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CD64 Expression Distinguishes Monocyte-Derived and Conventional Dendritic Cells and Reveals Their Distinct Role during Intramuscular Immunization

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Although most vaccines are administered i.m., little is known about the dendritic cells (DCs) that are present within skeletal muscles. In this article, we show that expression of CD64, the high-affinity IgG receptor FcγRI, distinguishes conventional DCs from monocyte-derived DCs (Mo-DCs). By using such a discriminatory marker, we defined the distinct DC subsets that reside in skeletal muscles and identified their migratory counterparts in draining lymph nodes (LNs). We further used this capability to analyze the functional specialization that exists among muscle DCs. After i.m. administration of Ag adsorbed to alum, we showed that alum-injected muscles contained large numbers of conventional DCs that belong to the CD8α+– and CD11b+–type DCs. Both conventional DC types were capable of capturing Ag and of migrating to draining LNs, where they efficiently activated naïve T cells. In alum-injected muscles, Mo-DCs were as numerous as conventional DCs, but only a small fraction migrated to draining LNs. Therefore, alum by itself poorly induces Mo-DCs to migrate to draining LNs. We showed that addition of small amounts of LPS to alum enhanced Mo-DC migration. Considering that migratory Mo-DCs had, on a per cell basis, a higher capacity to induce IFN-ɣ-producing T cells than conventional DCs, the addition of LPS to alum enhanced the overall immunogenicity of Ags presented by muscle-derived DCs. Therefore, a full understanding of the role of adjuvants during i.m. vaccination needs to take into account the heterogeneous migratory and functional behavior of muscle DCs and Mo-DCs revealed in this study. The Journal of Immunology, 2012, 188: 1751–1760.

D endritic cells (DCs) are central to adaptive immune defenses. Conventional DCs comprise DCs that spend their whole life in secondary lymphoid tissues (LT-DCs), as well as DCs that reside first in the parenchyma of nonlymphoid tissues, where they are known as interstitial DCs (Int-DCs), before migration to draining lymph nodes (LNs), where they are called migratory DCs (Mig-DCs). Int-DCs take up incoming Ags and carry them to draining LNs, where they excels in activating Ag-specific naïve T cells. Conventional DCs express CD11c and MHC class II (MHCII) molecules, and have been categorized as CD8α+– and CD11b+–type DCs, a dichotomy that takes into account phenotypic, developmental, and functional attributes (1–3).

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; DC, dendritic cell; Int-DC, interstitial DC; LN, lymph node; LT-DC, lymphoid tissue DC; MHCII, MHC class II; Mig-DC, migratory DC; Mig-Mo-DC, migratory monocyte-derived DC; Mo-DC, monocyte-derived DC.

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A unique bone marrow (BM)-derived precursor gives rise to conventional DCs and to monocytes (4). Two subsets of CD11c–MHCII+ monocytes coexist in the blood and are defined as Gr1+Ly-6Chigh and Gr1+Ly-6Clo (4). After extravasation into tissues, Gr1+Ly-6Chigh blood monocytes locally develop into CD11b+CD11c+MHCII+ macrophages and CD11b+CD11c+MHCII+ cells. On the basis of CD11c and MHCII expression, two DC attributes, the last cells are referred to as monocyte-derived DCs (Mo-DCs) (5, 6). Microbial infections trigger the massive recruitment of Gr1+Ly-6Chigh blood monocytes at the site of infection and their in situ differentiation into macrophages and Mo-DCs (7–10). Sterile stimuli including alum, an adjuvant composed of aluminum particles, also stimulate the recruitment of Gr1+Ly-6Chigh blood monocytes at the site of injection (11). Because of their identical CD11b+CD11c+MHCII+ phenotype, it has been difficult to distinguish the CD11b+–type DCs from the Mo-DCs that accumulate during infections and inflammation, and it remains thus to be established whether Mo-DCs possess the same LN-homing and Ag-presenting properties as conventional DCs.

