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

Christelle Langlet, Samira Tamoutounour, Sandrine Henri, Hervé Luche, Laurence Ardouin, Claude Grégoire, Bernard Malissen and Martin Guilliams

*J Immunol* 2012; 188:1751-1760; Prepublished online 18 January 2012; doi: 10.4049/jimmunol.1102744
http://www.jimmunol.org/content/188/4/1751

Supplementary Material

http://www.jimmunol.org/content/suppl/2012/01/18/jimmunol.1102744.DC1

References

This article cites 31 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/188/4/1751.full#ref-list-1

Why *The JI*?

Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The *Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
CD64 Expression Distinguishes Monocyte-Derived and Conventional Dendritic Cells and Reveals Their Distinct Role during Intramuscular Immunization

Christelle Langlet, Samira Tamoutounour, Sandrine Henri, Hervé Luche, Laurence Ardouin, Claude Grégoire, Bernard Malissen, and Martin Guilliams

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 subsets were capable of capturing Ag and of migrating to draining LNs, where they efficiently activated naive T cells. In muscles, conventional 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-γ-producing T cells than conventional DCs, the addition of LPS to alum enhanced the overall immunogenicity of Ags presented by muscle 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.
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-week-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 × 10^9 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 chimerism was determined.

Ag and adjuvants

Imject alum (Pierce Biochemicals) consists of a mixture of aluminum hydroxide and magnesium hydroxide. Imject alum was mixed with OVA (Invitrogen) 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-Ab1-E) (M5/114.15.2), PE-Cy5-conjugated anti-CD45.2 (104), allophycocyanin-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-Ly6C (AL21), biotin-conjugated anti–Ly-6C (AL21), Pacific Blue-conjugated anti-CD4 (RM4-5), PE-Cy7-conjugated anti-CD8 (53-6.7), Alexa 700-conjugated anti-CD8 (53-6.7), biotin-conjugated anti–CCR7 (4B12), and PE-conjugated anti-CD69 (H1.2F3) were from BD Pharmingen. Allophycocyanin-conjugated anti-Langerin (929F3) was purchased from Dendrites.

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 with PE-Cy5-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 described 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, CD5, and TCR Vα2. 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 (Hyglo). 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 glutamine, and 50 μM 2-ME. After 3 d 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 determined. For 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−CD11b+ subset (Fig. 1). Gr1+Ly-6C(high) 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-6C(high) 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−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 CD45.1+ 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−CD11b+ cells showed a CD45.1:CD45.2 cell ratio skewed toward cells of CD45.1 origin (Fig. 1). Therefore, CD24−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−CD11b+ cells, CD64 and Ly-6C permitted to distinguish a major Ly-6C−CD64+ and a minor Ly-6C+CD64− subset among CD24−CD11b+ cells (Fig. 1). The Ly-6C−CD64− subset present in competitive chimeric mice coexpressing CD45.1 and CD45.2 was lethally irradiated and reconstituted with a 1:1 mixture of BM cells isolated from wild-type CD45.1+ 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−CD11b+ cells showed a CD45.1:CD45.2 cell ratio skewed toward cells of CD45.1 origin (Fig. 1). Therefore, CD24−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−CD11b+ cells, CD64 and Ly-6C permitted to distinguish a major Ly-6C−CD64+ and a minor Ly-6C+CD64− subset among CD24−CD11b+ cells (Fig. 1). The Ly-6C−CD64− subset present in competitive chimeric mice was CD45.1+CD45.2− and the Ly-6C+CD64− subset was CD45.1−CD45.2+.
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 the coexpression of CD64 and Ly-6C, and are thus referred to as CD11b<sup>+</sup>CD64<sup>+</sup>Int-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(langerin)<sup>−</sup>CD8α<sub>low</sub>CD172α<sup>−</sup>, and that approximately half of them expressed CD103 (Fig. 2). In contrast, the CD11b<sup>+</sup>CD64<sup>−</sup>Int-DCs were CD172α<sup>+</sup> and lacked CD207, CD8α, 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 CD<sup>4+</sup> T cells that are occurred after injection of alum-adjuvanted Ags into 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-6C<sup>−</sup>m<sup>−</sup> 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
decreased (Fig. 5B). When compared with steady-state conditions, the numbers of CD24+CD11b2 and CD11b+CD642 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+CD11b2 and CD11b+CD642 Mig-DC subsets found in the dorsal LNs expressed high levels of CCR7 (Fig. 6A). Moreover, CD24+CD11b2 and CD11b+CD642 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 (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 (Fig. 6B).

