibited higher side-scatter properties than did SPM and were CD115<sup>+</sup>MHCII<sup>+</sup>CD93<sup>+</sup>CD205<sup>+</sup> (Fig. 1A), consistent with the LPM phenotype (10).

To characterize the functions of peritoneal DC, SPM, and LPM, cells were isolated by FACS and then analyzed for cytokine production in response to LPS in vitro. LPS stimulated all three cell types to secrete IL-1<sub>B</sub>, IL-9, IL-12(p70), IL-13, eotaxin, and IFN-<sub>γ</sub>, and there were only modest differences (<2-fold) across the three cell compartments (Fig. 1B, Supplemental Fig. 1). However, SPM produced significantly more MIP-1α and TNF-α than did either DC or LPM (Fig. 1B, Supplemental Fig. 1). LPM, in contrast, selectively produced abundant G-CSF, GM-CSF, and KC in response to LPS, whereas DC exclusively generated IL-12(p40) (Fig. 1B, Supplemental Fig. 1). DC and SPM were more potent sources of RANTES than were LPM, but SPM and LPM produced significantly more IL-1α, IL-6, IL-10, MCP-1, and MIP-1β than did DC (Fig. 1B, Supplemental Fig. 1). The discrete cytokine profiles of

![FIGURE 1. Effector functions of SPM and LPM.](http://www.jimmunol.org/)

(A) Flow cytometric gating strategy to identify peritoneal DC (R1: IgM<sup>+</sup>CD11c<sup>+</sup>SSC<sup>low</sup>), SPM (R2: IgM<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>hi</sup>F4/80<sup>low</sup>), and LPM (R3: IgM<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>hi</sup>F4/80<sup>hi</sup>) in peritoneal lavages of C57BL/6 mice. Cells in gate R4 (IgM<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>int</sup>SSC<sup>hi</sup>) were identified as eosinophils. Forward and side light scattering (FSC and SSC) properties and surface expression of CD11b, CD11c, MHCII (I-A/I-E), CD93, and CD205 (open graphs) for each compartment are shown (right panels). Filled graphs represent background staining by isotype-matched control Abs. (B) Cytokine signatures of sorted peritoneal DC, SPM, and LPM following in vitro exposure to LPS. The concentrations of various cytokines in the supernatants were determined by multiplex bead array. Data are summarized in a Venn diagram and detailed in Supplemental Fig. 1. Data represent three independent experiments (n = 3–7 samples/cell type). (C) Peritoneal cells were assessed for their ability to phagocytose apoptotic cells in vivo. pHrodo Red–labeled apoptotic thymocytes were injected i.p., and peritoneal lavages were performed 1 h later. Phagocytosis was measured as the frequency of pHrodo Red–labeled cells in the SPM, LPM, DC, and eosinophil compartments. Representative dot plots of background fluorescence by cells of untreated mice (upper panels) and plots of gated cells from a mouse injected with labeled apoptotic cells (lower panels). Data are representative of two individual experiments (n = 4 mice).
LPS-treated DC, SPM, and LPM suggest that these myeloid compartments contribute differently to inflammatory responses in the peritoneal cavity.

Resident phagocytes contribute to tissue homeostasis by clearing dying cells (6); consequently, we measured the capacity of peritoneal DC, SPM, and LPM to phagocytose apoptotic cells. Apoptotic thymocytes were labeled with pHrodo Red, a dye that fluoresces at increased intensity following transport into acidic lysosomes (18), and then injected i.p. into mice. After 1 h, mice were sacrificed, and peritoneal cells were analyzed by FACS for the presence of pHrodo Red. Frequencies of pHrodo Red⁺ cells in each macrophage compartment varied among mice, but in all animals the frequency of labeled LPM was greater than SPM (Fig. 1C). The ratio of labeled LPM/SPM was 3.8 ± 1.3 (geometric mean ± GSD, range 2.9–4.9), indicating that LPM exhibit greater phagocytic activity than do SPM. The frequency of labeled DC was only slightly higher than background, whereas eosinophils lacked label (Fig. 1C). Although both SPM and LPM have the capacity to engulf apoptotic cells, LPM are the more potent phagocytes.