Although skeletal muscles constitute the site of injection of most human vaccines, only limited data are available on the myeloid cells that are present in steady-state muscles and on the composition of the inflammatory cell infiltrate that results from i.m. immunization with Ags adsorbed to alum. For instance, in a recent study characterizing the effects of i.m. injection of adjuvanted Ags, the CD11c+CD11b+MHCII+ cells that were found in the injected muscles and the draining LNs were analyzed in “bulk” without attempting to distinguish conventional DCs and Mo-DCs among them (12). In this report, we characterized the conventional DCs and the Mo-DCs residing in the skeletal muscle, before and after i.m. immunization, and assessed their respective ability to activate naïve T cells in the draining LNs. By analyzing the expression of
CD64, the high-affinity IgG receptor FcγRI (13), we succeeded in distinguishing conventional DCs from Mo-DCs, and we determined that under steady-state conditions, skeletal muscles contain Mo-DCs and two subsets of conventional DCs. By combining the CD64 marker with a highly tractable anatomical configuration involving the tibialis posterior muscle and the draining dorsal LN (also called iliac LN), we demonstrated that alumn-injected muscles contain large numbers of conventional DCs that were capable of capturing Ag in the muscle and of migrating to the dorsal LN, where they efficiently activated naïve T cells. The Mo-DCs found in alumn-injected muscles were as numerous as conventional DCs, but only a small fraction migrated to draining LNs. We showed that Mig-Mo-DCs had, on a per cell basis, a higher capacity to induce IFN-γ-producing T cells than conventional DCs, and that the addition of small quantities of LPS to alumn-adjuvanted Ags increased the fraction of Mo-DCs capable of migrating to the dorsal LNs. Therefore, formulating adjuvants capable of enhancing the LN homing capability of Mo-DCs should lead to the development of more effective i.m. vaccines.

Materials and Methods

Mice

Mice were housed under specific pathogen-free conditions and handled in accordance with French and European directives. OT-I (14), OT-II (15), CCR2-/- (16), and CCR7-/- (17) mice have been previously described. All mice were maintained on a C57BL/6 background.

Generation of BM chimera

Seven- to 8-wk-old B6 (CD45.1 × CD45.2) mice were lethally irradiated with two doses of 550 rad each, 5 h apart, and then injected i.v. with 2 × 10^6 BM cells. BM cells were obtained from femurs and tibias of B6 (CD45.1) or CCR2-/- (CD45.2) mice. Seven to 8 wk after reconstitution, the level of chimera was determined.

Ag and adjuvants

Imject alum (Pierce Biocell) consists of a mixture of aluminum hydroxide and magnesium hydroxide. Imject alum was mixed with OVA (In vitro) and then stirred for at least 1 h. A total of 15 μl Imject alum (500 μg) containing 5 μg OVA (alum-OVA) with or without 250 ng LPS (Invivogen) was injected in each posterior tibialis muscle using a 26-gauge needle. In control experiments, each posterior tibialis muscle was injected with 15 μl Imject alum alone. I.m. injection of 15 μl PBS alone does not increase the numbers of myeloid cells found in steady-state posterior tibialis muscle, suggesting that the mechanical stress resulting from the injected volume does not contribute significantly to the cell recruitment noted in alumn-injected posterior tibialis muscle. In vivo stimulation of spleen DCs with LPS was performed by injecting 250 ng or 5 μg LPS i.v. Spleen DCs were analyzed by flow cytometry 24 h after LPS injection.

DC preparation

Mice were anesthetized and perfused with PBS before sacrifice. Posterior tibialis muscles were cut into small pieces and incubated for 1 h at 37°C and under constant agitation in RPMI medium containing 2% FCS, 1 mg/ml type II collagenase (Worthington Biochemical), and 150 μg/ml DNase I (Sigma-Aldrich). Undigested fibrous material was removed by filtration through a 70-μm nylon mesh (Becton Dickinson), and the resulting single-cell suspension was subjected to centrifugation on a Percoll gradient (Amersham-Pharmacia). DCs were isolated from LNs as previously described (18). In brief, LNs were cut into small pieces and digested for 20 min at room temperature with a mixture of type II collagenase (Worthington Biochemical) and DNase I (Sigma-Aldrich). The resulting cell suspension was treated with 5 mM EDTA to disrupt DC–T cell conjugates. Prior to sorting of the distinct DC subsets, undigested material was eliminated and light-density cells were enriched by centrifugation on a Optiprep solution (d = 1.32 g/ml; Abcys). For in vitro stimulation of DCs by LPS, light-density cells were isolated from LNs and further enriched for CD11c+ DCs by positive MACS selection (Miltenyi Biotec).

Flow cytometry

Cells were stained and analyzed using a FACS LSRII system (BD Biosciences). Data were analyzed with the BD FACSDiva software (BD Biosciences). Cell viability was evaluated using Sytox (Invitrogen) according to the manufacturer’s protocol. Allophycocyanin Cy7-conjugated anti-NK1.1 (PK136), allophycocyanin Cy7-conjugated anti-CD3 (17A2), allophycocyanin Cy7-conjugated anti-Ly-6G (1A8), allophycocyanin Cy7-conjugated anti-CD19 (6D5), PE-conjugated anti-CD64 (X54-5/7.1) were all from BioLegend; FITC-conjugated anti-CCR3 (S3103) was from R&D; PE-CY7-conjugated anti-CD11c (N418), Alexa 700-conjugated anti-MHCII (I-A/I-E) (M5/114.15.2), PE-CY5.5-conjugated anti-CD45.2 (104), allophycocyanocyanin-conjugated anti-CD45.1 (A20), PE-CY5-conjugated anti-CD24 (M1/69), and PE-CY5-conjugated anti-CD5 (53-7.3) were from eBioscience; and Pacific Blue-conjugated anti-CD11b (M1/70), biotin-conjugated anti-CD103 (M290), FITC-conjugated anti-Ly-6C (AL21), biotin-conjugated anti-Ly-6C (AL21), Pacific Blue-conjugated anti-CD4, PE-CY7-conjugated anti-CD5 (53-6.7), biotin-conjugated anti-CCR7 (4B12), and PE-CY5-conjugated anti-CD69 (H1.2F3) were from BD Pharmingen. Allophycocyanin-conjugated anti-Langerin (929F3) was purchased from Dendritics.

