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Inflammatory Spleen Monocytes Can Upregulate CD11c Expression Without Converting into Dendritic Cells

Scott B. Drutman, Julia C. Kendall, and E. Sergio Trombetta

Monocytes can differentiate into various cell types with unique specializations depending on their environment. Under certain inflammatory conditions, monocytes upregulate expression of the dendritic cell marker CD11c together with MHC and costimulatory molecules. These phenotypic changes indicate monocyte differentiation into a specialized subset of dendritic cells (DCs), often referred to as monocyte-derived DCs or inflammatory DCs (iDCs), considered important mediators of immune responses under inflammatory conditions triggered by infection or vaccination. To characterize the relative contribution of cDCs and iDCs under conditions that induce strong immunity to coadministered Ags, we analyzed the behavior of spleen monocytes in response to anti-CD40 treatment. We found that under sterile inflammation in mice triggered by CD40 ligation, spleen monocytes can rapidly and uniformly exhibit signs of activation, including a surface phenotype typically associated with their conversion into DCs. These inflammatory monocytes remain closely related to their monocyte lineage, preserving expression of CD11c, scavenging function, tissue distribution and poor capacity for Ag presentation characteristic of their monocyte precursors. In addition, 3–4 d after delivery of the inflammatory stimuli, these cells reverted to a monocyte-associated phenotype typical of the steady state. These findings indicate that, in response to anti-CD40 treatment, spleen monocytes are activated and express certain DC surface markers without acquiring functional characteristics associated with DCs. *The Journal of Immunology*, 2012, 188: 3603–3610.

The functional specializations of dendritic cells (DCs) and monocytes–macrophages have been a topic of much investigation, with recent focus on their developmental lineages as a way of understanding the relationships between these two cell types. Under steady state conditions, monocytes act as versatile cells that can convert into a variety of tissue-resident and lymphoid organ macrophage subsets. Under these same conditions, conventional DCs (cDCs) derive from a specialized precursor that shares a common progenitor to, but is distinct from, monocytes (1, 2). This lineage separation is paralleled by a divergence of functional specializations. The monocyte–macrophage lineage is specialized for robust Ag scavenging and secretion of inflammatory cytokines, but their capacity to convert internalized Ag into peptide–MHC complexes is poor. In contrast, cDCs are specialized for the efficient conversion of small amounts of captured Ag into peptide–MHC complexes, migration to T cell zones, and initiation of T cell responses (3, 4).

During inflammation, the plasticity of monocytes may also extend to the formation of certain subsets of dendritic cells (DCs), making it difficult to distinguish between these two lineages. Monocyte-derived DCs include TNF/iNOS-producing DCs and other inflammatory DCs (iDCs) described under microbial infec-

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Abbreviations used in this article: cDC, conventional dendritic cell; DC, dendritic cell; iDC, inflammatory dendritic cell; KO, knockout; MHC-II, MHC class II.

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Materials and Methods

*Mice*

C57BL/6 (B6), OT-I/RAG1 (OT-I), OT-II2.a/RAG1 (OT-II), and B6.SJL (CD45.1) mice were obtained from Taconic Farms. B6.129P2-Cd40<sup>tm1Kik</sup>/C211 mice were obtained from Taconic Farms. B6.129P2-Cd40<sup>tm1Kik</sup>/C211 mice were obtained from Taconic Farms.
(CD40 KO) were obtained from The Jackson Laboratory. Mice were housed under specific-pathogen–free conditions and maintained in compliance with institutional and federal regulatory guidelines. anti-CD40 mAb mediated inflammation was achieved by i.p. injection of 100 μg FGK4.5 mAb (Bio X Cell) or clone IC10 (LEAF grade, BioLegend). Rat IgG2a isotype control (LEAF grade, BioLegend) was used for control injections. Each injection of Ab (used to induce inflammation) or Ag (to study endocytosis or Ag presentation to T cells), contained undetectable levels of endotoxin, lower than 0.126 EU (∼13 pg) based on LAL test (Cambrex).

