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Monocyte-Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against *Leishmania*

Beatriz León,[1] María López-Bravo,[1] and Carlos Ardavín[1,*]
[1] Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología, Universidad Autónoma, 28049 Madrid, Spain
*Correspondence: ardavin@cnb.uam.es
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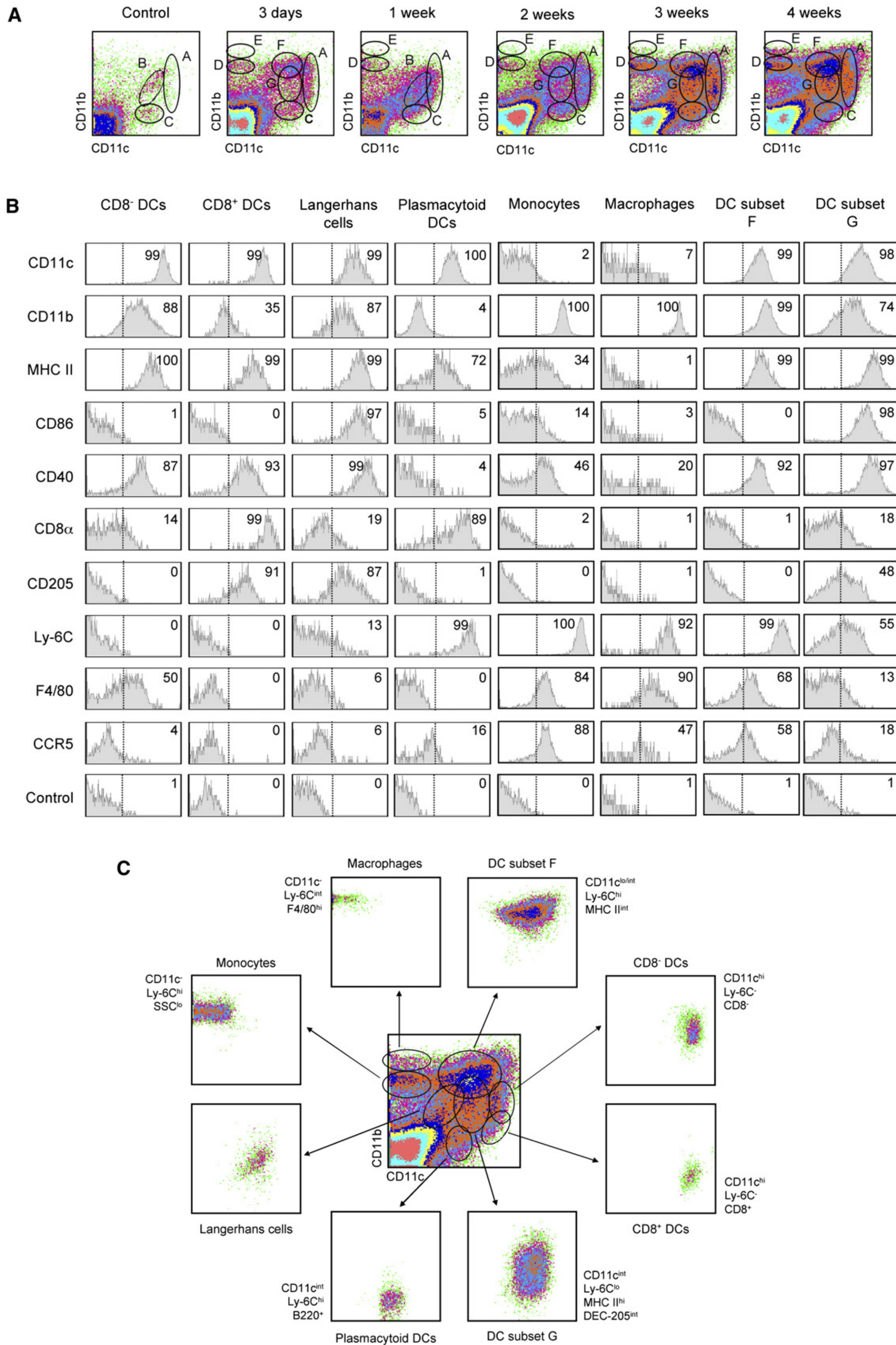
SUMMARY

Infection-induced inflammatory reactions involve a strong increase in dendritic cells (DCs) at the infection site and draining lymph nodes (dLNs). Whether inflammatory DCs are recruited to these locations or differentiate locally, and what their functional relevance is, remain unclear. Here we showed that during *Leishmania* infection, monocytes were recruited to the dermis and differentiated into "dermal monocyte-derived DCs," which subsequently migrated into the dLNs. In addition, monocyte recruitment to the dLNs resulted in the differentiation into "LN monocyte-derived DCs." Analysis of the kinetics of monocyte differentiation into DCs, susceptibility to infection, IL-12 production, and *L. major*-specific T cell stimulation potential suggest that dermal monocyte-derived DCs controlled the induction of protective T helper 1 responses against *Leishmania*. Thus, the demonstration of monocyte differentiation potential into DCs during in vivo infection and of local DC differentiation in inflammatory foci suggests that de novo formed monocyte-derived DCs are essential in T cell immunity against pathogens.

INTRODUCTION

Recent research suggests that differentiation of conventional dendritic cells (DCs), located in mucosal surfaces and lymphoid organs, does not occur in the bone marrow, but locally, in these peripheral locations (reviewed in Ardavin, 2003; Shortman and Naik, 2007). This hypothesis relies on the fact that conventional DC subpopulations existing in lymphoid and nonlymphoid organs are found neither in the bone marrow nor in the blood and that DC number increases considerably in the lymphoid organs during infection; in addition, DC precursor populations have been described in the circulation (reviewed in Ardavin, 2003). The concept of local DC differentiation implies the existence of circulating DC precursors that would be recruited to peripheral antigen-capture locations and lymphoid organs, where they would differentiate into DCs. Peripheral DC differentiation would therefore be responsible for the maintenance of the different DC subsets existing under steady state. A number of reports suggest that DC precursor recruitment and de novo DC formation could also take place in inflammatory foci and lymphoid organs during infection (Martin et al., 2002; Serbina et al., 2003; Yoneyama et al., 2001). Although different research groups have addressed the identity of DC precursors in steady state (reviewed in Shortman and Naik, 2007), the characterization of DC precursors responsible for de novo DC formation during infection has remained elusive. Studies describing the accumulation of DCs in lymphoid tissues during infection or showing in noninfectious inflammatory conditions that monocytes can differentiate into DCs (Le Borgne et al., 2006; Geissmann et al., 2003; Naik et al., 2006; Randolph et al., 1999; Serbina et al., 2003) suggest that DC differentiation from monocytes could occur in vivo during infection. However, this hypothesis was challenged by a report showing that monocyte differentiation into DCs induced by subcutaneous (s.c.) injection of latex microspheres was blocked if bacteria were present in the injection area (Rotta et al., 2003).

In order to provide new insights on de novo DC differentiation during in vivo infection, we used a murine experimental model of cutaneous leishmaniasis induced by *Leishmania major (L. major)* injection. Based on phenotypic studies, analysis of the kinetics of monocyte recruitment and differentiation into DCs, and monocyte transfer assays, our data demonstrate that during *L. major* infection, two de novo formed DC subsets were found in the popliteal lymph nodes (PO-LNs). These included DCs derived from monocytes recruited to the dermis that differentiated locally and migrated to the PO-LNs (dermal mo-DCs) and from monocytes recruited to the PO-LNs where they differentiated into DCs (LN mo-DCs). Dermal mo-DCs and LN mo-DCs were the DC subsets predominantly infected by *L. major*, and accordingly they were the only DC subsets that expressed IA^d-LACK peptide complexes. Among de novo formed monocyte-derived DCs, dermal mo-DCs displayed a higher capacity to produce IL-12 and to stimulate *L. major*-specific T cells, suggesting their essential role in the induction of protective T helper 1 (Th1) responses against *Leishmania*.