Intracellular staining

OT-I and OT-II T cells were harvested from dorsal LNs and incubated at 37°C for 6 h in the presence of PMA (5 ng/ml) and ionomycin (250 ng/ml). Monensin (Golgistop; BD Pharmingen) was added to the suspension for the last 5 h. Cells were stained using PE-CY5.5-conjugated anti-CD45.1, Alexa 700-conjugated anti-CD8, Pacific Blue-conjugated anti-CD4, PE-CY5-conjugated anti-CD5, and PE-conjugated anti-CD69 and then permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). Intracellular cytokines were detected by staining with PE-CY7-conjugated anti–IFN-γ (XMG1.2; BD Pharmingen).

Sorting of DCs

Light-density cells isolated from LNs draining the posterior tibialis muscle were stained with CD19, Ly-6C, MHCII, CD11c, CD11b, and CD64 Abs. DC subsets were sorted on a FACS Aria system (BD) using the gating strategies specified in Fig. 6 and Supplemental Fig. 2 and summarized in Fig. 3.

Culture of CFSE-labeled OT-I and OT-II T cells with DC subsets

OT-I and OT-II T cells were isolated from pooled LNs and spleen of OT-I or OT-II mice kept on a Rag-2-/- × B6 (CD45.1) background using a CD8+ and CD4+ T cell-negative isolation kit (Dynal, Invitrogen), respectively. Purity was determined by staining with CD4, CD8, CD45, and TCR Vb2. For CFSE labeling, purified OT-I and OT-II T cells were resuspended in PBS containing 2.5 mM CFSE (Molecular Probes) for 3 min at room temperature. The various sorted DC subsets were pulsed for 2 h at 37°C with or without endotoxin-free OVA (Hyglos). After washing, pulsed DCs were cultured with CFSE-labeled OT-I and OT-II T cells in 150 μl RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM glucose, and 50 μM 2-ME. After 3 days of culture, proliferation was measured by flow cytometry as a loss of CFSE staining. The percentages and the absolute numbers of OT-I and OT-II T cells that have proliferated were measured. In vivo studies, 10^6 CFSE-labeled OT-I and OT-II T cells were adoptively transferred into the specified mice. At the indicated times, single-cell suspensions were prepared from LNs draining the posterior tibialis muscle, and OT-I and OT-II T cells were analyzed by FACS.

Determination of cytokine concentration

The levels of IFN-γ present in the specified culture supernatants were measured using a CBA kit (BD Pharmingen).

Statistical analysis

Comparative experiments were tested for statistical significance using the unpaired Student t test in GraphPad Prism software (version 4.0; GraphPad).

Results

CD64 expression distinguishes Mo-DCs from CD11b+ type DCs

To characterize the DCs present in steady-state skeletal muscle, single-cell suspensions were prepared by enzymatic digestion and gentle dissociation of the tibialis posterior muscles. After excluding neutrophils, eosinophils, NK cells, B cells, and T cells, the remaining CD45+ cells were analyzed for CD11c and MHCII expression. DCs were identified using their CD11c+MHCII+...
phenotype and subdivided on the basis of CD24 and CD11b expression into a CD24+CD11b+ and a CD24− to +CD11b+ subset (Fig. 1). Gr1+Ly-6Chigh blood monocytes use the CCR2 chemokine receptor to egress from the BM into the circulation, and as a result, mice deficient in CCR2 show markedly reduced numbers of Gr1+Ly-6Chigh blood monocytes, tissue macrophages, and Mo-DCs under both steady-state and inflammatory conditions (19). In contrast, conventional DCs are not affected by CCR2 deficiency. Therefore, we used such differential CCR2 requirement to determine whether the CD24− to +CD11b+ subset present in the skeletal muscle was composed of both CCR2-dependent Mo-DCs and CCR2-independent conventional DCs.