Cells

Unless otherwise specified, all cells were washed and resuspended in PBE (PBS with 0.5% BSA, endotoxin free; Equitech-Bio; 1 mM EDTA). Splenocytes were digested with Liberase Blendzyme 2 (Roche Diagnostics) for 15 min in PBS at 21°C, passed through a 40-μm cell strainer, treated with ACK Buffer (Lonza) to remove red cells, and resuspended in PBE. For purification of cells for in vitro Ag presentation experiments or for transfer experiments, splenocytes were first enriched by magnetic negative depletion with biotinylated Abs against CD19 (MB19.1), CD3 (145-2C11), NK1.1 (PK136), Ly-6G(1A8), and erythroid cell marker (TER-119) Abs (eBioscience or BioLegend), followed by enrichment using the EasySep Biotin Selection Kit (StemCell Technologies). Cells were subsequently incubated 1.8 h with anti-CD40 mAb or with negative, PE negative), from cells with particles stuck to their surface to distinguish cells that completely internalized particles (fluorescein brightness units at 600 nm, then at 24˚C for 16 h with 1 mM isopropyl β-D-thiogalactoside (Sigma). Cells were lysed with lysozyme, sonication, and freeze–thaw cycles, and the his-tagged protein was affinity purified on Ni-Sepharose (Pharmacia). The resulting protein was further purified by ion exchange with Q-Sepharose (Pharmacia). The resulting protein had <1.26 EU/mg of endotoxin (≤125 pg/mg) by LAL test (Cambrex). For in vivo soluble Ag endocytosis assays, 2 mg GFP was injected i.v., and 30 min later splenocytes were collected and analyzed for Ag capture as compared with a similarly treated mouse not injected with Ag. Endotoxin-free OVA (prepared as described below for Ag-presentation assays) at 10 mg/ml in PBS was labeled using fluorescein-5-isothiocyanate (Invitrogen) according to the manufacturer’s instructions. Excess unincorporated fluorescein was removed over a Sephadex G-25 column (GE Healthcare) followed by several buffer exchanges with PBS using an Amicon Ultra-4 centrifugal filter device (Millipore) and filtration over a Polymixin-B-Sepharose column (Pierce). For endocytosis assays, mice were injected with 900 μg FITC-OVA, and 20 min later splenocytes were analyzed by flow cytometry to detect FITC-OVA uptake. Fluorescent particulate Ag was prepared by incubating 1.8 μm streptavidin-coated polystyrene microbeads (Spherotech) at 2.8×10^9/ml with 10 μg/ml Fluorescein-biotin (Invitrogen) in PBS for 30 min and washed in PBE. For 2-μm particle uptake assays, 1.4×10^8 particles were injected i.v. and 60 min later splenocytes were collected and analyzed for Ag capture. Cells were also stained with PE-conjugated anti-fluorescein (Invitrogen) to distinguish cells that completely internalized particles (fluorescein positive, PE negative), from cells with particles stuck to their surface (fluorescein positive, PE positive). For 5-μm particle endocytosis assays, 0.8×10^8 Fluoresbrite YG carboxylate microspheres (Polysciences) were injected i.v. and 30 min later splenocytes were collected and analyzed for Ag capture. Results are expressed as mean ± SD from the mean.

Endocytosis assays

Soluble GFP protein was prepared as described previously (31). The construct in pET-28 vector (Novagen) in BL21 Escherichia coli (Novagen) was grown in TB media (Invitrogen) at 37°C to a density of ∼0.1 absorbance units at 600 nm, then at 24˚C for 16 h with 1 mM isopropyl β-D-thiogalactoside (Sigma). Cells were lysed with lysozyme, sonication, and freeze–thaw cycles, and the his-tagged protein was affinity purified on Ni-Sepharose (Pharmacia). The resulting protein was further purified by ion exchange with Q-Sepharose (Pharmacia). The resulting protein had <1.26 EU/mg of endotoxin (≤125 pg/mg) by LAL test (Cambrex). For in vivo soluble Ag endocytosis assays, 2 mg GFP was injected i.v., and 30 min later splenocytes were collected and analyzed for Ag capture as compared with a similarly treated mouse not injected with Ag. Endotoxin-free OVA (prepared as described below for Ag-presentation assays) at 10 mg/ml in PBS was labeled using fluorescein-5-isothiocyanate (Invitrogen) according to the manufacturer’s instructions. Excess unincorporated fluorescein was removed over a Sephadex G-25 column (GE Healthcare) followed by several buffer exchanges with PBS using an Amicon Ultra-4 centrifugal filter device (Millipore) and filtration over a Polymixin-B-Sepharose column (Pierce). For endocytosis assays, mice were injected with 900 μg FITC-OVA, and 20 min later splenocytes were analyzed by flow cytometry to detect FITC-OVA uptake. Fluorescent particulate Ag was prepared by incubating 1.8 μm streptavidin-coated polystyrene microbeads (Spherotech) at 2.8×10^9/ml with 10 μg/ml Fluorescein-biotin (Invitrogen) in PBS for 30 min and washed in PBE. For 2-μm particle uptake assays, 1.4×10^8 particles were injected i.v. and 60 min later splenocytes were collected and analyzed for Ag capture. Cells were also stained with PE-conjugated anti-fluorescein (Invitrogen) to distinguish cells that completely internalized particles (fluorescein positive, PE negative), from cells with particles stuck to their surface (fluorescein positive, PE positive). For 5-μm particle endocytosis assays, 0.8×10^8 Fluoresbrite YG carboxylate microspheres (Polysciences) were injected i.v. and 30 min later splenocytes were collected and analyzed for Ag capture. Results are expressed as mean ± SD from the mean.
were analyzed by flow cytometry. In vivo, 1.0 mg OVA protein was injected i.v. into mice, and 30 min later cells were purified by cell sorting as described above. Various numbers of APCs were cocultured with 50,000 CFSE-labeled OT-II CD4+ T cells or OT-II CD4+ T cells in U-bottom 96-well plates. Sixty hours later, T cell proliferation was assessed using flow cytometry to measure the dilution of CFSE accompanying each T cell division. Cells were cultured in RPMI 1640 (Life Technologies) with 10% heat-inactivated FBS (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, 50 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 100 μM mercaptoethanol (Sigma) in a 5% CO2 37˚C incubator.

