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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 CD115, 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-

cations or adjuvant-induced peritonitis (1, 5–8). Monocyte-derived iDCs are characterized as DCs based on the expression of surface markers characteristic of cDCs in the spleen and lymph nodes, namely high surface expression of CD11c, as well as MHC-II and costimulatory molecules. Some iDCs were found to be dispensable for Ag presentation and T cell priming (9), whereas other iDCs were proposed to contribute to T cell stimulation (8, 10–12). Given the variety in inflammatory settings under which these iDCs arise, it is unclear whether the various iDCs reported represent related populations with common functional properties or they encompass a spectrum of different monocyte-derived cell types.

Because most studies describing conversion of monocytes into iDCs rely on processes that last several days or weeks, we sought to evaluate the conversion of monocytes into iDCs in vivo under conditions that induce potent Ag-specific immunity. We studied the response of mice to anti-CD40 treatment, which has proven efficacy to prime effective T cell responses in experimental animals (13–27) and has shown significant clinical potential (28–30). We found that induction of systemic inflammation in mice with an activating Ab against CD40 uniformly induced surface CD11c expression on Ly6CHi monocytes. These cells also expressed MHC class II (MHC-II) and costimulatory molecules typically associated with DC-like phenotypes ascribed to iDCs. However, these Ly6CHi-CD11cHi monocyte-derived iDCs share functional properties with their Ly6CHi-CD11cNeg precursors, not with Ly6CNeg-CD11cHi cDCs. In addition, this phenotypic change accompanies an increase in endocytic capacity, highlighting their activated monocyte phenotype. After 3–4 d, this Ly6CHi-CD11cHi monocyte-derived population reverts to a surface phenotype characteristic of monocytes, further supporting the continuity of the DC-independent lineage.

Materials and Methods

Mice

C57BL/6 (B6), OT-I/RAG1 (OT-I), OT-IIa/RAG1 (OT-II), and B6.SJL (CD45.1) mice were obtained from Taconic Farms. B6.129P2-Cd40tm1Kik/J
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 treated 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 sorted on a Dako MoFlo. Postsort analysis confirmed purity of >96% and viability of >95%. OT-I CD8+ or OT-II CD4+ T cells were isolated from the lymph nodes and spleens of OT-I/RAG1 knockout (KO) or OT-II/RAG1 KO mice by disruption through a 40-μm cell strainer, followed by negative selection using mouse CD8+ T cell or mouse CD4+ T cell enrichment kit, respectively (StemCell Technologies). Enriched T cells were pulse with 0.5 mM CFSE (Invitrogen) for 5 min, washed twice, and resuspended in complete RPMI 1640.

Monocyte transfer experiments

Ly6C+ monocytes (as identified in Fig. 1A) were purified from spleens of CD45.1 mice by cell sorting. Purified cells (5.0 × 10⁶) were injected i.v. into mice that were injected either with 100 μg anti-CD40 mAb or with control IgG or PBS as negative controls 5 min after cell transfer. At various time points after transfer, spleens were analyzed by flow cytometry, and the phenotypes of the endogenous (CD45.2) and transferred (CD45.1) cells were assessed.

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 spleens 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 × 10⁹/mg with 10 μg/ml Fluorescin-biotin (Invitrogen) in PBS for 30 min and washed in PBE. For 2-μm particle uptake assays, 1.4 × 10⁹ 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 × 10⁹ Fluoresbrite YG carboxylate microspheres (Polysciences) were 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 spleens 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 × 10⁹/mg with 10 μg/ml Fluorescin-biotin (Invitrogen) in PBS for 30 min and washed in PBE. For 2-μm particle uptake assays, 1.4 × 10⁹ 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 × 10⁹ Fluoresbrite YG carboxylate microspheres (Polysciences) were

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**FIGURE 1.** Ly6C+CD11c+ cells appear in the spleens of mice during CD40-mediated inflammatory responses. (A) Gating scheme for identification of spleen subsets by flow cytometry showing the Ly6C versus CD11c plots used for the identification of Ly6C+ monocytes, Ly6C+CD11c+ monocytes, and CD11b+ DCs. (B) Mice were injected with 100 μg anti-CD40 mAb, and spleen monocytes were analyzed at various time points after injection. (C) Identification of four populations in mice treated with anti-CD40 for 40 h. (D) Plots shown in (B) were gated on Ly6C+ cells (solid line) for analysis of CD11c expression levels over time and compared with the levels expressed by CD12cs (dotted line) from the same mouse. Data are representative of five experiments, three mice per group. Results are expressed as mean ± SD from the mean.
injected i.v., and 60 min later splenocytes were collected and analyzed for Ag capture.

Ag-presentation assays

OVA protein (grade IV; Sigma) was purified to remove any potential endotoxin contamination by ion exchange using Q-Sepharose (Pharmacia) as described previously (31). To assay presentation of Ag captured by cells 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-I CD8+ 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, 2 mM L-glutamine, and 100 μM mercaptoethanol (Sigma) in a 5% CO2 37°C incubator.