Mice coexpressing CD45.1 and CD45.2 were lethally irradiated and reconstituted with a 1:1 mixture of BM cells isolated from wild-type B6 mice and from CD45.2+ B6 mice that lacked CCR2. Those B6 (CD45.1) CCR2+ + B6 (CD45.2) CCR2− → B6 (CD45.1-CD45.2) competitive chimeras were analyzed 8 wk after BM transfer. Neutrophils, T cells, and B cells present in the blood of the chimeras developed irrespective of the presence of CCR2, indicating that engraftment of both BM was equally successful, and that the absence of CCR2 did not affect BM engraftment (data not shown). As expected, cells of CD45.1 origin represented >90% of blood monocytes and CD11b+CD11c+ MHCII+ muscle macrophages (Fig. 1 and data not shown). Among the CD11c+MHCII+ cells found in the muscle of the chimeras, the CD24+CD11b+ Int-DCs consisted of equal numbers of CD45.1 and CD45.2 donor cells, a result suggesting that they develop in a CCR2-independent manner and correspond to bona fide conventional DCs (Fig. 1). In contrast, the CD24− to +CD11b+ cells showed a CD45.1:CD45.2 cell ratio skewed toward cells of CD45.1 origin (Fig. 1). Therefore, CD24− to +CD11b+ cells were heterogeneous and contained both CCR2-dependent Mo-DCs and CCR2-independent CD11b+ type DCs.

Among the wealth of markers tested to distinguish CCR2-dependent and -independent CD24− to +CD11b+ cells, CD64 and Ly-6C permitted to distinguish a major Ly-6C−CD64− and a minor Ly-6C+CD64+ subset among CD24− to +CD11b+ cells (Fig. 1). The Ly-6C−CD64− subset present in competitive chi-
mera consisted of almost equal numbers of CD45.1 and CD45.2 donor cells (Fig. 1). Therefore, it corresponds to CCR2-independent conventional DCs and is referred to as CD11b<sup>+</sup>CD64<sup>+</sup> Int-DCs. Note that CD24<sup>+</sup>CD11b<sup>-</sup> Int-DCs also displayed a Ly-6C<sup>+</sup>CD64<sup>+</sup> phenotype (Fig. 1). In marked contrast, the Ly-6C<sup>-</sup>CD64<sup>+</sup> subset resembled muscle macrophages in that it was dominated by cells of CD45.1 origin (Fig. 1). Therefore, among the CD11b<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> cells found in skeletal muscle, CCR2-dependent Mo-DCs can be unequivocally identified by their expression of CD64 and Ly-6C, and are thus referred to as CD11b<sup>+</sup>CD64<sup>+</sup> Int-Mo-DCs.

**Characterization of conventional muscle DCs**

Further characterization of the CD24<sup>-</sup>CD11b<sup>-</sup> Int-DCs present in skeletal muscles showed that they were CD207<sup>(langerin)−</sup>CD8<sup>e</sup>low CD172α<sup>-</sup>, and that approximately half of them expressed CD103<sup>+</sup> (Fig. 2). In contrast, the CD11b<sup>−</sup>CD64<sup>−</sup> Int-DCs were CD172α<sup>+</sup> and lacked CD207, CD8<sup>x</sup>, and CD103 expression (Fig. 2). Therefore, based on their surface phenotype, the CD24<sup>-</sup>CD11b<sup>-</sup> Int-DCs and CD11b<sup>−</sup>CD64<sup>-</sup> Int-DCs identified in skeletal muscle correspond to CD8α<sup>-</sup>- and CD11b<sup>-</sup>-type DCs, respectively (1–3). The CD11c<sup>-</sup>MHCII<sup>+</sup> cells found in steady-state skeletal muscle are thus composed of CD8α<sup>-</sup>- and CD11b<sup>-</sup>-type Int-DCs and of Int-Mo-DCs (Fig. 3, steady-state muscle).

**Dorsal LNs primarily drain the tibialis posterior muscle**

To determine the anatomical sites where primary T cell activation occurred after injection of alum-adjuvanted Ags into the tibialis posterior muscle, CFSE-labeled OT-II CD4<sup>+</sup> T cells that had divided were injected into B6 mice. One day after transfer, OVA protein adsorbed to alum (alum-OVA) was injected in the tibialis posterior muscle of each hind leg. Three days later, OT-II T cells that had divided were found only in the inguinal, popliteal, and dorsal LNs (Fig. 4A), suggesting that those LNs drained the tibialis posterior muscle.