Monocyte tracking with beads

Experiments were performed as previously described (25, 29). Mice were injected i.v. with 50 μl (2.3 × 10⁶) Fluoresbrite Carboxylate YG 1.0-μm microspheres (Polysciences) resuspended in PBS. At various times after bead injection, mice were injected with 100 μg anti-CD40 mAb i.p. or with control IgG or PBS as negative controls. At various time points after injection, splenocytes were purified and the cells containing the particiles were analyzed by flow cytometry.

Flow cytometry

Cells were preincubated with 10 μg/ml 2.4G2 mAb (Bio X Cell) for 15 min at 4˚C in PBE, incubated with mAb conjugates for 30 min at 4˚C, washed in PBE, and resuspended in PBE with 0.5 μg/ml 7-aminoactinomycin-D (Invitrogen) 10 min before analysis. Data were collected on a FACSCanto (BD Biosciences) and analyzed with FlowJo software (TreeStar). Mean ± SD of multiple experiments was calculated using Prism software (GraphPad Software). Abs: TCR-β (H57-597), CD8 (53-6.7), CD4 (GK1.5), CD86 (GL-1), CD80 (16-10A1), IA/E (114.15.2), H2-Kb (AF6-88.5), CD4 (7.4), CD115 (AFS98), F4/80 (BM8), Mac-3 (M3/84), CD14 (Sal5-2), Mac-2 (m3/38), CD4 (1B11), CD45.1 (A20), CD45.2 (104), CD8α (53-6.7), CD4 (GK1.5), CD86 (GL-1), CD80 (16-10A1), IA/E (MS/114.15.2), H2-Kb (AF6-88.5), CD40 (HM40-3), and isotype control in corresponding fluors (mouse IgG2a, Rat IgG1, Rat IgG2a, Rat IgG2b, Armenian Hamster IgG, Armenian Hamster IgM) were purchased from eBioscience or BioLegend.

Intracellular TNF-α assay

Mice were injected with 100 μg anti-CD40 mAb i.p., and 40 h later splenocytes were isolated and cultured in complete RPMI 1640 in the presence of media alone, 200 ng/ml LPS (from Salmonella typhimurium; Sigma), 5 μg/ml anti-CD40 mAb, or 5 × 10⁶/ml heat-killed Listeria (strain 10402S), and 5 μg/ml brefeldin A (Invitrogen). Sixty minutes later, cells were first stained for surface markers (see flow cytometry above) and then for intracellular TNF-α using Cytotix/Cytoperm (BD Biosciences) according to the manufacturer’s instructions. TNF-α was detected using biotin–anti-TNF-α (MP6-XT22; BioLegend), followed by streptavidin-PE (Invitrogen). Biotin-rat IgG1 (BioLegend) was used as an isotype control.