Intracellular TNF-α

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 × 10^5/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 Cytofix/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 cut in acetone at 4°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% normal goat serum. Sections were then incubated with mAbs directed against Mac-2 (m3/38), CD11c (N418), Ly-6C (HK1.4), CD115 (AFS98), F4/80 (BM8), Mac-3 (M3/84), CD14 (Sa14-2), Mac-2 (m3/38), CD43 (1B11), CD45.1 (A20), CD45.2 (104), CD8a (53-6.7), CD4 (GK1.5), CD86 (GL-1), CD80 (16-10A1), IA/E (M5/114.15.2), H2-Kb (AF6-88.5), CD40 (HM40-3), and isotype control in corresponding fluorochromes (mouse IgG2a, Rat IgG1, Rat IgG2a, Rat IgG2b, Armenian Hamster IgG, Armenian Hamster IgM) were purchased from eBioscience or BioLegend.

FIGURE 2. Despite their high expression of CD11c, Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells maintain a surface phenotype similar to activated Ly6C<sup>hi</sup> monocytes. (A) Comparison of the surface expression of MHC and costimulatory molecules on Ly6C<sup>hi</sup> monocytes, Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells, and CD11b<sup>+</sup> cDCs from the spleen of the same mouse 40 h after anti-CD40 treatment. Populations were identified as shown in Fig. 1D. (B) Similar to (A), surface phenotype of Ly6C<sup>neg</sup> monocytes was analyzed in spleens of either control-treated mice or in mice treated with anti-CD40 for 40 or 90 h. Also shown is the surface phenotype of Ly6C<sup>neg</sup>-CD11c<sup>hi</sup> cells from the mice treated with anti-CD40 for 40 h. In all cases, the indicated surface marker staining (solid line) was compared with staining obtained with an isotype control (dashed line). Data are representative of four experiments, two mice per group.
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 (Fig. 1A). 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> cells was obtained with two different clones

**FIGURE 3.** *Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells arise from monocytes in vivo.* (A) Mice were injected with 1.0-μm YG beads, and 1 h later cells that had internalized beads (mostly monocytes) were analyzed and overlaid onto all cells. (B) Mice were injected with 1.0-μm YG beads and then either control-treated or injected with anti-CD40 mAb. To track the fate of the cells that had internalized beads, 24 or 40 h later, the phenotype of the bead-containing cells was analyzed and overlaid onto all the cells. (C) Similar to (A), but cells were examined 24 h after bead injection. (D) Similar to (B), but mice were injected with anti-CD40 24 h after injection of beads. (E) Spleen Ly6C<sup>hi</sup> monocytes were sorted to purity from CD45.1 mice using the gates shown in Fig. 1. (F) Approximately 5 × 10<sup>4</sup> purified monocytes as shown in (E) were injected into CD45.2 mice that were either control treated, or immediately injected with anti-CD40 mAb. Twenty-four or 40 h later, the phenotype of the endogenous and transferred spleen monocytes was analyzed by flow cytometry. Data are representative of three experiments, two mice per group. 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 Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells are also apparent in the CD11b versus CD11c plots commonly used to detect the appearance of iDCs (Supplemental Fig. 1B, 1C). This Ly6C<sup>hi</sup>-CD11c<sup>hi</sup>-CD11b<sup>hi</sup> 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: Ly6C<sup>hi</sup> monocytes, Ly6C<sup>neg</sup> monocytes, Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells, and CD11b<sup>+</sup> cDCs (Fig. 1C). A comparison of these four populations shows that Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells and CD11b<sup>+</sup> cDCs express similar levels of CD11c, whereas Ly6C<sup>neg</sup> monocytes express lower levels of CD11c and Ly6C<sup>hi</sup> monocytes do not express CD11c (Supplemental Fig. 1D). Although similar populations of Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> 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 Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> 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 Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> 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 Ly6C<sup>hi</sup> monocyte precursors than to cDCs (Fig. 2A). The Ly6C<sup>neg</sup> 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 Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells that shares surface markers with both cDCs and monocytes. For simplicity we will continue to refer to these monocyte-derived Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells as iDCs, although this population likely differs from previously described inflammatory monocyte-derived iDCs, as shown below.