Under steady-state conditions, Int-DCs constitutively migrate to draining LNs (20), and the resulting Mig-DCs can be distinguished from LT-DCs by their MHCII<sup>high</sup>CD11c<sup>inter</sup> to <sup>high</sup> phenotype (21). When identified on that basis, the Mig-DCs present in the inguinal and popliteal LNs were found to contain CD24<sup>-</sup>CD11b<sup>+</sup> cells that comprised a CD207<sup>+</sup> subset and CD11b<sup>−</sup>CD64<sup>−</sup> cells (Fig. 4B). Under steady-state conditions, CD207<sup>-</sup>MHCII<sup>high</sup>CD11c<sup>inter</sup> to <sup>high</sup> cells are found only in skin-draining LNs (21), their presence in the inguinal and popliteal LNs suggests that they both drained cutaneous territories in addition to the tibialis posterior muscle. In marked contrast, the Mig-DCs present in dorsal LNs were deprived of CD207<sup>+</sup> DCs and comprised only the migratory counterpart of the CD24<sup>+</sup>CD11b<sup>-</sup> and CD11b<sup>−</sup>CD64<sup>−</sup> Int-DCs identified in the tibialis posterior muscle (Figs. 3, steady-state LN, and 4B). Therefore, the dorsal LN primarily drained the tibialis posterior muscles and is thus particularly appropriate to track “in isolation” the dynamics of DC migration from the tibialis posterior muscle. Accordingly, the rest of our study focused on the tibialis posterior muscles and the dorsal LNs.

**Alum administration increases the generation of Int-DCs and Int-Mo-DCs**

We characterized next the inflammatory infiltrate induced by i.m. injection of alum-OVA and monitored its evolution at various time points after immunization. Importantly, CD64 expression kept its discriminatory power in the local inflammatory environment induced by i.m. alum administration and still permitted CCR2-independent CD11b<sup>+</sup>CD64<sup>−</sup> to low DCs to be distinguished from CCR2-dependent CD11b<sup>+</sup>CD64<sup>+</sup> Mo-DCs in the posterior tibialis muscle (Supplemental Fig. 1) and the dorsal LN (Supplemental Fig. 2). After i.m. alum-OVA injection, the number of CD24<sup>-</sup>CD11b<sup>-</sup> and CD11b<sup>−</sup>CD64<sup>-</sup> Int-DCs increased, peaked at day 6 postimmunization, and then decreased (Fig. 5A). When compared with steady-state conditions, the numbers of CD24<sup>-</sup>CD11b<sup>-</sup> and CD11b<sup>−</sup>CD64<sup>-</sup> Int-DCs present at the peak of the response had increased 19-fold and 9-fold, respectively (Fig. 5A). Commensurate with the large influx of Gr1<sup>+</sup>Ly-6Chigh monocytes observed in alum-injected muscle (data not shown), the numbers of CD11b<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> muscle macrophages and Int-Mo-DCs dramatically increased upon immunization (Fig. 5A and data not shown). At the peak of the response, 6 d after immunization, Int-Mo-DCs were 180 times more numerous than in steady-state conditions and their numbers decreased thereafter (Fig. 5A). Therefore, i.m. injection of alum-OVA enhanced the local accumulation of Int-Mo-DCs and of both CD24<sup>+</sup>CD11b<sup>-</sup> and CD11b<sup>−</sup>CD64<sup>-</sup> Int-DCs (Fig. 3, muscle injected with alum-adjuvanted Ags).

**CD11b<sup>+</sup>CD64<sup>−</sup> DCs dominate muscle-derived migratory cells**

After alum-OVA injection, the number of muscle-derived CD24<sup>+</sup>CD11b<sup>-</sup> and CD11b<sup>−</sup>CD64<sup>−</sup> Mig-DCs found in the draining dorsal LN reached a peak at day 1.5 postimmunization and then

**FIGURE 2.** Characterization of the DC subsets present in the posterior tibialis muscle. A, Flow cytometry analysis of single-cell suspensions prepared from steady-state posterior tibialis muscle. After excluding nonhematopoietic cells, NK cells, B cells, T cells, eosinophils, and neutrophils, the remaining CD45<sup>+</sup> cells were analyzed for CD11c and MHCII expression. CD11c<sup>−</sup>MHCII<sup>−</sup> cells were subdivided into CD24<sup>+</sup>CD11b<sup>−</sup> Int-DCs and CD11b<sup>+</sup> cells that were CD24<sup>−</sup> to <sup>+</sup>, CD24<sup>−</sup> to <sup>+</sup>CD11b<sup>+</sup> were further subdivided into CD64<sup>+</sup> Int-DCs and CD64<sup>−</sup> Mo-DCs. The percentages of cells found in each of the specified gates are indicated. B, Expression of CD8α, CD172α, CD103, and CD207 on CD24<sup>+</sup>CD11b<sup>−</sup> Int-DCs, CD11b<sup>−</sup>CD64<sup>−</sup> Int-DCs, and CD11b<sup>−</sup>CD64<sup>−</sup> Int-Mo-DCs gated as specified in A. Data shown are representative of three independent experiments.
decreased (Fig. 5B). When compared with steady-state conditions, the numbers of CD24+CD11b– and CD11b+CD64– Mig-DCs present at the peak of the response were 1.7- and 2.8-fold increased, respectively (Fig. 5B). Consistent with their LN-homing ability, the CD24+ CD11b– and CD11b+CD64– Mig-DC subsets found in the dorsal LNs expressed high levels of CCR7 (Fig. 6A). Moreover, CD24+CD11b– and CD11b+CD64– Mig-DCs were almost completely absent from the dorsal LNs of CCR7-deficient mice (Fig. 6B).