A
Control
3 days
1 week
2 weeks
3 weeks
4 weeks
CD11b
CD11c
A
B
C
D
E
F
G
B
CD8⁻ DCs
CD8⁺ DCs
Langerhans cells
Plasmacytoid DCs
Monocytes
Macrophages
DC subset F
DC subset G
CD11c 99 99 99 100 2 7 99 98
CD11b 88 35 87 4 100 100 99 74
MHC II 100 99 99 72 34 1 99 99
CD86 1 0 97 5 14 3 0 98
CD40 87 93 99 4 46 20 92 97
CD8α 14 99 19 89 2 1 1 18
CD205 0 91 87 1 0 1 0 48
Ly-6C 0 0 13 99 100 92 99 55
F4/80 50 0 6 0 84 90 68 13
CCR5 4 0 6 16 88 47 58 18
Control 1 0 0 0 0 1 1 1
C
Macrophages
CD11c⁻
Ly-6Cint
F4/80hi
DC subset F
CD11clo/int
Ly-6Chi
MHC IIint
Monocytes
CD11c⁻
Ly-6Chi
SSClo
CD8⁻ DCs
CD11chi
Ly-6C⁻
CD8⁻
Langerhans cells
CD8⁺ DCs
CD11chi
Ly-6C⁻
CD8⁺
Plasmacytoid DCs
CD11cint
Ly-6Chi
B220⁺
DC subset G
CD11cint
Ly-6Clo
MHC IIhi
DEC-205int
CD11b
CD11c

RESULTS

Changes in PO-LN DC Subpopulations

Injection of *L. major* in the footpad of C57BL/6 mice induced an inflammatory process causing a swelling reaction, both in the skin and draining PO-LNs. This inflammatory reaction was detectable 24 hr after injection, reached a maximal development after 4–5 weeks, and then declined progressively. In C57BL/6 mice, this led to healing of leishmaniasis at 5–6 weeks after infection. In contrast, in BALB/c mice that cannot control parasite infection and die of leishmaniasis, disease progression caused a continuous development of the inflammatory reaction both in the skin and PO-LNs (see Figures S1A and S1B in the Supplemental Data available online).

In order to investigate whether *L. major* infection caused quantitative and qualitative changes in the DC subsets existing in the PO-LNs, and whether de novo DC differentiation occurred, we first characterized the DC subpopulations present in the PO-LNs at different time points after infection. Uninfected PO-LN DC subpopulations included $CD8^-$ and $CD8^+$ DCs, Langerhans cells (LCs), dermal DCs (dDCs), and plasmacytoid (pDCs) (Figure 1A). These DC subsets, also found within infected PO-LNs, essentially displayed the same phenotypic characteristics as previously reported (Figure 1B; reviewed in Villadangos and Heath, 2005). We further examined all DC-related PO-LN subpopulations at 4 weeks after infection (Figure 1B). Although $CD8^-$ and $CD8^+$ DCs were negative for CD86, they expressed higher MHC II and CD40 than their control counterparts (not shown), suggesting that they had undergone a partial maturation process during infection. Interestingly, *L. major* infection was paralleled by the appearance of three new DC-related subpopulations, not detected in control PO-LNs. These included $CD11c^-$ cells expressing high CD11b that, based on their phenotype (Figures 1B and 1C) and previous data (León et al., 2004), corresponded to monocytes, and two $CD11c^+$ DC subsets (DC subsets F and G; Figures 1A and 1C), with different amounts of CD11b expression. As shown in Figure 1B, an extensive phenotypic study was performed by correlating the expression of the DC marker CD11c with cell markers that allowed the description of the different mouse DC subpopulations (reviewed in Ardavin, 2003). This led to a precise definition of all PO-LN DC subsets during *L. major* infection (Figure 1C) and to an accurate calculation of the absolute number of cells within each subset (Figure 2A). In this regard, it is important to emphasize, that in Figure 1A, regions A to G do not define exactly the indicated cell subsets, because these cannot be accurately characterized based only on the CD11c versus CD11b correlation.

DC subsets F and G were already detectable 3 days after infection and constituted quantitatively the main PO-LN DC subpopulations from week 3 onward. Their number dropped markedly at the end of the first week but increased again from week 2 (Figures 1C and 2A; Table S1). The phenotypic analysis of these DC subsets revealed that DCs of subset F were Ly-$6C^{high}$, displayed intermediate MHC II expression, and did not express CD86. DCs of the subset G were Ly-$6C^{low}$ and expressed high amounts of MHC II and CD86.

Figure 2A summarizes the variations in the absolute cell number for each DC-related PO-LN subpopulation, during *L. major* infection. $CD8^+$ and $CD8^-$ DC subsets increased significantly and progressively from day 3, reaching a peak during week 2. After this point, the number of $CD8^-$ DCs (that increased approximately 30-fold from day 3 to week 2) remained unchanged until week 4, whereas the number of $CD8^+$ DCs (that increased aproximately 15-fold from day 3 to week 2) dropped ~80% from week 2 to week 4. LCs and pDCs followed a similar kinetics as did $CD8^-$ DCs. Regarding the new subsets that appeared as a consequence of infection, i.e., monocytes and the DC subsets F and G, they all dramatically increased in number from day 1 to week 4 (~40-fold for monocytes and ~100-fold for DC subsets F and G), although, as pointed out above, their number dropped markedly at the end of the first week. At week 4 after infection, DC subsets F and G represented ~75% of all DCs present in the PO-LNs. Macrophages, a minor population in the PO-LNs, followed a similar kinetics as did $CD8^-$ DCs.

Thus, all DC subpopulations present in the PO-LNs increased in number during the first 2 weeks of infection, but only the new DC subsets F and G kept increasing at weeks 3 and 4. Cell-cycle analysis of PO-LN cells at 3 weeks after infection revealed that no significant cell proliferation was found within monocytes or DCs, whereas up to 20% cycling lymphocytes were detected (Figure 2B). As reported (reviewed in Sacks and Noben-Trauth, 2002), the immune response of C57BL/6 mice against *L. major* switched, between 2 and 3 weeks after infection, from a nonprotective Th2 response toward a protective Th1 response, as revealed by the IL-4 versus IFN-γ secretion pattern (Figure 2C). This Th2 to Th1 switch resulted in a decrease in the number of eosinophils after week 2 (Figure 2A), whereas eosinophils increased all along the disease in BALB/c mice (not shown). Thus, the late phases of the immune response against *L. major* infection were

Figure 1. Characterization of the PO-LN DC Subpopulations during *L. major* Infection

DC subpopulations were analyzed in the PO-LNs of C57BL/6 mice at different time points after subcutaneous infection of *L. major* promastigotes.

(A) Changes in the DC subpopulations present in the PO-LNs at different time points after *L. major* infection (A, $CD8^-$ and $CD8^+$ DCs; B, LCs and dDCs; C, pDCs; D, monocytes; E, macrophages; F, DC subset F; G, DC subset G, as indicated in [B] and [C]).

(B) Phenotypic analysis of PO-LN DC subpopulations at week 4 after infection. The percentage of cells with a fluorescence intensity over control staining is indicated.

(C) Definition of the DC subpopulations present in the PO-LNs at week 4 after infection based on the level of expression of the indicated markers. Data are representative of 5 independent experiments with similar results.

