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-γ-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: 000–000.

Dendritic 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 excite 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).

**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.
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 alun-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 alun-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 alun-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

Inject alum (Pierce Biochemicals) consists of a mixture of aluminum hydroxide and magnesium hydroxide. Inject alum was mixed with OVA (Invitrogen) and then stirred for at least 1 h. A total of 15 μl Inject 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 Inject 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 alun-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 Perciprep solution (d = 1.32 g/ml; Abcyn). 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 FACS DIVA 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 (33103) was from R&D; PE-CY7-conjugated anti-CD11c (N418), Alexa 700-conjugated anti-MHCII (I-Ab/E) (M5/114.15.2), PE-CY5.5-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 cBioScience; 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-CD6 (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-CY5.5-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 (XM61.2; 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 re suspended 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 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+ expression.
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<sup>high</sup> 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<sup>high</sup> 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<sup>+</sup> B6 mice and from CD45.2<sup>+</sup> B6 mice that lacked CCR2. Those B6 (CD45.1) CCR2<sup>+</sup> + B6 (CD45.2) CCR2<sup>−</sup>→ 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<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> muscle macrophages (Fig. 1 and data not shown). Among the CD11c<sup>+</sup>MHCII<sup>+</sup> cells found in the muscle of the chimeras, the CD24+CD11b<sup>+</sup> cells 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<sup>-</sup>CD11b<sup>-</sup> cells showed a CD45.1:CD45.2 cell ratio skewed toward cells of CD45.1 origin (Fig. 1). Therefore, CD24<sup>-</sup>CD11b<sup>-</sup> cells were heterogeneous and contained both CCR2-dependent Mo-DCs and CCR2-independent CD11b<sup>-</sup>-type DCs.

Among the wealth of markers tested to distinguish CCR2-dependent and -independent CD24<sup>-</sup>CD11b<sup>-</sup> cells, CD64 and Ly-6C permitted to distinguish a major Ly-6C<sup>−</sup>CD64<sup>−</sup> and a minor Ly-6C<sup>+</sup>CD64<sup>+</sup> subset among CD24<sup>-</sup>CD11b<sup>-</sup> cells (Fig. 1). The Ly-6C<sup>−</sup>CD64<sup>−</sup> subset present in competitive chimeras was almost entirely composed of donor-derived cells, indicating that CCR2 is required for the development of the Ly-6C<sup>−</sup>CD64<sup>−</sup> subset.
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+CD64−Int-DCs. Note that CD24+CD11b−Int-DCs also displayed a Ly-6C−CD64+ phenotype (Fig. 1). In marked contrast, the Ly-6C+CD64−subset resembled muscle macrophages in that it was dominated by cells of CD45.1 origin (Fig. 1). Therefore, among the CD11b+CD11c+MHCI+ 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+CD64+Int-Mo-DCs.

Characterization of conventional muscle DCs

Further characterization of the CD24+CD11b−Int-DCs present in skeletal muscles showed that they were CD207(langerin)+CD8αlo−CD172α−, and that approximately half of them expressed CD103 (Fig. 2). In contrast, the CD11b+CD64−Int-DCs were CD172α+ and lacked CD207, CD8α, and CD103 expression (Fig. 2). Therefore, based on their surface phenotype, the CD24+CD11b−Int-DCs and CD11b+CD64−Int-DCs identified in skeletal muscle correspond to CD8α+ and CD11b+ type DCs, respectively (1–3).

The CD11c+MHCI+ cells found in steady-state skeletal muscle are thus composed of CD8α− and CD11b+ 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+ T cells that are specific for H-2I−Ab−OV A complexes were adoptively transferred with steady-state conditions, the numbers of CD24+CD11b−Int-DCs dominate muscle-derived migratory cells

After alum-OVA injection, the number of CD11b+CD64−Int-DCs present at the peak of the response had increased 19-fold and 9-fold, respectively (Fig. 5A). When compared with steady-state conditions, the numbers of CD24+CD11b− and CD11b+CD64−Int-DCs increased, peaked at day 6 postimmunization, and then decreased (Fig. 5A). 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+CD64−to low DCs to be distinguished from CCR2-dependent CD11b+CD64+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+CD11b+ and CD11b+CD64−Int-DCs increased, peaked at day 6 postimmunization, and then decreased (Fig. 5A). Thus, 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.