Origins and maintenance of the peritoneal DC, SPM, and LPM compartments

The mononuclear phagocyte system is thought to depend on hematopoiesis for the maintenance of tissue phagocytes, with circulating monocytes serving as cellular intermediates between BM progenitors and tissue-resident macrophages (1). However, reports of in situ proliferation suggest that some macrophage compartments have the capacity for self-replenishment (20). To quantify in situ proliferation by peritoneal DC, SPM, and LPM, we measured frequencies of BrdU-labeled cells in these myeloid compartments 3 h after BrdU administration. The frequencies of BrdU⁺ DC, SPM, and LPM were 0.7, 1.2, and 2.4%, respectively, whereas autologous frequencies of the BrdU⁺ BM hematopoietic stem cell (Lin⁻ c-Kit⁺ CD16/32⁺) compartments were 18 and 39%, respectively (Fig. 2A). Peritoneal DC, SPM, and LPM undergo little proliferation in the steady-state.

We next monitored the appearance of BrdU label in peritoneal myeloid compartments after BrdU injection as an indication of differentiation from proliferating precursors. The frequency of BrdU⁺ DC and SPM increased after BrdU administration, peaking at days 4–6 and 6–10, respectively, before gradually returning to background levels (Fig. 2B). DC and SPM acquired BrdU label, regardless of BrdU injection route (day 6 i.p. versus i.v.: 17.7 versus 17.2% for DC, 5.8 versus 5.4% for SPM, p > 0.05 for both), indicating that the appearance of BrdU label in these populations after i.p. injection was not due to local inflammation. We conclude that peritoneal DC and SPM are continuously produced from proliferating precursors and have tissue half-lives of only a few days.

In contrast to DC and SPM, BrdU labeling was not detectable in the LPM compartment over a span of 14 d after the BrdU pulse (Fig. 2B), suggesting that LPM, in steady-state conditions, are dependent on hematopoietic stem cells (4, 5). To determine the relationship between BM hematopoiesis and peritoneal DC, SPM, and LPM, we irradiated BM hematopoietic stem and progenitor cells (HSPC: Lin⁻ c-Kit⁺ Sca-1⁺ c-Kit⁺ CD16/32⁺) and granulocyte/macrophage progenitor (Lin⁻ Sca-1⁻ c-Kit⁺ CD16/32⁺) compartments were harvested as controls. The mean (± SD) frequency of BrdU⁺ cells in each compartment is shown. (B) BrdU transit through peritoneal DC, SPM, and LPM compartments after a single BrdU pulse. The mean (± SD) frequencies of BrdU⁺ cells in the DC, SPM, and LPM compartments at intervals after BrdU administration. Data represent two independent experiments (n = 2–4 mice/data point). (C) CX3CR1 expression by peritoneal DC, SPM, and LPM. GFP fluorescence by cells from Cx3cr1CreKO mice (open graphs) and C57BL/6 controls (filled graphs) (upper panels), GFP fluorescence by peritoneal DC, SPM, and LPM from Cx3cr1CreWT Rosa26R-FGFP mice (open graphs) and control Cx3cr1WTWT Rosa26R-FGFP mice (filled graphs) (lower panels). Graphs are representative of results in two independent experiments (n = 3–4 mice/genotype). (D) Reconstitution of peritoneal DC, SPM, and LPM following irradiation and BM transfer. C57BL/6.CD45.1 BM cells were adoptively transferred into irradiated C57BL/6.CD45.2 hosts. Representative graphs show frequencies of donor-derived (CD45.1⁺) cells in peritoneal DC, SPM, and LPM compartments 4–5 wk after reconstitution. Mean (± SD) frequencies of CD45.1⁺ cells in each compartment (right panel). Data represent two independent experiments (n = 4 mice).

Cx3cr1CreRosa26R-FGFP mice, which identify both active and past expression of Cx3cr1, ~90% of DC and nearly all SPM and LPM were GFP⁺ (Fig. 2C). We conclude that most peritoneal DC and all SPM are short-lived, recent descendants of Cx3cr1⁺ precursors, whereas LPM exhibit a more distant ontogenic relationship with a Cx3cr1⁺ progenitor.

Although macrophages are commonly thought to differentiate from circulating monocytes (1), recent reports indicate that some tissue-resident macrophages arise from Cx3cr1⁺ precursors in the yolk sac and are maintained independently of hematopoietic stem cells (4, 5). To determine the relationship between BM hematopoiesis and peritoneal DC, SPM, and LPM, we irradiated...
C57BL/6 mice and reconstituted them with C57BL/6.CD45.1 BM. Four weeks after BM reconstitution, ~90% of peritoneal DC and ~80% of SPM were CD45.1+, indicative of hematopoietic origin. Although previous studies (21, 22) and our BrdU-transit study (Fig. 2B) indicate that the LPM compartment is maintained independently of hematopoiesis in the steady-state, >70% of LPM in chimeric mice were CD45.1+ (Fig. 2D), demonstrating a pathway of differentiation from BM precursors to LPM. We conclude that, in steady-state conditions, the peritoneal DC and SPM compartments are sustained through hematopoiesis, whereas LPM are maintained independently of BM. However, the cytotoxic effects of radiation activate a pathway of hematopoietic differentiation that replenishes the LPM compartment.