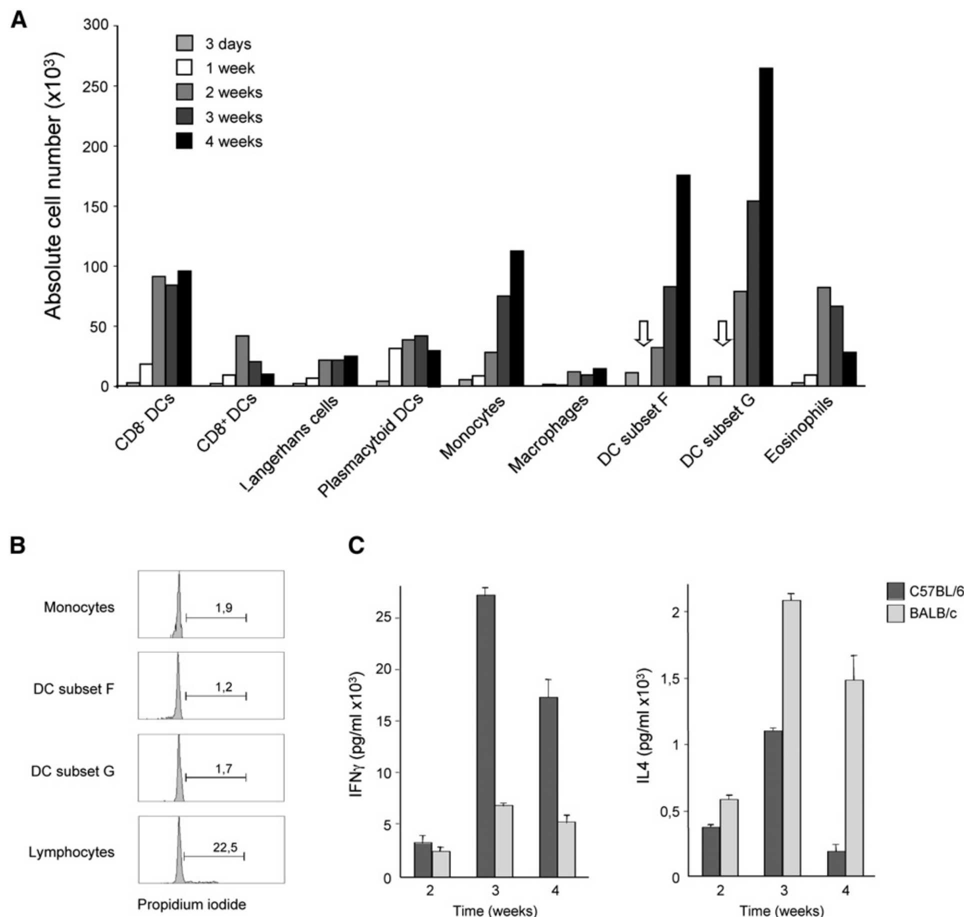


Figure 2. Kinetics of PO-LN Subpopulations during *L. major* Infection

The kinetics of the immune response against *L. major* was determined by analyzing the variations in the absolute number within the different cell populations present in the PO-LNs at different time points after infection, the cell-cycle status of monocytes and monocyte-derived DCs, and the T helper response polarization.

(A) Changes in the absolute number of each PO-LN DC subpopulation along *L. major* infection. White arrows indicate the reduction in DC subsets F and G occurring 1 week after infection.

(B) Cell-cycle analysis of monocytes, DC subsets F and G, and total lymphocytes from the PO-LNs at week 4 after infection. Numbers indicate the percentage of cells in the S + G/M stages of the cell cycle as assessed by propidium iodide staining.

(C) Kinetics of Th1 (IFNγ production) versus Th2 (IL-4 production) response during *L. major* infection in C57BL/6 and BALB/c mice.

Error bars represent the SD of triplicate cultures. Data are representative of 5 (A) and 3 (B and C) independent experiments with similar results.

characterized by an important increase in monocytes and DC subsets F and G.

Analysis of DC Subpopulations Present in the Inflamed Skin

The phenotype of monocytes and DC subsets F and G led us to speculate that DC subset F could represent monocyte-derived DCs in an immature stage, whereas DC subset G would correspond to monocyte-derived DCs that have undergone a maturation process. This postulate was the basis of our working hypothesis, summarized in Figure S2, proposing that DC subset F would correspond to immature DCs differentiated from monocytes directly recruited to the PO-LNs. In contrast, because the phenotype reported for dDCs after migration to the LNs (Itano et al., 2003) coincides with that of DC subset G, this subset could correspond to DCs differentiated from monocytes recruited to the dermis that have subsequently migrated to the PO-LNs. To demonstrate this hypothesis, we analyzed whether monocytes were recruited to the dermis and whether monocyte-derived DCs were detected in the dermis during *L. major* infection.

Dermis-infiltrating cells (identified as CD45-positive cells) were characterized by correlating the expression of CD11c and Ly-6C with that of CD11b, MHC II, CD86, DEC-205, or F4/80. At week 3 after infection (corresponding to the peak of inflammation), the dermis-infiltrating cells included monocytes, macrophages, neutrophils, and DCs (Figure 3A). DCs could be further subdivided in Ly-6C^{high} and Ly-6C^{int} fractions, the latter displaying a more mature phenotype (Figure 3B). Ly-6C^{int} DCs infiltrating the dermis were phenotypically similar to subset G DCs detected in

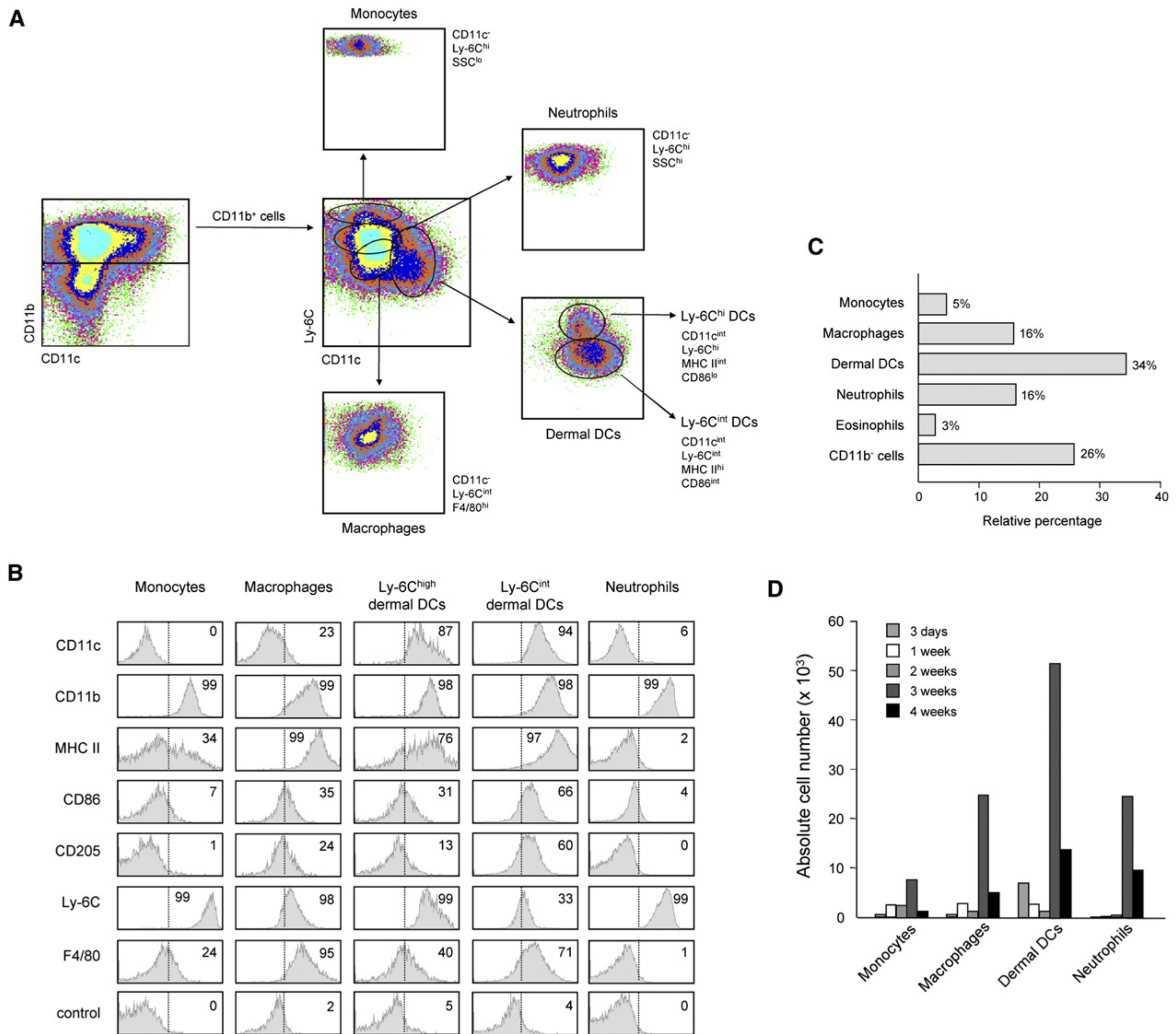


Figure 3. Analysis of Dermis-Infiltrating Cells during *L. major* Infection

Dermis-infiltrating cells were analyzed in the footpad of C57BL/6 mice at different time points after subcutaneous infection with *L. major* promastigotes.