Alum-OVA i.m. injection also triggered a transient appearance of small numbers of Ly-6C+CD64+ cells in the dorsal LNs that likely corresponded to the migratory counterpart of the Int-Mo-DCs and are thus denoted as Mig-Mo-DCs (Fig. 5B). Consistent with that view and akin to Mig-DCs, those Ly-6C+CD64+ cells expressed CCR7 and were absent from the dorsal LNs of CCR7-deficient mice that have been immunized with alum-OVA (Fig. 6A, 6B). In addition, a small population of Ly-6ChighCD64high cells temporarily appeared among the MHCIIhighCD11cint to high cells present in the dorsal LNs of immunized mice (Figs. 5B, 6A, 6B). These cells were CCR7+ and their presence was unaffected by the lack of CCR7 (Fig. 6A, 6B). They likely corresponded to Mo-DCs that differentiated in situ from blood monocytes without any contribution from the muscle and that is denoted as LT-Mo-DCs. CD11b+CD11c+MHCII+ muscle macrophages and neutrophils have not been represented for the sake of simplicity.

FIGURE 3. Phenotype of DCs and Mo-DCs found in skeletal muscle and of their migratory counterparts found in draining LNs. Based on the marker combinations specified in the key shown at the bottom, three distinct subsets can be identified among the CD11c+MHCII+ cells present in mouse steady-state skeletal muscles. They correspond to CD8α+ Int-DCs, CD11b+ Int-DCs, and Int-Mo-DCs. Under steady-state conditions, CD8α+ Int-DCs and CD11b+ Int-DCs are the sole DC subsets capable of migrating to LNs draining skeletal muscles. i.m. injection of alum-adjuvanted Ags enhances the generation of both Int-DCs and Int-Mo-DCs and confers to a small fraction of Int-Mo-DCs the ability to migrate to draining LNs in a CCR7-dependent manner. When combined to alum, LPS enhances the LN-tropic properties of Int-Mo-DCs. LNs that drain skeletal muscles injected with alum-adjuvanted Ags contain a small population of Mo-DCs that differentiated in situ from blood monocytes without any contribution from the muscle and that is denoted as LT-Mo-DCs. CD11b+CD11c+MHCII+ muscle macrophages and neutrophils have not been represented for the sake of simplicity.
OT-II responses peaked at day 6 postimmunization and subsequently decreased (Supplemental Fig. 3B). To determine the relative Ag-presenting capacities of the various Mig-DC subsets identified in the dorsal LNs of immunized mice, CD24+CD11b−Mig-DCs, CD11b+CD64−Mig-DCs, and Mig-Mo-DCs were sorted from dorsal LNs 1.5 d after immunization with alum-OVA and analyzed for their respective capacity to induce the proliferation of CFSE-labeled OT-I and OT-II T cells. CD11b+CD64−Mig-DCs were superior in presenting OVA to OT-II cells (Fig. 7A). Interestingly, CD24+CD11b−Mig-DCs, CD11b+CD64−Mig-DCs, and Mig-Mo-DCs were equally capable of cross-presenting OVA as documented by the robust CD8+ OT-I T cell proliferation they induced (Fig. 7A). Therefore, all the muscle-derived DCs that migrated to the dorsal LNs were capable of taking up OVA and cross-presenting it. Their capacity to cross-present OVA did not depend on prior alum treatment because CD24+CD11b− and CD11b−CD64−Mig-DCs isolated from steady-state dorsal LNs and pulsed with OVA were also capable of efficiently cross-presenting OVA (Fig. 7B). In contrast, LT-DCs and LT-Mo-DCs sorted from dorsal LNs of mice immunized i.m. with alum-OVA failed to activate OT-I and OT-II T cells (data not shown). Therefore, because of their numerical advantage (Fig. 5B) and their capacity to efficiently induce both CD4+ and CD8+ T cell responses (Fig. 7A), the CD11b+CD64−Mig-DCs likely accounted for the bulk of the T cell response triggered by i.m. immunization with alum-adjuvanted OVA.

**FIGURE 4.** Identification of the LNs that drain the posterior tibialis muscle. A, CFSE-labeled OT-II T cells were adoptively transferred into B6 mice 1 d before i.m. injection of alum-OVA. Three days after immunization, the specified LNs and the spleen were harvested and the extent of CFSE dilution determined among OT-II cells. Data are representative of at least three independent experiments involving groups of three to five mice. B, The MHCIIHIGH subset to both Mig-DCs present in inguinal, dorsal, and popliteal steady-state LNs were analyzed for CD24 versus CD11b and CD207 versus CD11b expression. Data are representative of two independent experiments.