**Altered phenotype and function of peritoneal macrophages in Cebpb−/− mice**

Studies in Spic−/− mice highlight the specificity of transcription factors for the generation of distinct tissue macrophage compartments (13). In addition to Spic, transcription factors implicated in macrophage differentiation include Cebpb (24), Egr1 (25), If35 (26), Maf (27), MafB (27), Mif (28), and Nfκb (29). Microarray analysis of transcription factor expression by tissue macrophages revealed that Cebpb is highly expressed in peritoneal macrophages (F4/80hiCD115+B220−MHCII+ cells, consistent with the LPM phenotype) (30). Using quantitative RT-PCR, we confirmed that Cebpb transcription was high in LPM, whereas levels were intermediate in SPM, low in peritoneal DC, and undetectable in B cells (Fig. 3A). Similar results were obtained from analysis of CEBPB protein levels by flow cytometry (Fig. 3B).

We next investigated peritoneal macrophages in Cebpb−/− mice. Viability of Cebpb−/+ pups with the C57BL/6 genetic background is low (31); therefore, we produced Cebpb−/− mice by crossing heterozygous C57BL/6 and 129/Sv parents, generating C57BL/6×129/Sv.F1 mice (Fig. 3C). CD11b+ cells from Cebpb−/− mice were comparable in numbers and phenotype in C57BL/6 and C57BL/6×129/Sv.F1 Cebpb−/− mice (data not shown).

C/EBPB deficiency severely affected peritoneal macrophage compartments. In Cebpb−/− mice, the overall number of peritoneal macrophages (IgM+CD11c−CD11b+ cells) was only 35% of that in Cebpb+/* mice (p = 0.01) (Fig. 3B, 3C). Although F4/80 labeling revealed SPM and LPM as discrete populations in Cebpb−/− and Cebpb−/+ mice, distinct F4/80hi and F4/80low macrophage populations were not found in Cebpb−/− mice (Fig. 3B). Instead, we noted a homogenous population of cells exhibiting low to intermediate levels of F4/80 (F4/80low and F4/80int, respectively), with <10% of cells falling into the F4/80hi gate (Fig. 3B). Compared with control littersmates, the numbers of F4/80low and F4/80int peritoneal macrophages in Cebpb−/− mice were increased 6- and 25-fold, respectively (p = 0.01), whereas the number of F4/80hi macrophages was reduced >95% (p = 0.01) (Fig. 3B, 3C). In Cebpb−/− mice, peritoneal DC numbers were modestly, but not significantly, increased (Fig. 3C), whereas the number of CD11b+ SSCc cells, identified as eosinophils, was elevated 10-fold over controls (Supplemental Fig. 3A).

We analyzed Cebpb−/− macrophages for MHCII and CD93, markers that distinguish SP and LPM in control animals. Peritoneal macrophages of Cebpb−/− mice were indistinguishable from MHCII staining into a F4/80+MHCIIhi compartment (similar to SP in control mice), a F4/80−MHCIIhi population, and a F4/80hi population exhibiting levels of MHCII that were low and similar to LPM in Cebpb−/+ animals (Fig. 3E). The frequency of F4/80low MHCIIhi SP-like cells was 4-5-fold higher in Cebpb−/− mice than in control animals, with a concurrent reduction in the frequency of F4/80lowMHCIIlow LPM-like cells (Fig. 3E). However, unlike wild-type (WT) LPM, F4/80int macrophages in Cebpb−/− mice did not express CD93 (Fig. 3E). Moreover, Cebpb−/− F4/80int macrophages exhibited high levels of CD115, similar to a transitional population of cells in WT mice (Fig. 3E). Overall, C/EBPB deficiency was associated with more peritoneal macrophages bearing the SPM phenotype (F4/80lowMHCII+CD115+MHCIIhi), a CD115hi macrophage population sharing characteristics of both SPM (CD115−) and LPM (MHCII+MHCII−), but not cells exhibiting the complete LPM phenotype (F4/80−MHCII+CD115−MHCIIhi).