(A) Definition of the main cell populations present in the dermis 3 weeks after infection based on the expression of the indicated markers.

(B) Phenotypic analysis of dermis-infiltrating cell populations 4 weeks after infection. The percentage of cells with a fluorescence intensity over control staining is indicated.

(C) Relative percentage represented by each dermis-infiltrating cell population 3 weeks after infection. Data are representative of 4 independent experiments with similar results.

(D) Changes in the absolute number of the dermis-infiltrating cell populations at different time points after infection.

the PO-LNs, although the latter displayed higher MHC II and CD86 expression. At the peak of the inflammatory reaction, DCs constituted the most numerous cell population, accounting for up to 35% of CD45$^+$ dermal cells; both macrophages and neutrophils represented ~15%, and monocytes ~5% (Figure 3C). The remaining 30% included eosinophils, T, and NK cells. The absolute number of dermal monocytes, macrophages, DCs, and neutrophils increased dramatically from week 2 to 3 of infection and decreased from week 4 (Figure 3D).

Therefore, *L. major* infection induced the recruitment of monocytes to the dermis and a strong increase in the absolute number of DCs in this location. Both processes were coincident with the increase in the number of monocytes and DC subsets F and G in the PO-LNs and with the onset of the Th1 response. Taking into account the phenotype of dermal and PO-LN monocytes, DC subsets F and G, and Ly-6C^{high} and Ly-6C^{int} DCs found in the dermis, our data suggest that Ly-6C^{int} DCs would result from partial maturation of Ly-6C^{high} DCs. Partially mature Ly-6C^{int} DCs would complete their maturation after migration to the PO-LNs, where they would constitute the DC subset G. DC subset F would result from the local differentiation of monocytes recruited to the PO-LNs.

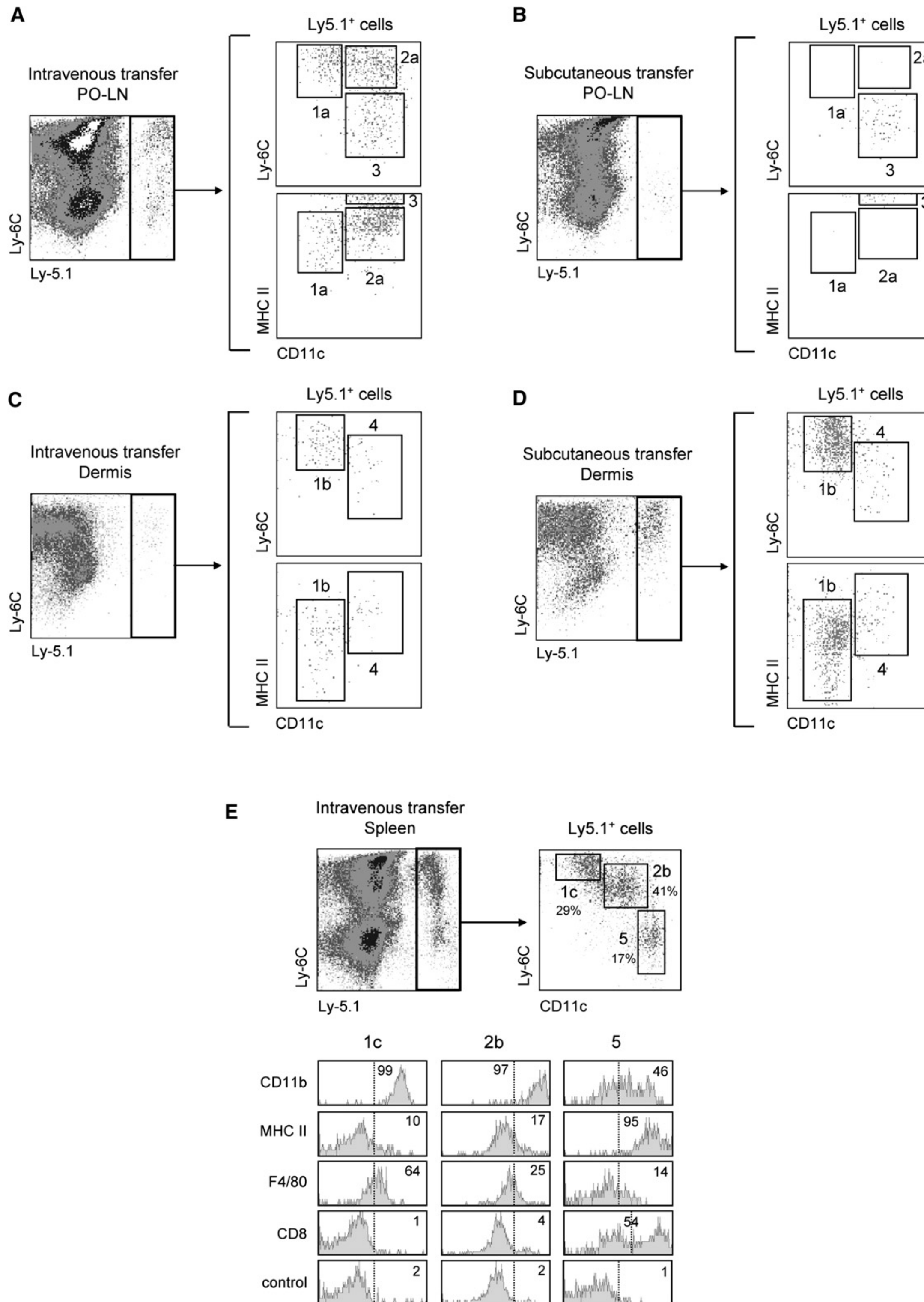


Figure 4. Monocyte Recruitment and Differentiation during *L. major* Infection

In vivo differentiation of monocytes isolated from C57BL/6 Ly 5.1 Pep^{3b} mice and transferred intravenously or subcutaneously into C57BL/6 Ly 5.2 mice at week 4 after infection with *L. major*, was analyzed in the dermis and PO-LNs.

(A and B) Differentiation of DCs from monocytes transferred intravenously (A) or subcutaneously (B) 4 weeks after infection, analyzed in the PO-LNs.

(C and D) Differentiation of DCs from monocytes transferred intravenously (C) or subcutaneously (D) 4 weeks after infection, analyzed in the dermis.

Analysis of In Vivo Monocyte Recruitment and De Novo DC Differentiation

Monocyte transfer experiments were performed to address whether DC subsets F and G derived from monocytes recruited to the PO-LNs and skin, respectively. Monocytes from Ly-5.1^{+} mice were transferred, either intravenously or subcutaneously, into Ly-5.2^{+} mice, 3–4 weeks after *Leishmania* infection, i.e., coincident with the peak of the Th1 response. The PO-LNs and dermis were analyzed 72 hr after monocyte transfer for the presence of Ly-5.1^{+} monocyte-derived cells (Figure 4A). After intravenous (i.v.) transfer, Ly5-1^{+} cells present in the PO-LNs included CD11c^{low}, Ly-6C^{high}, MHC II^{int} cells (population 1a), CD11c^{int}, Ly-6C^{high}, MHC II^{int} cells (population 2a), and CD11c^{int}, Ly-6C^{low}, MHC II^{high} cells (population 3). Previous data (León et al., 2004) indicate that population 1a most likely corresponds to monocytes in an early phase of differentiation into DCs. Populations 2a and 3 displayed the same phenotype as endogenous DC subsets F and G, respectively (Figure 1B). When PO-LNs were analyzed for the presence of Ly-5.1^{+} cells after monocyte s.c. transfer, only CD11c^{int}, Ly-6C^{low}, MHC II^{high} DCs, phenotypically identical to population 3 in Figure 4A or to the endogenous DC subset G were detected (Figure 4B).