Confocal microscopy

Spleens were immersed in OCT media (Tissue-Tek) and frozen in an isopentane-liquid nitrogen bath; 10-μm cryosections were performed in acetone for 5 min at -20˚C. All subsequent steps were performed at room temperature. Sections were dried for 1 h, rehydrated in PBS for 10 min, and blocked with 5% goat serum and 5 μg/ml anti FcR-mAb (2.4G2; Bio X Cell). T cells were stained with 2 μg/ml rabbit anti-CD3 (Dako) for 1 h, followed by 0.16 mg/ml HRP conjugated anti-rabbit (Jackson Immunoresearch) for 1 h. T cells were first stained for surface markers (see flow cytometry above) and then for intracellular TNF-α using Cytotix/Cytoperm (BD Biosciences) according to the manufacturer’s instructions. TNF-α was detected using biotin–anti-TNF-α (MP6-XT22; BioLegend), followed by streptavidin-PE (Invitrogen). Biotin-rat IgG1 (BioLegend) was used as an isotype control.
gated CD11c (N418; BioLegend) for 1 h, and monocytes were stained with 0.6 μg/ml FITC-conjugated anti-Ly6C (HK1.4; Biolegend) for 1 h followed by 2 μg/ml Alexa488-conjugated goat anti-fluorescein (Invitrogen) for 1 h. Control stains were performed with normal rabbit serum or isotype controls labeled with Alexa647 or FITC. Sections were mounted with Prolong Gold (Invitrogen) and imaged with a Zeiss Plan Apochromat 10× 0.45NA objective on a Zeiss LSM510 microscope.

Results

**Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> monocytes accumulate in the spleen of mice after treatment with anti-CD40 mAb**

We focused on the two major populations of monocytes in the spleen: Ly6C<sup>Hi</sup>-CD11c<sup>Neg</sup> monocytes and Ly6C<sup>Neg</sup>-CD11c<sup>Low</sup> monocytes. Unlike monocytes, cDCs are Ly6C<sup>Neg</sup> and express high levels of CD11c, a marker that is typically used to identify these cells (Fig. 1A). To analyze the behavior of monocytes and the potential formation of iDCs in vivo under conditions that induce strong Ag-specific immunity, we studied mice treated with an agonistic Ab against CD40, which has proven efficacy to prime effective T cell responses in experimental animals (13–27).

We found that induction of systemic inflammation in mice with an anti-CD40 mAb induced CD11c expression on Ly6C<sup>Hi</sup> monocytes in the spleen, reaching levels comparable to cDCs in the same mouse within 40 h (Fig. 1B, 1C). The same induction of CD11c expression on Ly6C<sup>Hi</sup> monocytes was obtained with two different clones of anti-CD40 mAbs (Fig. 1D, 1E). The numbers inside plots represent the percentage of cells in the adjacent gate.
of agonistic anti-CD40 mAb, whereas this conversion did not occur in mice that lacked CD40 (Supplemental Fig. 1A).

These Ly6CHi-CD11cHi cells are also apparent in the CD11b versus CD11c plots commonly used to detect the appearance of iDCs (Supplemental Fig. 1B, 1C). This Ly6CHi-CD11cHi-CD11bHi phenotype is typical of iDCs described under various inflammatory stimuli. In mice treated with anti-CD40 for 40 h, we could identify four populations for further comparison: Ly6Chi monocytes, Ly6cneg monocytes, Ly6chi-CD11cHi cells, and CD11b+ cDCs (Fig. 1C). A comparison of these four populations shows that Ly6CHi-CD11cHi cells and CD11b+ cDCs express similar levels of CD11c, whereas Ly6cneg monocytes express low levels of CD11c and Ly6CHi monocytes do not express CD11c (Supplemental Fig. 1D). Although similar populations of Ly6CHi-CD11cHi cells could be detected in other organs after anti-CD40 treatment (Supplemental Fig. 1E) we focused our analysis on spleen-derived monocytes because of the abundance of these cells and to be able to establish a direct comparison with the well-characterized cDCs from that organ.

In addition to high levels of CD11c, these Ly6CHi-CD11cHi cells also expressed MHC-II and costimulatory molecules at levels similar to cDCs from the spleen of the same mouse, which is another surface phenotype typical of iDCs (Fig. 2A). Although these Ly6CHi-CD11cHi cells shared some surface characteristics with cDCs, the expression levels of other markers such as F4/80, Mac-2, Mac-3, CD14, and CD115 remained closer to their Ly6CHi monocyte precursors than to cDCs (Fig. 2A). The Ly6cneg monocytes in the spleen of the same animals retained lower MHC-II expression under the same conditions (Fig. 2B). These results show that treatment of mice with anti-CD40 mAb induces the appearance of a population of Ly6CHi-CD11cHi cells that shares surface markers with both cDCs and monocytes. For simplicity we will continue to refer to these monocyte-derived Ly6CHi-CD11cHi cells as iDCs, although this population likely differs from previously described inflammatory monocyte-derived iDCs, as shown below.