The absence of F4/80lowCD93+ macrophages in Cebpb−/− mice suggested a defect in LPM generation. Alternatively, LPM may be normal in Cebpb−/− mice but fail to express F4/80 and CD93 as the result of dysregulation of the corresponding genes. To determine whether F4/80lowMHCIIhi LPM-like cells in Cebpb−/− mice function as LPM, we compared the phagocytic activity of F4/80lowMHCIIhi SP-like cells and F4/80lowMHCIIhi LPM-like cells in Cebpb KO mice. Following injection of pHrodo Red-labeled apoptotic cells, the ratio of labeled LPM/SPM was 3.5 (geometric mean ± GSD; range, 2.6–5.0) in Cebpb+/+ littersmates, similar to that in BL/6 mice (Fig. 3F). However, in Cebpb−/− mice, the ratio of labeled F4/80lowMHCIIhi LPM-like cells to F4/80lowMHCIIhi SP-like cells was only 1.6 ± 1.2 (range, 1.5–1.9) (p ≤ 0.05, Cebpb−/− versus Cebpb+/+, BL/6). Based on phenotypic and functional analyses of Cebpb−/− peritoneal macrophages, we conclude that C/EBPB is necessary for the generation of LPM.

C/EBPB is induced by inflammatory stimuli and affects cytokine production in several macrophage types, including peptone-elicited macrophages (32), alveolar macrophages (33), splenic macrophages (34), and other subtypes (35, 36). Peritoneal macrophages of Cebpb KO mice also exhibited altered cytokine production. Overall, LPS-induced cytokine responses of SPM-like (IgM+CD11clowCD11b+SSCchlMHCIIhi) and LPM-like (IgM+CD11clowCD11b+SSCchlMHCIIlow) cells from Cebpb−/− mice were decreased compared with WT SP and LPM, respectively (Supplemental Fig. 2). In both SP-like and LPM-like cells of Cebpb−/− mice, the cytokines most affected included IL-6, G-CSF, and MIP-1α (Supplemental Fig. 2). However, both SP-like and LPM-like macrophages of KO mice produced more IL-12 (p40) than did corresponding WT cells, consistent with observations in other macrophage subtypes (35). MCP-1 secretion was also elevated in Cebpb−/− macrophages compared with control cells (Supplemental Fig. 2). As observed in other macrophages, C/EBPB plays an integral role in the cytokine responses of peritoneal macrophages.

C/EBPB deletion in lysozyme M–expressing cells blocks LPM generation

C/EBPB is expressed by many cell types (14, 31, 37); consequently, we determined whether C/EBPB acts intrinsically for LPM generation. LysMCreWT mice delete floxed alleles in granulocytes and macrophages (38). Using LysMCreWT Rosa26R-FGFP mice, we confirmed that, in the peritoneal cavity, Cre recombines activity is primarily restricted to SP and LPM, although ~35% of DC were also GFP+ (Fig. 4A).

The peritoneal phenotype of LysMCreWT Cebpbfl/fl mice was similar to that of Cebpb−/− mice, despite differences in genetic background (C57BL/6 for LysMCreWT Cebpbfl/fl mice; 129/SvxC57BL/6F1 for Cebpb−/− mice) and general versus conditional deletion of Cebpb. In LysMCreWT Cebpbfl/fl mice, F4/80hi LPM-like cell numbers were reduced >95% (p ≤ 0.01) and F4/80low SP-like cell numbers were increased 7-fold (p ≤ 0.01) compared with controls (Fig. 4B, 4C). As in Cebpb−/− mice, LysMCreWT Cebpbfl/fl mice lacked peritoneal...
macrophages bearing the LPM phenotype of MHCIIlowCD93+ (Fig. 4B). We conclude that Cebpb deficiency in macrophages is sufficient to prevent LPM generation.

Constitutive peritoneal eosinophilia in Cebpb−/− mice

Although Cebpb−/− mice exhibited altered peritoneal macrophage populations, peritoneal B cell numbers (1.3 ± 1.0 × 10⁶, 1.7 ± 1.4 × 10⁶, and 0.7 ± 0.3 × 10⁶) were not different in Cebpb+/+; Cebpb−/−, and Cebpb−/- mice (respectively; p > 0.05 for all). However, a population of CD11bSSC62 cells was greatly expanded (>10-fold, p < 0.05) (Supplemental Fig. 3A, 3B). Similar to Cebpb−/- mice, the number of CD11bSSC62 cells was also significantly elevated in the peritoneal cavity of LysMCreWT/Cebpbfl/fl mice (Supplemental Fig. 3B). The CD11bSSC62 cells were Siglec F+/CD115+ (Supplemental Fig. 3C); when isolated, they exhibited eosinophil morphology (data not shown). The peritoneal eosinophilia of Cebpb−/- mice and LysMCreWT/Cebpbfl/fl mice was not associated with increased eosinophil numbers in the BM or blood (Supplemental Fig. 3D), suggesting that their abundance in the peritoneal cavity is not due to enhanced eosinopoiesis.