Analysis of the dermis after i.v. or s.c. monocyte transfer (Figures 4C and 4D) revealed that Ly5-1^{+} monocyte-derived cells included CD11c^{low}, Ly-6C^{high}, MHC II^{int} cells (population 1b), and CD11c^{int}, Ly-6C$^{int-high}$, MHC II$^{int-high}$ cells (population 4). Population 1b was equivalent to population 1a found in the PO-LNs after i.v. monocyte transfer. Population 4 had a phenotype equivalent to that described for endogenous dDCs (Figures 3A and 3B) and consequently included DCs expressing from intermediate to high levels of Ly-6C and MHC II.

Results from monocyte transfer experiments represent the first formal demonstration of monocyte differentiation into DCs during an in vivo infectious process. In addition, they strongly support that population 2a, and more likely the DC subset F, corresponds to DCs differentiated from monocytes recruited to the PO-LNs through high endothelial venules (HEVs). Finally, these data demonstrate that population 3, and therefore most likely the DC subset G, correspond at least in part to DCs differentiated in the dermis from monocytes that had subsequently migrated to the PO-LNs through the lymphatics. Nevertheless, we could not exclude that a fraction of cells included in population 3 could derive from population 2a, as a result of a maturation process.

The analysis of the spleen (Figure 4E), an organ not receiving afferent lymphatic vessels, revealed that cells derived from i.v. transfer of monocytes included CD11c^{low}, Ly-6C^{high}, MHC II^{int} cells (population 1c; equivalent to 1a and 1b) and CD11c^{int}, Ly-6C^{high}, MHC II^{int} cells (population 2b; equivalent to 2a), but not cells corresponding to population 3 (and therefore to DC subset G). This result favors the hypothesis that DC subset F corresponds to DCs differentiated from monocytes recruited from the blood to the PO-LNs. Intravenous transfer of monocytes also appeared to generate splenic DCs equivalent to endogenous CD8^{+} and CD8^{-} DCs (population 5).

In conclusion, our data derived from monocyte-transfer assays support that infection-induced inflammatory processes promote the recruitment to antigen-capture areas and associated lymphoid organs of monocytes that subsequently differentiate locally into DCs. According to our working hypothesis, de novo formed DCs found in the PO-LNs would include DCs generated in situ from monocytes recruited through the HEVs (that will be referred to as LN mo-DCs), and DCs differentiated from monocytes recruited to the dermis through dermal venules that would have undergone a process of maturation and migration to the PO-LNs (these DCs will be referred to as dermal mo-DCs).

Analysis of Cell Subsets Infected by *L. major*

The fact that de novo formed dermal mo-DC and LN mo-DC subpopulations increased dramatically along weeks 3 and 4 of infection, concomitantly with the Th2-Th1 switch, prompted us to investigate their role in the induction of T cell immunity against *L. major*. In this sense, it has been demonstrated that protective immune responses against *L. major* rely on the induction of Th1 responses against the *L. major* immunodominant LACK antigen that in turn involve the presentation by infected DCs of a LACK-derived peptide in the context of MHC II molecules to specific CD4^{+} T cells (Mougneau et al., 1995).

We first analyzed which PO-LN DC subsets were infected by injecting *L. major* red fluorescent DsRed^{+} promastigotes, which allow the detection of the cells having internalized the parasite. In the PO-LNs, ~1% of the cells were infected at week 4 after infection, and those included essentially monocytes, macrophages, LN mo-DCs, and dermal mo-DCs (Figure 5A). The percentage of infected cells within each of these subsets was ~5%–7%, whereas up to 20% of macrophages were infected. The percentage of infected cells among CD8^{-} DCs, CD8^{+} DCs, LCs, pDCs, and CD11b-negative cells (including T cells, B cells, and NK cells) was undetectable (Figure 5B). Analysis of the footpad revealed that the percentage of cells infected among dermal-infiltrating cells was significantly higher than in the PO-LNs (~50%). These included monocytes, macrophages, dermal mo-DCs, neutrophils, and eosinophils. A high percentage of all these cell types was infected: ~40% of dermal monocytes and eosinophils, 70% of dermal macrophages and neutrophils, and 90% of dermal mo-DCs (Figure 5C). In contrast, less than 5% of dermal CD11b-negative cells (mainly T cells and NK cells) were infected. With regard to dermal mo-DCs, the fact that more than 90% of them were infected in the

(E) Differentiation and phenotype of DCs derived from monocytes transferred intravenously 4 weeks after infection, analyzed in the spleen. Numbers represent the percentage of cells with a fluorescence intensity over control staining.
Data are representative of 4 (A–D) and 3 (E) independent experiments with similar results.

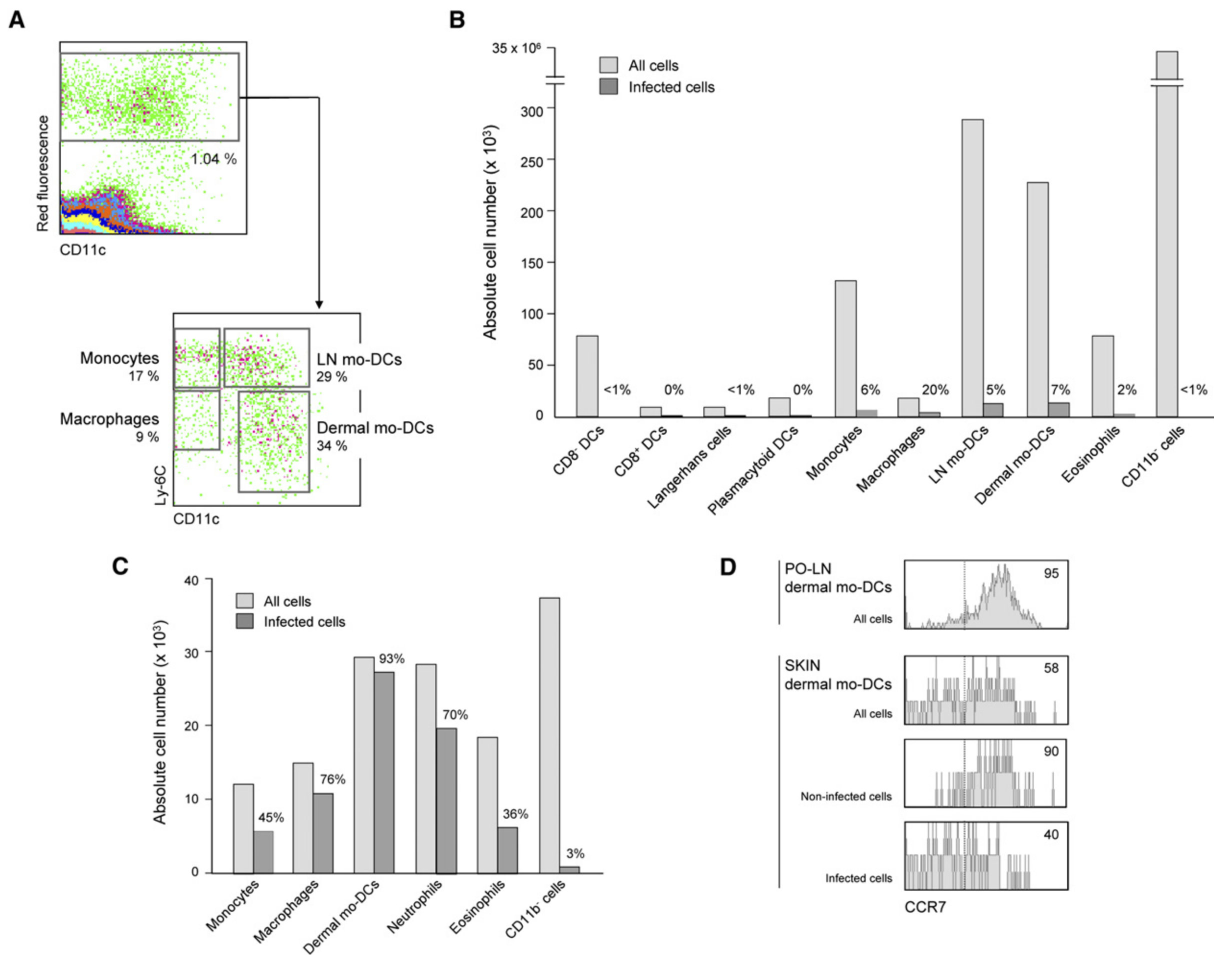


Figure 5. Analysis of DC Subsets Infected by *L. major*

Cells infected by *L. major* were analyzed in the PO-LNs and dermis of C57BL/6 mice 4 weeks after subcutaneous injection of red fluorescent DsRed$^+$ *L. major* promastigotes.