**Spleen Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells derive from monocytes**

The appearance of Ly6C<sup>hi</sup> cells expressing high levels of CD11c following injection of anti-CD40 suggested that Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells were the result of upregulation of CD11c on Ly6C<sup>hi</sup>-CD11c<sup>neg</sup> monocytes in the spleen. However, these results could not exclude the possibility that the Ly6C<sup>hi</sup>-CD11c<sup>neg</sup> 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 Ly6C<sup>hi</sup> and Ly6C<sup>neg</sup> monocytes, and to a lesser degree with cDCs (Fig. 3A, Supplemental Fig. 2). When control-treated mice (uninflamed) were injected with 2.0-μm fluorescent particles; 60 min later, bead capture by splenocytes was analyzed by flow cytometry. Percentages represent the cells that internalized one or more beads. (A) Similar to (A), but 5.0-μm particles were used. (C) Similar to (A) and (B), but mice were injected with soluble GFP protein. After 30 min, GFP capture was analyzed by comparing the fluorescence signal of cells from a mouse injected with GFP (solid line) compared with a mouse similarly treated with anti-CD40 but not injected with GFP (dashed line). Spleen populations were gated as described in Fig. 1. The mean fluorescence intensity difference between GFP-injected mice and control mice is shown in each panel, ±SD. (D) The same experimental approach as in (C), but mice were injected i.v. with FITC-OVA [instead of GFP as used in (C)]. Data are representative of three experiments, three mice per group. Results are expressed as mean ± SD from the mean.

Because bead tracking in situ cannot selectively label the Ly6C<sup>hi</sup> monocytes without additional manipulations such as clodronate liposomes (33), we sorted Ly6C<sup>hi</sup> 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 Ly6C<sup>hi</sup> monocytes into Ly6C<sup>neg</sup> 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 Ly6C<sup>hi</sup> monocytes, which paralleled the induction of CD11c expression on the endogenous Ly6C<sup>hi</sup> population in the recipient (Fig. 3F). These results indicate that the Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells formed under inflammation induced by anti-CD40 treatment were a result of upregulation of CD11c on splenic CD11c<sup>neg</sup> monocytes.

**FIGURE 4.** Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells retain the scavenging capacity of monocytes. (A) Comparison of the capacity of Ly6C<sup>hi</sup> monocytes, Ly6C<sup>hi</sup>-CD11c<sup>hi</sup> cells, and CD11b<sup>+</sup> cDCs to internalize particulate Ags. Mice that had been treated with anti-CD40 mAb 24 h earlier were injected with 2.0-μm fluorescent particles; 60 min later, bead capture by splenocytes was analyzed by flow cytometry. Percentages represent the cells that internalized one or more beads. (B) Similar to (A), but 5.0-μm particles were used. (C) Similar to (A) and (B), but mice were injected with soluble GFP protein. After 30 min, GFP capture was analyzed by comparing the fluorescence signal of cells from a mouse injected with GFP (solid line) compared with a mouse similarly treated with anti-CD40 but not injected with GFP (dashed line). Spleen populations were gated as described in Fig. 1. The mean fluorescence intensity difference between GFP-injected mice and control mice is shown in each panel, ±SD. (D) The same experimental approach as in (C), but mice were injected i.v. with FITC-OVA [instead of GFP as used in (C)]. Data are representative of three experiments, three mice per group. Results are expressed as mean ± SD from the mean.
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 Ly6C−/−-CD11c+ 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 Ly6C+ monocytes transferred into CD40−/− recipients could not be induced to form Ly6C−/−-CD11c+ 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).

Ly6C+−/−CD11c+ cells functionally resemble activated monocytes

The apparent activation of Ly6C+ monocytes in mice treated with anti-CD40 led to a surface phenotype with similarities to both cDCs and their Ly6C+ 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 Ly6C+ monocytes, iDCs (Ly6C+−/−CD11c+), 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, Ly6C+ monocytes showed a higher phagocytic capacity compared with cDCs (Fig. 4A). In these same mice, the Ly6C+−/−CD11c+ 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 Ly6C+ monocytes could still internalize these large particles, which could not be internalized by cDCs (Fig. 4B). In contrast to cDCs, the Ly6C+−/−CD11c+ 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 endocytic probe (GFP [Fig. 4C] or FITC-OVA [Fig. 4D]). Ly6C+ monocytes exhibited a markedly higher endocytic activity than cDCs, whereas Ly6C+−/−CD11c+ 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, Ly6C+−/−-CD11c+ (iDCs), and Ly6C+−/−CD11c−/− (Ly6C+ 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 Ly6C+−/−CD11c+ or Ly6C+−/−CD11c−/− 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 Ly6C+−/−CD11c+ and Ly6C+−/−CD11c−/− 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>H</sup>-CD11c<sup>H</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>H</sup>-CD11c<sup>H</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>H</sup>-CD11c<sup>H</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>H</sup>-CD11c<sup>H</sup> cells ultimately convert into Ly6C<sup>Neg</sup>

monocytes

We considered the possibility that the conversion of Ly6C<sup>H</sup> monocytes to a DC-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>H</sup>-CD11c<sup>H</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>H</sup>-CD11c<sup>H</sup> cells could indicate their further conversion into cDC-like population, we performed adoptive transfer experiments for longer periods, after Ly6C<sup>H</sup>-CD11c<sup>H</sup> cells had apparently disappeared. Interestingly, the transferred Ly6C<sup>H</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>H</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>H</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>H</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 monocytic 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

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