Spleen Ly6CHi-CD11cHi cells derive from monocytes

The appearance of Ly6CHi cells expressing high levels of CD11c following injection of anti-CD40 suggested that Ly6CHi-CD11cHi cells were the result of upregulation of CD11c on Ly6CHi-CD11cNeg monocytes in the spleen. However, these results could not exclude the possibility that the Ly6CHi-CD11cNeg population was disappearing and simultaneously replaced by unrelated populations with increasingly higher levels of CD11c. To distinguish between these alternatives, we used a bead-labeling protocol for tracking monocytes in situ (32–34). One hour after injection, beads were associated primarily with Ly6CHi and Ly6cneg monocytes, and to a lesser degree with cDCs (Fig. 3A, Supplemental Fig. 2). When control-treated mice (uninflamed) were analyzed 24 or 40 h after injection of beads without any inflammatory treatment, beads were associated almost exclusively with Ly6cneg monocytes, and there were almost no more bead-associated Ly6CHi monocytes (Fig. 3B, Supplemental Fig. 2), which was previously shown to reflect the conversion of the Ly6CHi to Ly6cneg monocytes (34–36). However, when mice were additionally treated with anti-CD40 only 15 min after bead injection, bead-associated Ly6CHi-CD11cHi cells could be identified easily, indicating the conversion of monocytes to Ly6CHi-CD11cHi cells (Fig. 2B). In these experiments, both Ly6CHi monocytes and Ly6cneg monocytes contained beads at the time of anti-CD40 administration, making it possible that both monocyte populations contributed to the Ly6CHi-CD11cHi cells.

To investigate the contribution of Ly6cneg monocytes to the Ly6CHi-CD11cHi population, mice were injected with beads and then rested for 24 h to allow the bead-labeled Ly6CHi monocytes to convert to bead-labeled Ly6cneg monocytes, as described previously (34) (Fig. 3C, Supplemental Fig. 2). These mice with beads almost exclusively limited to Ly6cneg monocytes were then injected with anti-CD40. Forty hours after treatment, beads were also found within Ly6CHi-CD11cHi cells, suggesting that Ly6cneg monocytes contribute to the formation of the Ly6CHi-CD11cHi population (Fig. 3D).

Because bead tracking in situ cannot selectively label the Ly6CHi monocytes without additional manipulations such as clodronate liposomes (33), we sorted Ly6CHi monocytes from CD45.1 mice and adoptively transferred them i.v. into syngeneic CD45.2 recipients (Fig. 3E). When recipient mice were control treated, we observed the subsequent conversion of these transferred Ly6CHi monocytes into Ly6cneg monocytes (Fig. 3F), as described previously (34–36). In contrast, when inflammation was induced with anti-CD40 after adoptive transfer, we observed upregulation of CD11c on the transferred Ly6CHi monocytes, which paralleled the induction of CD11c expression on the endogenous Ly6CHi population in the recipient (Fig. 3F). These results indicate that the Ly6CHi-CD11cHi cells formed under inflammation induced by anti-CD40 treatment were a result of upregulation of CD11c on splenic CD11cNeg monocytes.
To evaluate the role of CD40 on the responding splenic monocytes, we prepared mixed bone-marrow chimeras between wild type and CD40−/− mice (Supplemental Fig. 3). We found that ligation of CD40 on bone marrow-derived cells is required for the induction of Ly6CHi-CD11cHi cells, and the formation of this population is further enhanced when CD40 is also expressed on somatic cells (Supplemental Fig. 3A). In addition, wild type Ly6CHi monocytes transferred into CD40−/− recipients could not be induced to form Ly6CHi-CD11cHi cells, indicating that CD40 ligation on the monocytes alone is insufficient, but a variety of cell types contribute to the inflammatory response to anti-CD40 (Supplemental Fig. 3B).