The altered peritoneal macrophage and eosinophil populations raised the possibility that C/EBPβ deficiency provokes inflammation; aged Cebpb−/- mice routinely develop an inflammatory pathology mediated by IL-6 (39). We examined the peritoneal lavage fluids of Cebpb−/- mice for neutrophils and inflammatory monocytes (Gr-1+Ly-6B+ cells) as evidence of local inflammation but found neutrophil and inflammatory monocyte numbers to be similar in Cebpb−/- and Cebpb−/- mice (Supplemental Fig. 3E).

We next analyzed the sera of Cebpb+/+ and Cebpb−/- mice for evidence of systemic inflammation. Multiplex analysis of sera did not reveal any differences in the concentrations of 23 cytokines, including IL-6 (Supplemental Fig. 3F). There was no evidence that altered macrophage and eosinophil populations in the peritoneal cavity of Cebpb−/- mice were due to ongoing inflammation.

**FIGURE 3.** Effect of C/EBPβ deficiency on peritoneal macrophages. (A) Peritoneal B cells (IgM+) and peritoneal DC, SPM, and LPM were sorted (gated as in Fig. 1A) and analyzed for C/EBPβ, GAPDH, and β-actin transcripts by quantitative RT-PCR. We measured C/EBPβ and GAPDH transcription relative to β-actin and then normalized to the average value of the peritoneal DC compartment. Values represent the geometric mean (± GSD) from three individual sorts. (B) C/EBPβ protein in SPM, LPM, peritoneal DC, and B cells. Intracellular staining of peritoneal cells with anti-C/EBPβ (open graphs) or isotype control (filled graphs) is shown. Data are representative of two individual experiments (n = 2). (C) Representative flow cytometric analyses of peritoneal macrophages in Cebpb+/+ and Cebpb−/- mice. Upper panels: IgM+ and CD11c+ cells have been excluded as in Fig. 1A; SPMCD11b+ cells are gated as total macrophages (R1). F4/80 and CD11b expression by total macrophages of Cebpb+/+ and Cebpb−/- mice (lower panels); populations were subdivided based on F4/80 staining intensity (R2: SPM, R3: F4/80int, and R4: LPM). (D) Mean (± SD) numbers of total macrophages (Mφ), F4/80+ SPM, F4/80+ macrophages, and F4/80+ LPM from Cebpb−/-, Cebpb−/-, and Cebpb−/- mice. Data represent nine independent experiments (n = 6–8 mice/genotype). *p ≤ 0.05, **p ≤ 0.01 versus Cebpb−/- mice. (E) Phenotypic analysis of total macrophages (R1) macrophages from Cebpb−/- mice (upper panels) and Cebpb−/- mice (lower panels). In all dot plots, F4/80 is shown on the x-axis, and MHCII, CD93, and CD115 are shown on the y-axis. Data are representative of two or three experiments (n ≥ 3 mice/Ab panel). (F) The phagocytic capacity of Cebpb−/- macrophages (upper panels) and Cebpb−/- macrophages (lower panels) was assessed following i.p. injection of pHrodo Red–labeled apoptotic thymocytes. Macrophages were gated based on F4/80 and MHCII expression, as in (E, left panels). SPM and SPM-like macrophages were gated as F4/80+MHCIIm+ SPMLPM-like macrophages were due to ongoing inflammation.
C/EBPβ deficiency affects alveolar macrophages but not macrophage populations in spleen, lymph node, liver, kidney, or skin

To determine whether C/EBPβ plays a general role in macrophage generation, we analyzed Cebpb KO and littermate controls for blood monocytes and resident macrophages of the spleen, mesenteric lymph nodes, skin, kidneys, liver, and lungs. Frequencies of F4/80+/SSClo monocytes in blood were similar in Cebpb b/Wb and Cebpb b/Wt animals (Fig. 5A). C/EBPβ deficiency affects alveolar macrophages but not peritoneal and lung macrophages.

C/EBPβ is dispensable for the general role for peritoneal and lung macrophages.

**SPM are precursors for LPM**

The abundance of SPM-like and F4/80int macrophages but dearth of LPM in Cebpb b KO mice (Figs. 3, 4) suggested an ontogenic relationship between SPM and LPM, as well as a differentiative block in the absence of C/EBPβ. When Cebpb b/w SPM from GFP+ mice (42) were adoptively transferred into the peritoneal cavities of Cebpb−/− mice, the frequency of F4/80low donor cells gradually decreased, whereas the frequency of F4/80hi LPM-like cells concomitantly increased; by day 8, >85% of GFP+ cells were F4/80 hi, and most of these cells were MHCIILowCD93−, identifying them as LPM (Fig. 6A, 6B). C/EBPβ is required intrinsically for SPM differentiation into LPM.