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+ cells were analyzed for CD11c and MHCI expression. CD11c+MHCI+ cells were subdivided into CD24+CD11b−Int-DCs and CD11b+ cells that were CD24− to +. CD24− to −CD11b+ were further subdivided into CD64+Int-DCs and CD64−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+CD11b−Int-DCs, CD11b+CD64−Int-DCs, and CD11b+CD64+Int-Mo-DCs gated as specified in A. Data shown are representative of three independent experiments.
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 (Fig. 6B).

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–OV A 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+CD11b\(^2\) Mig-DCs, CD11b+CD64\(^2\) 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\(^2\) 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.
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. To validate these findings in vivo, we injected B6 mice with muscle-derived conventional Mig-DCs (Fig. 8). Myeloid cells present in the tibialis posterior muscle (muscle) and dorsal LNs of B6 and CCR7-deficient mice were analyzed 1.5 d after i.m. alum-OVA injection. Absolute numbers of CD24+CD11b+ DCs, CD11b+CD64+ DCs, Mo-DCs, and LT-Mo-DCs were determined. Data are representative of three independent experiments involving three to five mice, and error bars shown in B correspond to the SEM.

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+ 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 CD8α+-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 CD8α+-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+MHCII+ 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-desacetyl-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+Mig-DCs and CD11b+CD64+ Mig-DCs and Mig-Mo-DCs were sorted from dorsal LNs 1.5 d after injection and pulsed with endotoxin-free OVA for 2 h. OT-I cells were cultured with the specified OVA-pulsed DC subsets and after 3 d of culture, the concentration of IFN-γ was determined in culture supernatants. D, OT-I cells were transferred into B6 mice 1 d before i.m. injection of OVA adsorbed to alum (alum-OVA) or of OVA plus LPS adsorbed to alum (alum-OVA-LPS). Six days after immunization, the OT-I cells present in the dorsal LNs were isolated and restimulated in vitro with PMA and ionomycin for 6 h and IFN-γ production was assessed by intracellular staining. The percentages of CFSE− IFN-γ+ OT-I T cells are shown. Data are representative of three independent experiments, and the error bars shown in A and C correspond to the SEM. *p < 0.03 versus alum-OVA.

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FIGURE 8. Addition of LPS to alum increases the LN-homing properties of Int-Mo-DCs. B6 mice were injected i.m. with alum alone (alum), alum plus LPS (alum LPS), or left untreated (steady state). A and B, Histograms show the absolute numbers of the specified cells (see key) found in the posterior tibialis muscles (A) and the dorsal LNs (B) before (steady state) or 1.5 (D1.5) and 6 (D6) days after treatment. Pie charts show the relative percentage of the specified cells at the peak of DC accumulation in the posterior tibialis muscles (day 6; A) and in the dorsal LNs (day 1.5; B). C, B6 mice were injected i.m. with alum plus LPS. CD24+ CD11b+ Mig-DCs and CD11b+CD64+ Mig-DCs and Mig-Mo-DCs were sorted from dorsal LNs 1.5 d after injection and pulsed with endotoxin-free OVA for 2 h. OT-I cells were cultured with the specified OVA-pulsed DC subsets and after 3 d of culture, the concentration of IFN-γ was determined in culture supernatants. D, OT-I cells were transferred into B6 mice 1 d before i.m. injection of OVA adsorbed to alum (alum-OVA) or of OVA plus LPS adsorbed to alum (alum-OVA-LPS). Six days after immunization, the OT-I cells present in the dorsal LNs were isolated and restimulated in vitro with PMA and ionomycin for 6 h and IFN-γ production was assessed by intracellular staining. The percentages of CFSE− IFN-γ+ OT-I T cells are shown. Data are representative of three independent experiments, and the error bars shown in A and C correspond to the SEM. *p < 0.03 versus alum-OVA.

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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