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+-type Int-DCs, CD11b+-type Int-DCs, and Int-Mo-DCs. Under steady-state conditions, CD8α+-type Int-DCs and CD11b+-type 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.

Ag-presenting capacity of muscle-derived Mig-DCs and Mig-Mo-DCs

Injection of alum-OVA in the posterior tibialis muscle triggered the extensive in vivo proliferation of adoptively transferred CD4+ OT-II T cells and CD8+ OT-I T cells, the latter being specific for H-2 Kb–OVA complexes (Supplemental Fig. 3A). Both OT-I and
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+CD11b2 Mig-DCs, CD11b+CD642 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+CD642 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.

**Mig-Mo-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.

---

**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 MHCIId expression in 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.
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 muscles at 1.5 and 6 d after immunization showed that addition of LPS to alum had a positive impact on the T cell-priming capacity of Int-Mo-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+ 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⁺ T cells. In addition, the few muscle Mo-DCs that migrated to the dorsal LNs efficiently cross-primed CD8⁺ T cells. Our results are thus consistent with several recent reports (10, 23) that challenge the view that CD8⁺-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⁺-type DCs to induce cross-priming of naive CD8⁺ 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⁺ CD11c⁺ MHCI⁺ 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 (3-0-desacyl-4'-monophosphoryl lipid A) increased the

**FIGURE 7.** Functional analysis of muscle-derived CD24⁺CD11b⁻ Mig-DCs, CD11b⁺CD64⁻ Mig-DCs, and Mig-Mo-DCs. A, B6 mice were immunized with alum-OVA, and the specified cells were sorted from the dorsal LNs 1.5 d later using the gating strategy specified in Fig. 6 and Supplemental Fig. 2. CFSE-labeled OT-I and OT-II T cells were cultured with the sorted DC subsets and after 3 d analyzed for the percentage of proliferating T cells. B and C, The specified DC subsets were sorted from the dorsal LNs of mice that were left untreated (steady state) or that received an i.m. injection of alum alone (alum) 1.5 d before sorting. Each sorted subset was pulsed with endotoxin-free OVA for 2 h. B, OVA-pulsed DCs were cultured with CFSE-labeled OT-I T cells and after 3 d of culture, T cell proliferation was evaluated. C, OVA-pulsed DCs were cultured with OT-I or OT-II T cells and after 3 d of culture, the concentration of IFN-γ was determined in culture supernatants. B and C, The very small numbers of Mig-Mo-DCs found in steady-state dorsal LNs prevented their analysis. Data are representative of three independent experiments, and the error bars shown in C correspond to the SEM.
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.

Acknowledgments
We thank M. Malissen, L. Leserman, A.-M. Schmitt-Verhulst, A. Didierlaurent, M. Jenkins, M. Plantiga, B.N. Lambrecht, and A. Malzac for dis-
cussion and advice. We thank M. Barad, P. Grenot, and A. Zouine for assistance with flow cytometry.

Disclosures

The authors have no financial conflicts of interest.