Ly6CHi-CD11cHi cells functionally resemble activated monocytes

The apparent activation of Ly6CHi monocytes in mice treated with anti-CD40 led to a surface phenotype with similarities to both cDCs and their Ly6CHi monocyte precursors; therefore, we wanted to determine whether these iDCs exhibit functional characteristics of cDCs. To this end, we compared the endocytic capacity and Ag-presenting capacities of Ly6CHi monocytes, iDCs (Ly6CHi-CD11cHi), and cDCs, all of them isolated from the same mice treated with anti-CD40. To examine their phagocytic capacity in situ, mice were injected i.v. with 2-μm fluorescent particles. As expected, Ly6CHi monocytes showed a higher phagocytic capacity compared with cDCs (Fig. 4A). In these same mice, the Ly6CHi-CD11cHi cells also demonstrated a higher phagocytic capacity than did cDCs, similar to their monocyte precursors. Interestingly, this phagocytic capacity was enhanced compared with their monocyte precursors, suggesting that activation of this function accompanied their phenotypic transformation (Fig. 4A). When similar experiments were performed using larger (5 μm) fluorescent beads, we found that Ly6CHi monocytes could still internalize these large particles, which could not be internalized by cDCs (Fig. 4B). In contrast to cDCs, the Ly6CHi-CD11cHi cells exhibited a similar or enhanced ability to capture large particles compared with their monocyte precursors (Fig. 4B). A similar result was also observed after injection of soluble proteins as an endocytosis probe (GFP [Fig. 4C] or FITC-OVA [Fig. 4D]). Ly6CHi monocytes exhibited a markedly higher endocytic activity than cDCs, whereas Ly6CHi-CD11cHi cells exceeded both of these populations (Fig. 4C, 4D).

Having established the endocytic capacity of these three cell populations, we next evaluated their capacity to present the internalized Ags to T cells (Fig. 5A). To this end, mice treated with anti-CD40 were injected i.v. with 1 mg OVA, and 30 min later the spleen cDCs, Ly6CHi-CD11cHi (iDCs), and Ly6CHi-CD11cNeg (Ly6CHi monocytes) cells were isolated by cell sorting and cocultured with OT-I or OT-II T-cells. We found that, despite their comparatively weaker endocytic capacity (Fig. 4), cDCs were much more effective than Ly6CHi-CD11cHi or Ly6CHi-CD11cNeg monocytes in presenting Ag on both MHC-I and MHC-II and stimulating cognate T cells. cDCs induced strong T cell proliferation, even at low cDC:T cell ratios. Under these same conditions, in which the Ly6CHi-CD11cHi and Ly6CHi-CD11cNeg monocytes had shown high levels of Ag capture (Fig. 4), both populations induced little, if any, CD4+ or CD8+ T cell proliferation (Fig. 5A).

**FIGURE 5.** Ly6CHi-CD11cHi cells retain functional properties of monocytes. (A) Despite a large capacity for Ag capture, Ly6CHi-CD11cHi cells do not present the internalized Ag to T cells, a feature similar to monocytes but in contrast to dendritic cells. Mice were treated with anti-CD40 and 40 h later injected i.v. with 1 mg OVA protein; 30 min after OVA injection, splenocytes were harvested and Ly6CHi-CD11cHi cells, Ly6CHi monocytes, and CD11bCHi-CD11cHi cDCs were isolated from the same spleen and separately cocultured with CFSE-labeled OT-I or OT-II T-cells. We found that, despite their high levels of Ag capture (Fig. 4), both populations induced little, if any, CD4+ or CD8+ T cell proliferation (Fig. 5A).

**FIGURE 6.** Similar to Ly6CHi monocytes, Ly6CHi-CD11cHi cells ultimately differentiate into Ly6CHiNeg monocytes. (A) The disappearance of Ly6CHi-CD11cHi cells 90 h after anti-CD40 treatment correlates with an increase in Ly6CHiNeg monocytes. (B) Similar to Fig. 3F, purified CD45.1 monocytes were injected into CD45.2 mice, which were immediately injected with anti-CD40 mAb; 90 h later, the phenotype of the endogenous and transferred splenic monocytes was analyzed by flow cytometry. (C) Similar to Fig. 3B, mice were injected with 1.0-μm YG beads and then immediately injected with anti-CD40 mAb; 90 h later the phenotype of the bead-containing cells was analyzed and overlaid over all the cells. Data are representative of three experiments, two mice per group.