However, when SPM were transferred into Cebpb b/w or Cebpb b−/− mice, only 20–40% of transferred cells were recovered as F4/80hi cells (Fig. 6A). Moreover, <30% of the F4/80hi donor cells bore the LPM phenotype of MHCIILowCD93−, and the majority still expressed MHCIi, a characteristic of SPM, even 8 d after transfer (Fig. 6A). The efficient generation of LPM from transferred SPM in Cebpb b KO, but not wild-type, hosts suggests a difference in the peritoneal microenvironment, perhaps relating to the presence or absence of endogenous LPM.

Resident peritoneal macrophages are reported to proliferate as a means of self-replenishment (20). Because LPM are missing in...
Cebpb−/− mice (Fig. 3), the appearance of LPM in Cebpb−/− mice after SPM transfer might represent proliferation of LPM contaminants in the adoptive transfer and not differentiation from SPM. Therefore, we transferred GFP+ LPM i.p. into Cebpb KO and control mice; if LPM proliferate in Cebpb−/− recipients, then we expected to recover more GFP+ LPM from Cebpb−/− hosts than from CEBPβ+ hosts. Transferred LPM retained the LPM phenotype, even in Cebpb−/− hosts (Fig. 6C). However, the numbers of donor LPM recovered from CEBPβ-sufficient and -deficient hosts were similar, even 30 d posttransfer (Fig. 6D). The equal recovery of donor LPM from KO and WT hosts excludes the possibility of expansion by LPM contaminants in adoptive-transfer experiments.

Discussion

In this study, we reveal a crucial role for the CEBPβ transcription factor in LPM generation. Adoptively transferred SPM efficiently differentiate into LPM in Cebpb−/− hosts, which lack endogenous LPM, but rarely generate LPM in WT hosts (Fig. 6). We conclude that SPM are targets of demand-driven differentiation signals that maintain macrophage homeostasis in the peritoneal cavity. Our findings uncover a novel, tissue-selective role for CEBPβ in the generation of peritoneal and lung macrophages and may provide insight into a general mechanism that sustains macrophage populations in tissues.

The heterogeneity of macrophages in different tissues implies specific developmental pathways to control resident phagocyte differentiation. For example, mice deficient for the transcription factor Spic specifically lack splenic red pulp macrophages (13), a population that is intact in Cebpb−/− mice (Fig. 5); reciprocally, Cebpb−/− mice lack LPM, yet peritoneal macrophages are normal in Spic−/− mice (13). Therefore, CEBPβ and Spic-C must represent two independent pathways of tissue macrophage generation. Given that liver macrophage, marginal zone macrophage, and metallophilic macrophage populations are intact in both Cebpb−/− and Spic−/− mice, these populations must be products of other differentiation pathways.

CEBPβ has well-characterized roles in monocyte/macrophage responses to inflammation and stress (43). Moreover, CEBPβ has been implicated in the polarization and/or function of myeloid cells exhibiting immunosuppressive properties, including myeloid-derived suppressor cells (44) and M2 macrophages (45). Although these reports define the importance of CEBPβ in the generation and/or function of myeloid cells during pernicious conditions, our study reveals novel roles for CEBPβ in the production of macrophage compartments in unperturbed tissue. CEBPβ is expressed by macrophages in several tissues but is distinctively elevated in LPM and alveolar macrophages (30). The selective sensitivity of LPM and alveolar macrophages to CEBPβ deficiency (Fig. 5) may reflect shared requirements for CEBPβ gene targets for differentiation and/or survival in the peritoneal cavity and lung.

In general, three mechanisms have been implicated in the maintenance of tissue macrophage compartments: proliferation in situ by resident macrophages (20), recruitment and maturation of circulating precursors (46), and mobilization of hematopoietic progenitors to the periphery and their subsequent proliferation and differentiation (47). Inflammation activates all of these mechanisms, and most studies have relied on inflammatory stimuli or cytotoxic agents to study macrophage replenishment. However, it is unclear which mechanism(s) is active for macrophage maintenance. We show in a genetic model of macrophage deficiency, with no evidence of inflammation, that the absence of a terminally differentiated macrophage compartment (LPM) is sufficient to induce the maturation of the immediate precursor (SPM) (Fig. 6A). The absence of endoge-