**FIGURE 5.** Effect of i.m. alum-OVA injection on the generation and migration of Int-DCs and Mo-DCs. Kinetics of accumulation of CD24+CD11b− and CD11b+CD64− DCs and of Mo-DCs in the tibialis posterior muscle (A) and the draining dorsal LN (B) in response to alum-OVA injection. LT-Mo-DCs found in alum-injected dorsal LNs correspond to Mo-DCs that differentiated in situ from blood monocytes (see Fig. 6). Values correspond to the absolute numbers of specified cell types per pair of tibialis posterior muscles (A) and dorsal LNs (B). Data are representative of at least three independent experiments involving groups of three to five mice, and error bars correspond to the SEM.

CD64−Mig-DCs are the most efficient inducers of IFN-γ–producing T cells

We determined next whether the Mig-DC subsets that reached the dorsal LNs of untreated mice and of mice that had received an i.m. injection of alum alone 1.5 d before analysis differed in their capacity to induce effector cytokine production in Ag-responsive T cells. Because i.m. injection of alum alone sufficed to induce the migration of Mig-Mo-DCs to the dorsal LN (Supplemental Fig. 2), it allowed us to compare Mig-Mo-DC function with that of the CD24+CD11b− and CD11b+CD64−Mig-DCs that concomitantly reached the dorsal LN. Sorted Mig-DCs and Mig-Mo-DCs were pulsed with OVA and cultured with OT-I and OT-II T cells. After 3 d of culture, the concentration of IFN-γ produced by T cells was determined in culture supernatants. When compared with the two Mig-DC subsets, Mig-Mo-DCs induced OT-I T cells to produce 3-fold more IFN-γ, and although not statistically significant, a similar trend occurred with OT-II T cells (Fig. 7C). Therefore, the Mig-Mo-DCs that reached the dorsal LNs of mice that had received an i.m. injection of alum alone are, on a per cell basis, the most efficient inducers of IFN-γ–producing T cells among muscle-derived Mig-DCs.
Addition of LPS to alum-OVA increases the LN-homing properties of Int-Mo-DCs

Mig-Mo-DCs represent a minor fraction of the muscle-derived Mig-DCs that reached the dorsal LNs after alum treatment (Fig. 5B). Maneuvers aiming at increasing the LN-homing properties of the Int-Mo-DCs found in inflamed muscles should thus permit the exploitation of their higher IFN-γ-inducing capacity as compared with muscle-derived conventional Mig-DCs. Cheong and colleagues (23) recently showed that i.v. administration of LPS induced a massive differentiation of monocytes into Mo-DCs that were capable of priming naive T cells. Along that line, we analyzed whether the addition of a small quantity of LPS to alum enhanced the LN-homing properties of Int-Mo-DCs. B6 mice were injected i.m. with alum alone, alum plus LPS, or left untreated. Analysis of the posterior tibialis muscle at 1.5 and 6 d after immunization showed that addition of LPS to alum had no major effect on the number and the relative percentage of the conventional DCs and Mo-DCs that were recruited in the muscle as compared with treatment with alum alone (Fig. 8A). In contrast, analysis of the dorsal LNs at the peak of Mig-DC accumulation showed that addition of LPS to alum led to the presence of 3-fold more CD64+ Mig-Mo-DCs (Figs. 3, + LPS, and 8B).

To demonstrate that the LPS-induced increase in CD64+ cells was not due to upregulation of CD64 expression by CD8α+- or CD11b+ -type conventional DCs, we isolated DCs from the LNs of untreated mice and stimulated them with various doses of LPS in vitro. As shown in Supplemental Fig. 4A, LPS treatment did not induce a measurable upregulation of CD64 on CD8α+ - and CD11b+ -type DCs. To validate these findings in vivo, we injected LPS i.v. and measured its effect on spleen DCs (Supplemental Fig. 4B). Whereas LPS injection induced the conversion of splenic CD8α+ monocytes into MHCII+CD11c+CD64+ Mo-DCs, it did not concomitantly induce upregulation of CD64 expression by spleen CD8α+ - and CD11b+ -type DCs. Altogether, these experiments indicate that CD64 expression permits to distinguish Mo-DCs from conventional DCs even under the strong DC activation conditions that are reached upon LPS stimulation.

Considering that the Mig-Mo-DCs that reached dorsal LNs after alum plus LPS treatment kept a higher functional potency than muscle-derived Mig-DCs (Fig. 8C), their increased representation in dorsal LNs should result in an increase in the numbers of IFN-γ-producing T cells that are induced in vivo. Consistent with that hypothesis, the addition of LPS to alum-adjuvanted OVA resulted in increased percentages of IFN-γ-producing OT-I T cells in the dorsal LNs at the peak of the response (Fig. 8D). Therefore, by increasing the relative representation of Mig-Mo-DCs, addition of LPS to alum-adjuvanted Ags had a positive impact on the T cell responses that unfold in the LNs draining the site of i.m. immunization.