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Having established the endocytic capacity of these three cell populations, we next evaluated their capacity to present the internalized Ags to T cells (Fig. 5A). To this end, mice treated with anti-CD40 were injected i.v. with 1 mg OVA, and 30 min later the spleen cDCs, Ly6CHi-CD11cHi (iDCs), and Ly6CHi-CD11cNeg (Ly6CHi monocytes) cells were isolated by cell sorting and cocultured with OT-I or OT-II T-cells. We found that, despite their comparatively weaker endocytic capacity (Fig. 4), cDCs were much more effective than Ly6CHi-CD11cHi or Ly6CHi-CD11cNeg monocytes in presenting Ag on both MHC-I and MHC-II and stimulating cognate T cells. cDCs induced strong T cell proliferation, even at low cDC:T cell ratios. Under these same conditions, in which the Ly6CHi-CD11cHi and Ly6CHi-CD11cNeg monocytes had shown high levels of Ag capture (Fig. 4), both populations induced little, if any, CD4+ or CD8+ T cell proliferation (Fig. 5A).
In agreement with their high scavenging and poor Ag presentation activities, Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> monocyte-derived cells were found predominantly in the red pulp of the spleen of inflamed mice, but they were essentially absent from T cell areas, in contrast with cDCs (Fig. 5B). Finally, we examined the ability of each population to produce inflammatory cytokine TNF-α after further stimulation. Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> cells, cDCs, and resident spleen monocytes all had the capacity to produce TNF-α upon restimulation in vitro (Supplemental Fig. 4). In conclusion, although the surface phenotype of Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> monocyte-derived cells shares some markers with cDCs, they share other surface markers, similar scavenging, and poor Ag-presenting characteristics of their monocyte precursors, and they consequently do not appear to acquire functional properties associated with cDCs.

**Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> cells ultimately convert into Ly6C<sup>Neg</sup> monocytes**

We considered the possibility that the conversion of Ly6C<sup>Hi</sup> monocytes to a cDC-like phenotype could take longer, and we consequently followed the fate of these cells at later time points after delivery of anti-CD40 stimulation. We found that up to 5 d after injection of anti-CD40, a decrease in Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> cells correlated with an increase in Ly6C<sup>Neg</sup>-CD11c<sup>Neg</sup> monocytes (Fig. 6A). To determine whether this reduction in the numbers of Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> cells could indicate their further conversion into cDC-like population, we performed adoptive transfer experiments for longer periods, after Ly6C<sup>Hi</sup>-CD11c<sup>Hi</sup> cells had apparently disappeared. Interestingly, the transferred Ly6C<sup>Hi</sup> monocytes, which convert into Ly6C<sup>Neg</sup>-CD11c<sup>Neg</sup> cells after 24 to 40 h (Fig. 3F), subsequently converted into Ly6C<sup>Neg</sup>-CD11c<sup>Neg</sup> monocytes (Fig. 6B); this is similar to the fate of Ly6C<sup>Hi</sup> monocytes in the steady state (Fig. 3F) (34–36). In addition, when we mapped the fate of these cells using the bead tracking techniques as in Fig. 3B, we also found that the Ly6C<sup>Neg</sup>-CD11c<sup>Neg</sup> cells had converted into Ly6C<sup>Neg</sup> monocytes at later time points (Fig. 6C).

**Discussion**

Our results indicate that under sterile systemic inflammatory conditions induced with anti-CD40, Ly6C<sup>Hi</sup> monocytes induce CD11c expression together with other surface markers typically associated with their acquisition of DC-like phenotype. However, such monocyte-derived CD11c<sup>Hi</sup> cells retain many characteristics of monocytes, including several surface markers, a high endocytic capacity, and inefficient conversion of internalized Ags into peptide–MHC complexes for T cell stimulation. In addition, these cells follow the fate of their monocycte lineage, ultimately converting into Ly6C<sup>Neg</sup> monocytes. These findings demonstrate that the upregulation of CD11c by monocytes and the conversion to DCs can be distinct processes; therefore, under certain inflammatory conditions, the expression of CD11c, MHC-II, and costimulatory molecules is insufficient to identify a population with similar properties as cDCs. Although not observed in this study, the expression of CD11c on other cell types, such as NK cells (37–39), may also be subject to regulation under inflammatory conditions.

The results presented in this and previous studies have shown that the material scavenged by monocyte and some monocyte-derived iDCs is apparently not presented to T cells efficiently (3, 9, 40). Given the abundance of iDCs and their vigorous scavenging capacity, it is important to establish the fate of such scavenged material, which can be transferred to other cell types, including Ag-presenting cDCs, even as preformed pMHC complexes (3, 41–46).