(A) Characterization of the PO-LN cell populations infected by *L. major* at week 4 after injection of red fluorescent parasites; infected cells were analyzed after gating on red fluorescent cells.

(B) Absolute number and relative proportion of cells infected by *L. major* for the indicated PO-LNs cell types, at week 4 after injection of red fluorescent parasites.

(C) Absolute number and relative proportion of cells infected by *L. major* for the indicated dermal cell types, at week 4 after injection of red fluorescent parasites.

(D) Expression of the chemokine receptor CCR7 by PO-LN and skin dermal mo-DCs at week 4 after infection. The percentage of cells with a fluorescence intensity over control staining is indicated. Data are representative of 4 independent experiments with similar results.

skin, whereas only ~10% were infected in the PO-LNs, could imply that noninfected dermal mo-DCs migrated more efficiently to the PO-LNs than those infected. In support of this hypothesis, whereas all dermal mo-DCs present in the PO-LNs were positive for the chemokine receptor CCR7 (controlling DC migration to peripheral LNs), among dermal mo-DCs present in the skin, those not infected by *L. major* were CCR7$^+$, whereas ~60% of those infected did not express this receptor (Figure 5D). Alternatively, it could be hypothesized that the lower percentage of infected dermal mo-DCs found in the PO-LNs could be related to a stronger microbicidal potential determined by DC maturation, as reported for infected macrophages after activation (reviewed in Sacks and Noben-Trauth, 2002).

Capacity of De Novo Formed Monocyte-Derived DCs to Induce T Cell Responses against *L. major*

The finding that, among DC subsets present in the PO-LNs at late phases of infection, only LN mo-DCs and dermal mo-DCs were infected by *L. major* suggests that these de novo formed monocyte-derived DCs could be responsible for T cell immunity against the parasite. To address this issue, we determined the capacity of LN mo-DCs and dermal mo-DCs, purified from PO-LNs at 4 weeks after infection, to induce T cell responses against

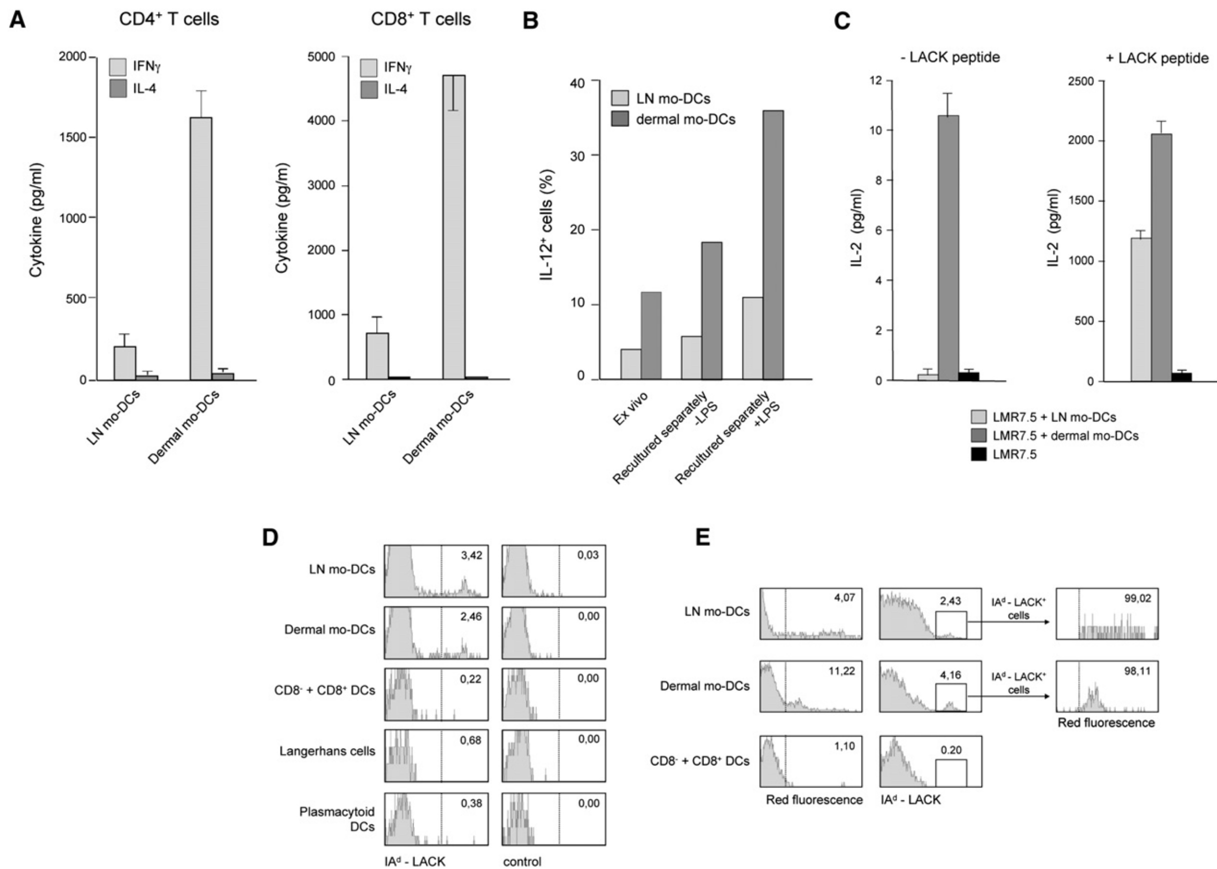


Figure 6. Analysis of the Potential of Monocyte-Derived DCs to Induce *L. major*-Specific T Cell Responses

LN mo-DCs and dermal mo-DCs purified from PO-LNs of C57BL/6 (A and B) or B10.D2 (C–E) mice, at 4 weeks after infection, were anlayzed for their capacity to induce T cell responses against *L. major*.

(A) Capacity of LN mo-DCs versus dermal mo-DCs to induce IFNγ and IL-4 secretion by $CD4^+$ and $CD8^+$ T cells; DCs and T cells were isolated from the PO-LNs of C57BL/6 mice at week 4 after infection. Error bars represent the SD of triplicate cultures.

(B) IL-12 p70 production by LN mo-DCs and dermal mo-DCs isolated from C57BL/6 mice at week 4 after infection determined by intracellular staining with IL-12 p70 antibodies.

(C) Capacity of dermal mo-DCs versus LN mo-DCs isolated from the PO-LNs of B10.D2 mice, at week 4 after infection, to stimulate the production of IL-2 by the LACK peptide 158-163-specific LMR7.5 T cell hybridoma. Error bars represent the SD of triplicate cultures.

(D) Detection of IA^d-LACK complexes by the mAb 2C44 at the cell surface of the indicated DC subsets isolated from the PO-LNs of B10.D2 mice, at week 4 after infection.

(E) Correlation between IA^d-LACK expression and *L. major* infection analyzed in the indicated DC subsets isolated from the PO-LNs of B10.D2 mice, at week 4 after infection. The percentage of cells with a fluorescence intensity over control staining is indicated.

Data are representative of 4 (A and B) and 3 (C–E) independent experiments with similar results.