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**FIGURE 5.** Survey of tissue macrophages in Cebpb−/− mice. (A) Single-cell suspensions of blood, peritoneal cavity, spleen, mesenteric lymph nodes, skin (ear), kidney, liver, and lungs of Cebpb+/+ and Cebpb−/− mice were stained for CD45, F4/80, and CD11b. Viable CD45+ cells were gated for analysis of F4/80+ macrophage/monocyte populations. (B) Viable CD45+ lung cells from Cebpb+/+ and Cebpb−/− mice were analyzed for the presence of alveolar macrophages using Siglec F and CD11c (upper panels) and side scatter (SSC) and autofluorescence (lower panels). Flow plots are representative of two individual experiments (n = 3 mice/genotype).
nous LPM in Cebpb−/− mice was not associated with proliferation by adoptively transferred LPM (Fig. 6D), suggesting SPM differentiation as an important, if not dominant, mechanism for macrophage replenishment. Thus, the Cebpb−/− mouse provides a potential tool to dissect mechanisms of macrophage homeostasis in the peritoneal cavity without the additional variables of inflammatory or cytotoxic treatments.

Although pathways of differentiation from hematopoietic progenitors to SPM and LPM were evident in BM-reconstitution experiments (Fig. 2D), the ontogenic relationship between SPM and LPM was revealed only in Cebpb−/− mice (Fig. 6A, 6B), in which LPM are scarce (Fig. 3C). Only in Cebpb−/− mice were we able to observe efficient generation of LPM from transferred SPM (Fig. 6A). The recovery of donor-derived LPM after SPM transfer into Cebpb−/− or Cebpb+/+ hosts (Fig. 6B) indicates that SPM-to-LPM differentiation is mediated by an extrinsic signal that is scarce in control mice but abundant in Cebpb−/− animals. The absence of endogenous LPM in Cebpb−/− mice may increase the availability of differentiation factors that act on transferred SPM. Alternatively, LPM in C/EBPβ-sufficient hosts may directly inhibit the maturation of transferred SPM. Precursor inhibition by cellular progeny is the hallmark of a process controlled by a demand-driven mechanism.

The notion that a demand-driven process directs the differentiation of transferred SPM in Cebpb−/− hosts is consistent with observations that adoptively transferred monocytes fail to generate lung macrophages unless endogenous tissue macrophages are first depleted (46). Our observations suggest that circulating CX3CR1+ precursors (likely monocytes) continuously seed the peritoneum to generate SPM. In steady-state conditions, SPM reside in tissue for only a few days before dying or leaving the peritoneal cavity (Fig. 2B). However, if LPM numbers decrease, as a result of inflammation or ablation via chemical or radioactive treatments, SPM readily mature into LPM. A reduction in LPM density may promote the recruitment of circulating precursors to increase the pool of precursors that differentiate into SPM and then LPM. Because SPM fail to form LPM in the absence of C/EBPβ, we postulate that demand-driven signals of recruitment and differentiation are constitutively active in Cebpb−/− mice, resulting in an accumulation of SPM and undeveloped LPM. This hypothesis is consis-
tent with the recent findings of Yona et al. (5), who observed monocyte-derived cells in the LPM compartment 8 wk after i.p. injection of thioglycollate.

Although macrophages are generally thought to arise from circulating monocytes (1), recent studies indicate that many tissue macrophage compartments arise from yolk sac–derived precursors and persist into adulthood independently of hematopoiesis (reviewed in Ref. 48). Schulz et al. (4) found that, in general, F4/80 expression by tissue macrophages correlated with yolk sac (F4/80<sup>hi</sup>) versus hematopoietic (F4/80<sup>low</sup>) descent, although the origins of peritoneal macrophages were not addressed in that study. Our BrdU-uptake and lineage-tracking studies (Fig. 2) support the hypothesis that F4/80<sup>hi</sup> LPM are long-lived and require little hematopoietic input for steady-state maintenance (5, 21, 22), whereas F4/80<sup>low</sup> SPM are short-lived products of hematopoiesis. However, in adoptive-transfer experiments, we observed that a small percentage (<10%) of SPM transferred into wild-type mice acquired the F4/80<sup>hi</sup>CD11c<sup>lo</sup>CD93<sup>hi</sup> phenotype of LPM (Fig. 6A), suggesting some degree of LPM generation from SPM in steady-state conditions. Our findings do not eliminate the possibility that LPM originate from yolk sac precursors; if they do, then observations of LPM phenotype cells in BM chimeras (Fig. 2D) and SPM adoptive-transfer experiments (Fig. 6A) indicate a pathway in which a yolk sac–derived macrophage compartment can be replaced by products of hematopoiesis. Demand-driven processes of differentiation from hematopoietic precursors may represent a general mechanism for the replacement of tissue macrophages of yolk sac origin.