Discussion

Collectively, our data established that conventional DCs and Mo-DCs are present in steady-state and alum-injected muscles. The CD24+CD11b- and CD11b+CD64+ Int-DCs identified in the skeletal muscle corresponded to CD8α+ - and CD11b+ -type DCs, respectively (1, 2, 24). Therefore, the CD8α+ - and CD11b+ -type DC dichotomy also applies to the skeletal muscle. CD8α+ -type DCs are generally considered to be the sole DC subset able to present exogenous Ag on MHC class I molecules (25), a property called cross-presentation. Upon injection of alum-adjuvanted OVA into the tibialis posterior muscle, we found, however, that the CD8α+ - and CD11b+ -type DCs that migrated to the dorsal LNs...
were both capable of cross-presenting OVA to CD8<sup>+</sup> T cells. In addition, the few muscle Mo-DCs that migrated to the dorsal LNs efficiently cross-primed CD8<sup>+</sup> T cells. Our results are thus consistent with several recent reports (10, 23) that challenge the view that CD8<sup>a</sup>+-type DCs prevail over CD11b+-type DCs and Mo-DCs in Ag cross-presentation. Therefore, our data suggest that i.m. vaccines do not need to specifically target CD8<sup>a</sup>+-type DCs to induce cross-priming of naive CD8<sup>+</sup> T cells.

Kool and colleagues (11) recently demonstrated that i.p. administration of alum induces the rapid recruitment of Ly-6Chigh monocytes to the peritoneal cavity and their local differentiation into Mo-DCs. In this study, we showed that i.m. injection with a dose of alum similar to that used by Kool and colleagues also induced the local recruitment of monocytes and their differentiation into Mo-DCs. However, only a small percentage of those Mo-DCs acquired the ability to migrate to the draining LNs. We demonstrated that this migration depended on CCR7 expression, as for conventional DCs. Interestingly, addition of LPS to alum enhanced the numbers of muscle Mo-DCs endowed with LN-homing properties. As a result, LPS addition increased the representation of Mo-DCs among the muscle-derived CD11b<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> cells that reached the draining LNs. Considering that Mig-Mo-DCs were capable of inducing IFN-γ production by T cells with a higher potency than muscle-derived Mig-DCs, spiking alum-adjuvanted OVA with LPS increased the global T cell stimulatory capacity of the migratory cells that reached the LNs draining the treated muscle. Therefore, addition of TLR4 agonists to alum-based vaccines should increase their immunogenicity. Consistent with that view, addition of the TLR4 agonist MPL (3-O-desacyl-4′-monophosphoryl lipid A) increased the
immunogenicity of alum-based vaccines in mice, monkeys, and humans (26, 27). A recent study based on i.p. immunization also demonstrated that addition of MPL to alum was required to induce the differentiation of CD8+ T cells into cytotoxic effectors (28). In these studies, the alum to MPL dosage differed, however, from the alum to LPS dosage used in this study. Whether the potentiating effect of MPL addition observed in these studies was due to increased migration of Mo-DCs to draining LNs remains to be formally established. Moreover, considering that successful vaccines should aim at activating both the cellular and humoral arms of immunity, an important question for future investigation is whether the three muscle-derived migratory CD11c+MHCII+ DC subsets identified in this study (Fig. 3) promote redundant or complementary functions in the setting of i.m. vaccination.

In conclusion, adjuvants are thought to promote immune responses by recruiting DCs to the vaccination site, by increasing the delivery of Ags to DCs, or by activating DCs to produce cytokines and provide activating signals to T cells (29–31). By using the expression of CD64 to distinguish CD11b+ type DCs from Mo-DCs, our data revealed that some adjuvants can also enhance the LN-homing property of muscle Mo-DCs and thereby have a positive impact on the T cell responses that ensue. Therefore, under some conditions, Mig-Mo-DCs can be mobilized to augment adaptive immune responses. Considering that the blood precursors of Mo-DCs are more numerous than those of conventional DCs (4), the possibility to develop adjuvants capable of triggering the local mobilization of Ly-6Chigh blood monocytes and of concomitantly enhancing the LN-homing properties of the resulting Mo-DCs should thus boost T cell responses. The comprehensive experimental system we have developed for studying the heterogeneous DC populations present in the skeletal muscle and their migratory counterparts present in draining LNs (Fig. 3) should thus contribute to a better understanding of the mode of action of adjuvants used in i.m. immunization and ultimately to the generation of more efficient vaccines.

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Disclosures

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References


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