L. major. We first analyzed their potential to induce the secretion of Th1 and Th2 cytokines by preactivated $CD4^+$ or $CD8^+$ T cells. Dermal mo-DCs were more efficient than LN mo-DCs in promoting IFNγ production by $CD4^+$ and $CD8^+$ T cells (Figure 6A). The higher T cell stimulatory potential of dermal mo-DCs was likely due to their higher MHC II, and particularly CD86 expression. Low amounts of IL-4 production by $CD4^+$ T cells were induced either by LN mo-DCs or dermal mo-DCs, indicating that both DC subsets promoted a Th1 bias of the response. To ascertain whether the stronger IFNγ production induced by dermal mo-DCs correlated with differences in IL-12 production, PO-LN LN mo-DCs and dermal mo-DCs were analyzed ex vivo for IL-12 production by intracellular staining at week 4 after infection. Approximately 10% dermal mo-DCs were positive for IL-12, whereas only 2%–3% of LN mo-DCs produced this cytokine (Figure 6B). Production of IL-12 by other PO-LN DC subsets was undetectable (not shown). To determine whether this differential ex vivo IL-12 production potential implied a stronger IL-12 production after in vitro stimulation, dermal mo-DCs and LN mo-DCs were cultured in the absence or presence of LPS. Culture in the absence of LPS promoted a moderate increase in $IL\text{-}12^+$ dermal mo-DCs and LN mo-DCs that was further increased after LPS treatment. The ratio between $IL\text{-}12^+$ dermal mo-DCs and $IL\text{-}12^+$ LN mo-DCs was similar in all the experimental conditions analyzed, indicating that the former have a higher potential to produce IL-12 before and after in vitro activation. Thus, our data on induction of IFN-γ production by $CD4^+$ T cells

and IL-12 production suggest that dermal mo-DCs could play a major role in the induction of *L. major*-specific Th1 responses.

To provide a stronger support to this hypothesis, we analyzed the capacity of dermal mo-DCs and LN mo-DCs, isolated from week 4 PO-LNs, to stimulate IL-2 production by the LMR7.5 T cell hybridoma, specific for the LACK antigen-derived 158-163 (LACK 158-163 peptide), in the context of I-A^d MHC II molecules. For these experiments, B10.D2 mice (I-A^d) were used, because C57BL/6 mice (used in this report for all the other experiments) express the I-A^b MHC allele nonrecognized by LMR7.5 cells. B10.D2 and C57BL/6 mice develop a similar immune response against *L. major*, regarding kinetics of infection, DC subsets infected, Th1 bias of the response, monocyte recruitment, and differentiation and DC subsets present in the PO-LNs and dermis (data not shown). As shown in Figure 6C, dermal mo-DCs, but not LN mo-DCs, induced IL-2 production by LMR7.5 cells. Positive control experiments revealed that LACK 158-163 peptide-pulsed dermal mo-DCs and LN mo-DCs could induce the stimulation of LMR7.5 T cells, although dermal mo-DCs were more effective. We next analyzed the potential of the different PO-LN DC subsets to present IA^d-LACK complexes to CD4$^+$-specific T cells, at week 4 after infection. For this purpose, we used the 2C44 mAb that specifically recognizes MHC II (IA^d)-LACK 158-163 peptide (IA^d-LACK) complexes at the cell surface. Only LN mo-DCs and dermal mo-DCs expressed IA^d-LACK complexes at the cell surface (Figure 6D); this result is in accordance with our data on susceptibility to infection revealing that LN mo-DCs and dermal mo-DCs were specifically infected by *L. major*. The finding that none of the other DC subsets analyzed (i.e., CD8$^-$ DCs, CD8$^+$ DCs, LCs, and pDCs) expressed IA^d-LACK complexes excluded the possibility that any of these DC subsets had acquired the capacity to present IA^d-LACK complexes as a result of IA^d-LACK complex transfer from infected LN mo-DCs or dermal mo-DCs to noninfected DCs. These data indicated that only those DC subsets that were susceptible to *L. major* infection had the capacity to present LACK-derived peptides, and conversely that DCs expressing IA^d-LACK complexes were infected by the parasite. To further support this hypothesis, the correlation between IA^d-LACK expression and infection by *L. major* was analyzed 4 weeks after infection of B10.D2 mice with red fluorescent parasites. As shown in Figure 6E, LN mo-DCs and dermal mo-DCs were the only DC subsets that were infected by *L. major* and expressed IA^d-LACK complexes. These experiments also demonstrate that all the LN mo-DCs and dermal mo-DCs expressing IA^d-LACK complexes were infected by *L. major*. Taken together, our data on the susceptibility to infection, expression of IA^d-LACK complexes at the cell surface, IL-12 production potential, and T cell stimulatory capacity strongly support the concept that dermal mo-DCs that derive from monocytes recruited to the infection site and that have migrated to the PO-LNs are essential for the induction of *L. major*-specific, Th1-biased T cell responses.

DISCUSSION

Results presented in this report provide a formal demonstration of the potential of monocytes to differentiate into DCs in vivo during infectious processes, the de novo local differentiation of DCs in inflammatory sites of infection and dLNs, and the essential role that inflammatory de novo formed monocyte-derived DCs can play in the induction of T cell responses against pathogens.

Although the capacity of monocytes to differentiate into DCs during in vivo infection is controversial, a number of studies had described the accumulation of DCs during infectious processes, or shown in noninfectious inflammatory models that monocytes can differentiate into DCs in vivo. During *Listeria monocytogenes* infection, de novo generation of splenic DCs was reported, and newly formed DCs were proposed to derive from monocytes recruited to the spleen (Serbina et al., 2003). Adjuvant-induced DC accumulation in epithelial tissues was preceded by CCR6- and CCL20-dependent monocyte recruitment; these newly recruited DCs were essential for CD8$^+$ T cell crosspriming against a protein antigen (Le Borgne et al., 2006). In contrast, experiments aiming to reproduce an infection-mediated inflammatory process, based on the s.c. injection of latex microspheres (Randolph et al., 1999), supported that cutaneous inflammatory monocytes differentiated locally into DCs that subsequenlty migrated to the dLNs. Two later studies have reported the differentiation of monocytes into DCs in the peritoneal cavity (Geissmann et al., 2003) and spleen (Naik et al., 2006), respectively, during experimental inflammatory reactions. However, the concept that monocytes could behave as DC precursors during infection was challenged by a report demonstrating that monocyte differentiation into DCs was blocked if bacteria were present in the area of injection of the latex microspheres (Rotta et al., 2003). These results are in accordance with in vitro data showing that activation of monocytes by pathogens or TLR ligands blocks their capacity to differentiate into DCs and promotes their differentiation into macrophages (reviewed in Palucka and Banchereau, 1999). Thus, it could also be speculated that during *L. major* infection, monocytes could differentiate into DCs after engagement of GM-CSF receptors in the absence of interaction of parasites with TLR receptors on the monocytes. Differentiation of dermal mo-DCs and LN mo-DCs from monocytes most likely occurred because of low parasitemia during late phases of *L. major* infection in C57BL/6 mice. In agreement with the inhibition of DC differentiation from monocytes that occurred in the presence of bacteria (Rotta et al., 2003), blockade of monocyte differentiation into DCs determined by parasite-induced monocyte activation could also be the basis for our observation of the strong reduction in dermal mo-DCs and LN mo-DCs after the first week of infection.

On the other hand, our results indicate that the appearance and increase in number of dermal mo-DCs and LN mo-DCs occurring during *L. major* infection was due to continuous de novo local differentiation of DCs from

monocytes recruited to the dermis and PO-LNs and not to the recruitment of preformed DCs. In this regard, an increase in the number of DCs in infection-induced inflammatory areas and dLNs has been described in three different reports (Martin et al., 2002; Yoneyama et al., 2001; Zhao et al., 2003). Importantly, these studies did not address whether DC increase was due to DC recruitment or to local differentiation of DCs. A possible role for monocytes in this process was supported by previous reports describing the generation of splenic DCs during *Listeria* infection (Serbina et al., 2003) and adjuvant-induced accumulation of DCs in epithelial tissues (Le Borgne et al., 2006). Interestingly, monocytes have been demonstrated to represent LC precursors both under steady state and during inflammation (Ginhoux et al., 2006; Merad et al., 2002).

L. major infection determined an important increase in pDC, $CD8^{-}$ DC, and $CD8^{+}$ DC subpopulations, although their role remains to be addressed. In contrast to conventional DCs, pDCs can be recruited to lymphoid organs and inflammatory areas (Bendriss-Vermare et al., 2004; Diacovo et al., 2005), suggesting that the increase in pDCs occurring during *L. major* infection could result from blood pDC recruitment to the PO-LNs, although whether de novo local differentiation of pDCs occurred has to be analyzed. Regarding $CD8^{-}$ and $CD8^{+}$ DCs, whether during *L. major* infection these DC subsets derived from monocytes or other DC precursors has to be clarified. Our results challenge the hypothesis that DCs generated during inflammation constitute an independent DC category, unrelated to resident DCs and pDCs. In this sense, DCs generated during pathogen-induced inflammatory reactions would include monocyte-derived DCs subsets not found in noninflammatory conditions (such as dermal mo-DCs and LN mo-DCs), as well as resident DC subsets existing in control conditions (i.e., $CD8^{-}$ DCs, $CD8^{+}$ DCs, and pDCs).