In prior studies describing monocyte-derived DCs, it has not been easy to distinguish between fully converted monocytes and functional DCs (47, 48). In previously described models relying on peritonitis induced by long-term effects of emulsified adjuvants or microbial infections, it has been difficult to establish a direct precursor-product relationship between monocytes and cDCs without ruling out alterations in the properties of cDC or pre-DC progenitors (7, 48). A recent study has shown that components from Gram-negative bacteria such as LPS can induce the conversion of blood monocytes into Ag-presenting monocyte-derived DCs migrating to lymph nodes (8). Given that conversion of monocytes into Ag-presenting DCs does not appear to be complete under certain inflammatory conditions with similar components derived from Gram-negative bacteria (49) or under sterile inflammation as reported in this study, it will be important to define the conditions that induce the full conversion of monocytes into iDCs capable of T cell stimulation.

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**Disclosures**

The authors have no financial conflicts of interest.

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**Legends for Supplemental Figures**

**Supplemental Figure 1. A.** Anti-CD40 antibody treatment is mediated by CD40. WT mice were either left untreated, injected with 100 μg RatIg2a isotype control, or with 100 μg of anti-CD40 (clone IC-10), or anti-CD40 (clone FGK4.5). Additionally CD40-/- mice were injected with anti-CD40 (FGK4.5). Forty eight hours later, spleens were analyzed for populations of interest. Cells were gated as described in figure 1A. **B.** Appearance of Ly6Chi-CD11cHi cells in CD11b v. CD11c plots. Gating scheme, similar to figure 1A, except cells are only gated though the first 5 gates and displayed on a CD11c v. CD11b plot. **C.** Appearance of Ly6Chi-CD11cHi cells in CD11b v. CD11c plots at various time points after injection of mice with 100 μg anti-CD40 mAb. **D.** Comparison of surface CD11c levels of cDCs, Ly6CHi monocytes, Ly6CNeg monocytes, and Ly6CHi-CD11cHi cells. **E.** Similar populations of Ly6CHi-CD11cHi cells can be detected in the bone marrow (BM), mesenteric lymph nodes (MLN) and the blood of mice treated with anti-CD40 mAb.

**Supplemental Figure 2. Fate of Ly6CNeg monocytes using bead labeling.** **A.** Mice were injected with fluorescent beads and then at the indicated times monocytes (lineage negative, CD11b+, CD115+ cells) were analyzed for bead content. **B.** Similar to experiments in figure 3, mice were injected with fluorescent beads and then analyzed as indicated.
Supplemental Figure 3. Requirements for CD40 expression on monocyte activation in response to anti-CD40. (A). Wild type or CD40/-/- mice were treated with anti-CD40 mAb for 24 hours to verify the specificity of the anti-CD40 and the requirement of CD40 for the response. Mixed bone-marrow chimeras were prepared by lethally irradiating wt or CD40/-/- mice and reconstituting them with bone marrow cells from wt or CD40/-/- as indicated. Mice were subsequently treated with anti-CD40 mAb and the spleens analyzed after 24 hr. (B). Ly6C<sup>hi</sup> monocytes were isolated from wt mice (CD45.1) and transferred into CD40/-/- recipients (CD45.2). The recipient mice were either control-treated (PBS or rat IgG control, left column) or treated with anti-CD40 mAb (right column). 24 hours after treatment spleens were harvested for analysis as indicated.

Supplemental Figure 4. Production of TNF-α by populations in the spleens of mice 40 hrs after anti-CD40 treatment. Mice were treated with anti-CD40 for 40 hrs to induce the appearance of Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells. Splenocytes were harvested and cultured in the presence of Brefeldin A (5 μg/ml) in either media alone, media with LPS (200 ng/ml), media with anti-CD40 mAb (5 μg/ml), or HK Listeria (5x10<sup>7</sup>/ml) for 60 minutes. Cells were then analyzed for intracellular TNF-α by flow cytometry.
Supplemental Figure 2

A

Time after bead injection: 1h 24h 40h 48h

all monocytes

% cells

Bead + monocytes

% cells

Ly6C

B

Time after bead injection: 1h 24h 40h 48h 64h 88h 138h

cells w/ beads

cells w/ beads

all cells

overlay

CD11c

CD11c

CD11c

CD11c

CD11c

CD11c

CD11c

CD11c
Supplemental Figure 3

A

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B

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