Our analysis of peritoneal leukocytes extends previous observations of functional heterogeneity among macrophage and DC populations in the peritoneal cavity (10, 49). Peritoneal DC, SPM, and LPM were distinct in their capacities to phagocytose apoptotic thymocytes (LPM > SPM > DC; Fig. 1C), DC, SPM, and LPM also differed in cytokine responses to LPS (Fig. 1B). Some of our observations are consistent with those of Dioszeghy et al. (49), who analyzed cytokines produced by F4/80<sup>hi</sup>CD11b<sup>+</sup> cells (LPM) and F4/80<sup>low</sup>CD11b<sup>+</sup> cells (likely representing a mixture of SPM). SPM produced more G-CSF and less IL-12p40 than did F4/80<sup>low</sup> B cells. Our findings do not eliminate the possibility that LPM from the recent finding of eosinophil support for macrophage homeostasis in the LPM compartment 8 wk after i.p. injection of thioglycollate.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Cytokine profiles of SPM, LPM, and DC following *in vitro* exposure to LPS.

SPM, LPM, and DC were sorted from peritoneal lavages of C57BL/6 mice and then stimulated overnight with LPS. Supernatants were analyzed for cytokines using a multiplex cytokine array. The mean+SD concentration of each cytokine is shown (n=3-7 per cohort). Statistical significance between LPS-treated cohorts is designated by letters above each bar; cohorts exhibiting significant differences (P<0.05) bear different letter designations. In cohorts labeled with an asterisk, some or all of the samples gave readings that exceeded the range of the standard curve; for these samples, the maximum value of the standard curve was used.
Supplemental Figure 2: Cytokine profiles of peritoneal macrophages of C/EBPβ+/+ and C/EBPβ−/− mice following in vitro treatment with LPS. IgM−CD11c−CD11bhiSSC$h$MHCIihi cells (“SPM”) and IgM−CD11c−CD11bhiSSC$h$MHCIilow cells (“LPM”) were sorted from peritoneal lavages of C/EBPβ+/+ (open bars) and C/EBPβ−/− (closed bars) mice and then stimulated overnight with LPS. Supernatants were analyzed for cytokines using a multiplex cytokine array. The mean±SD concentration of each cytokine is shown (n=4 per cohort). * P≤0.05, ** P≤0.01.

Supplemental Figure 2: Cain et al.
Supplemental Figure 3. Peritoneal eosinophilia but minimal inflammation in naïve C/EBPβ−/− mice. (A) Representative CD11b/SSC plots of IgM−CD11c− cells in C/EBPβ+/+ and C/EBPβ−/− mice.
(B) The mean (± SD) numbers of IgM−CD11c− cells in C/EBPβ+/+, C/EBPβ+−/−, and C/EBPβ−/− mice (left panel) and in LysMWT/WT/C/EBPβ−/− and LysMCre/WT/C/EBPβfl/fl mice (right panel). * P<0.05, ** P<0.01. (C) Isotype control (gray histograms) vs. CD115 and Siglec F staining (open histograms) of IgM−CD11c−CD11bintSSChi cells in C/EBPβ+/+ and C/EBPβ−/− mice. (D) Mean (± SD) numbers of Ly6G−CD11b+SSChi eosinophils in the bone marrow (2 femurs+2 tibiae) and blood of C/EBPβ+/+, C/EBPβ+−/−, and C/EBPβ−/− mice. n=4 mice per genotype. (E) C/EBPβ+/+ and C/EBPβ−/− mice were analyzed for peritoneal neutrophils and inflammatory monocytes (Gr-1+Ly-6B+ cells) by FACS. Data are representative of 4 independent experiments, n=3-7 mice. (F) The mean (± SD) concentrations of 23 cytokines in the sera of C/EBPβ+/+ and C/EBPβ−/− mice are shown. Data represent one experiment, n=3 mice per genotype.

Supplemental Figure 3: Cain et al.
Supplemental Figure 4. Histological analysis of macrophage compartments in the spleen and mesenteric lymph nodes of C/EBPβ heterozygous mice. (A) Spleen sections from C/EBPβ-sufficient and deficient mice were stained for F4/80 (red), MOMA-1 (green), SIGN-R1 (pink), B220 (blue), and TCRβ (yellow). (B) Sections of mesenteric lymph nodes from C/EBPβ-sufficient and deficient mice were stained for F4/80 (red), MOMA-1 (green), and B220 (blue). Scale bar = 100 μm.