Dermal mo-DCs and LN mo-DCs were, together with monocytes and macrophages, the cell subsets predominantly infected by *L. major*. This observation is in agreement with previous studies demonstrating that *L. major* internalization is mediated by complement receptors, such as CD11b and CR1, mainly expressed by cells of the monocytic-macrophage lineage, that have the capacity to sustain parasite replication (reviewed in Sacks and Noben-Trauth, 2002). It can be speculated that dDCs infected together with dermal monocytes and macrophages during early phases of infection would release amastigotes that would in turn infect, in the dermis, newly recruited monocytes and de novo formed dermal mo-DCs. Transmission of infection to monocytes recruited to the PO-LNs and to newly formed LN mo-DCs would occur after migration of infected dDCs and dermal mo-DCs. However, we cannot exclude that at early infection phases free promastigotes could reach the PO-LNs, in agreement with recent reports (Baldwin et al., 2004; Iezzi et al., 2006). According to this hypothesis, within the PO-LNs, a proportion of both dermal mo-DCs and LN mo-DCs were continuously infected, from week 2 until the last phases of infection. Infection was not detectable in the other PO-LN DC subsets, i.e., pDCs, $CD8^{-}$ DCs, and $CD8^{+}$ DCs. Accordingly, IA^{d}-LACK complexes were detected exclusively on dermal mo-DCs and LN mo-DCs, which consequently were the only DCs endowed with the potential to activate LACK-specific T cells. These data suggest that dermal mo-DCs are responsible for the induction of protective T cell responses against *L. major*, and therefore that inflammatory, de novo formed, monocyte-derived DCs could have an essential role in T cell immunity against pathogens.

Recent studies have addressed the identity of the DC subpopulations responsible for immune responses against pathogens. A role for either LCs, dermal DCs, interstitial DCs, $CD8^{-}$ DCs, or $CD8^{+}$ DCs in the induction of T cell immunity against viral, bacterial, or parasitic infections has been reported, although data were in some cases contradictory, probably resulting in part from the experimental models employed (reviewed in Villadangos and Heath, 2005). Concerning DCs responsible for the induction of T cell responses against *L. major*, although LCs were considered to play a major role in this process (Moll et al., 1993), mice not expressing MHC class II molecules on LCs displayed a healing phenotype (Lemos et al., 2004). On the other hand, T cell immunity against *L. major* was proposed to depend on resident $CD11b^{+}$ DCs (Filippi et al., 2003), which would have been infected by parasites transported to the PO-LNs through the lymphatics (Iezzi et al., 2006). Importantly, based on their phenotype, these so-called resident DCs could correspond to LN mo-DCs.

Discrepancy between these reports and our data supporting an essential role for migratory dermal mo-DCs in T cell immunity against *L. major* probably reflects that in the reports cited above, the early phases of infection were analyzed, whereas our results refer to later phases of infection that determined the Th2-Th1 switch in the T cell response. It is important to take into account that the experimental model employed in most reports on *L. major* infection (including the articles cited above and this report) involved supraphysiologic parasite doses. Thus, at early phases of infection, free parasites could gain access to the dLNs and infect resident DCs, whereas at late phases of infection, amastigotes released in the dermis from previously infected cells would infect dermal mo-DCs, which in turn would be responsible for carrying the infection to the LNs and inducing the activation of *L. major*-specific Th1 responses. Based on experiments performed with low parasite doses, reproducing more closely the physiological infection (Belkaid et al., 2000), it has been postulated that at high parasite doses, the early phases of infection involved an anomalous transport of the parasite to the LNs and a nonphysiologic immune response. In contrast, even after injection of high parasite doses, the late phases of infection would be comparable to the physiogical infection conditions.

In summary, our data strongly suggest that under physiological conditions, dermal mo-DCs, which could represent the inflammatory counterparts of dDCs, were responsible for the induction of protective Th1 responses against *L. major*. Interestingly, an essential role in the induction of virus-specific T cell responses for dDCs or interstitial DCs

(claimed to be the equivalent to dDCs in mucosal tissues) has also been reported during infection by influenza virus and herpes virus (Allan et al., 2006; Zhao et al., 2003). The new concept of de novo local DC differentiation in inflammatory foci opens a new area of research related to the influence of local mediators on the acquisition of the functional specialization of newly formed DCs.

EXPERIMENTAL PROCEDURES

C57BL/6, BALB/c, and B10.D2 mice were purchased from Harlan (Bicester, UK). C57BL/6 Ly 5.1 Pep^{3b} mice were purchased from Jackson (Bar Harbor, ME) and bred under SPF conditions. For monocyte transfers, donors were C57BL/6 Ly 5.1 Pep^{3b} mice and recipients were C57 BL/6 Ly 5.2 mice. *L. major* promastigotes (WHOM/IR/173 strain) and red fluorescent $DsRed^+$ *L. major* promastigotes (kindly provided by N. Glaichenhaus, Université de Nice-Sophia Antipolis, France) were grown as described (Filippi et al., 2003). Mice were injected with 2×10^6 stationary-phase promastigotes in the hind footpad. Lesion size was determined by measuring the thickness of the footpad at the site of injection.

A detailed description of the methods used for the preparation of cell suspensions and the purification of monocytes, DC subpopulations, and T cells is included in the Supplemental Data. In transfer experiments, 3–5 $\times 10^6$ purified monocytes were injected intravenously or subcutaneously into C57 BL/6 Ly 5.2 recipient mice that were infected 3–4 weeks before with *L. major*. Cells derived from transferred monocytes were analyzed as Ly-5.1^+ cells by flow cytometry 72 hr after transfer, in the dermis, PO-LNs, and spleen.

For the analysis of the kinetics of Th1/Th2 response (Figure 2C), $CD4^+$ T cells from the PO-LNs of *L. major*-infected C57BL/6 or BALB/c mice were analyzed for the production of IFNγ and IL-4, after 48 hr stimulation with anti-CD3ε (clone 145-2C11; Pharmingen) and anti-CD28 (37.51; Pharmingen) mAbs, by means of mouse IFNγ and IL-4 ELISA kits (Pharmingen).

For the analysis of the capacity of LN mo-DCs and dermal mo-DCs to induce the secretion of IFNγ and IL-4 by preactivated $CD4^+$ or $CD8^+$ T cells (Figure 6A), $CD4^+$ and $CD8^+$ T cells, as well as dermal mo-DCs and LN mo-DCs, were isolated from the PO-LNs of C57BL/6 mice at week 4 after infection, cocultured at a 10:1 ratio for 96 hr, and analyzed for the production IFNγ and IL-4 by ELISA.

For the analysis of the capacity of LN mo-DCs and dermal mo-DCs to stimulate the LACK 158-163 peptide-specific T cell hybridoma LMR7.5, LMR7.5 cells (kindly provided by N. Glaichenhaus, Université de Nice-Sophia Antipolis) were cocultured with dermal mo-DCs or LN mo-DCs from PO-LNs of B10.D2 mice at week 4 after infection, at a 5:1 ratio for 24 hr, in the absence or presence of 1 μM LACK 158-163 peptide, and analyzed for the production IL-2 with a mouse IL-2 ELISA kit (Pharmingen).

Flow cytometry analysis of PO-LN and skin cell subpopulations was performed after triple or quadruple staining by correlating Ly-6C, CD11b, and CD11c expression (or alternatively DEC-205, MHC II, and CD11c expression) with the expression of CD40, CD86, B220, CD4, CD8α, F4/80, CD45, Ly-5.1, CCR5, CCR7, IA^d-LACK complexes, or IL-12 p40/p70. A detailed description of the flow cytometry methods employed is included in the Supplemental Data.

Supplemental Data

Supplemental Data include two figures, one table, and Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/26/4/519/DC1/.

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