References

SUPPLEMENTAL FIGURE 1. The CD64 marker retains its discriminatory power in the inflammatory environment induced by i.m. alum administration. B6 (CD45.1) CCR2+→B6 (CD45.1-CD45.2) competitive chimeras were used 8 wk after reconstitution and injected i.m. with alum-OVA. The CD11c+MHCII+ cells present in the tibialis posterior muscle were analyzed 1.5 day after OVA-alum injection. (A) As observed in steady state muscle, CD11c+MHCII+ cells comprise both CD24+CD11b–Int-DCs and CD24–to+CD11b+ cells. Under those conditions, CD24+CD11b–Int-DCs were negative for both Ly-6C and were CD64–to+low whereas CD24–to+CD11b+ cells comprised Ly-6C–CD64–to+low Int-DCs and Ly-6C+CD64+Int-Mo-DCs. The percentages of cells found in each of the specified gates are indicated. Positioning of the CD64–to+low gate was based on staining of the CD24+CD11b–Int-DCs. (B) The CD24+CD11b–Int-DCs, CD11b+CD64–to+low Int-DCs and CD11b+CD64+Int-Mo-DCs present in the tibialis posterior muscle were analyzed for the percentage of CD45.1+ and CD45.2+ cells they contained. In the experiment shown, due to a slight overlap in the gates corresponding to CD11b+CD64+Int-Mo-DC and CD11b+CD64–to+low Int-DCs, the later cells displayed a CD45.1–CD45.2 ratio slightly higher than that of the CD24+CD11b–Int-DCs. Regardless of this operational limitation, the cells recorded in the CD11b+CD64+ gate remained, however, of pure monocyte origin. Data are representative of 4 chimeric mice corresponding to 3 independent experiments.
SUPPLEMENTAL FIGURE 2. CD64 expression still permitted to distinguish the CD11b^+CD64^- Mig-DCs and the CD11b^+CD64^+ Mig-Mo-DCs that reach dorsal LN following i.m. alum administration. B6 (CD45.1) CCR2^+ + B6 (CD45.2) CCR2^- → B6 (CD45.1-CD45.2) competitive chimeras were used 8 wk after reconstitution and injected i.m. with alum-OVA. Cells from the dorsal LN were analyzed 1.5 day after i.m. alum-OVA injection. (A) After excluding NK, T and B cells and the Ly-6C^{high} LT-Mo-DCs (see Fig. S6), CD11c^{int to high}MHCI^{hi} Mig-DCs were identified (purple cluster in left panel) and subdivided into CD24^+CD11b^- Mig-DCs and CD24^-to-^+CD11b^+ cells (middle panel). CD24^-to-^+CD11b^+ cells were further divided into Ly-6C^-CD64^- Mig-DCs and Ly-6C^-CD64^+ Mig-Mo-DCs (right panel). The percentages of cells found in each of the specified gates are indicated. Positioning of the CD64^- gate was based on CD64 staining of CD24^-CD11b^- Mig-DCs. (B) The CD24^-CD11b^- Mig-DCs, CD11b^-CD64^- Mig-DCs and CD11b^-CD64^- Mig-Mo-DCs present in the tibialis posterior muscle were analyzed for the percentage of CD45.1^+ and CD45.2^+ cells they contained. Data are representative of at least 3 independent experiments involving groups of 3 to 5 mice.
SUPPLEMENTAL FIGURE 3. Intramuscular alum-OVA injection elicits \textit{in vivo} proliferation of OVA-specific CD4$^+$ and CD8$^+$ T cells. CD45.1$^+$, CFSE-labeled OT-I and OT-II T cells were cotransferred into B6 [CD45.2] mice 1 day before i.m. alum-OVA injection. At days 3, 6, 9 and 12 after immunization, the CD8$^+$ OT-I and CD4$^+$ OT-II cells present in the dorsal LN were analyzed for the extent of CFSE dilution ($A$) and yield ($B$) of proliferating cells. Data are representative of at least 3 independent experiments involving groups of 3 to 5 mice and error bars shown in ($B$) correspond to the SEM.
**SUPPLEMENTAL FIGURE 4.** CD64 expression distinguishes Mo-DCs from conventional DCs even in conditions of strong DC activation. (A) Mig-DCs isolated from the LNs of steady-state mice were left unstimulated or stimulated *in vitro* with the specified doses of LPS. The expression of CD64 on CD8α⁺- and CD11b⁺-type DCs was evaluated 24h later by flow-cytometry. (B) Mice were injected i.v. with LPS (250 ng or 5 μg) or left untreated. 24h after LPS injection, the conversion of splenic Ly-6C⁺ monocytes into MHCI⁺CD11c⁺ Mo-DCs was evaluated and the CD64 expression of Mo-DCs compared to that of CD8α⁺- and CD11b⁺-type LT-DCs found in the spleen. Data are representative of at 2 independent experiments.