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Characterization of Human Afferent Lymph Dendritic Cells from Seroma Fluids

Barbara Morandi,* Irene Bonaccorsi,†‡ Mario Mesiti,§ Romana Conte,¶ Paolo Carrega,** Gregorio Costa,** Raffaella Iemmo,‡ Stefania Martini,¶ Soldano Ferrone,# Claudia Cantoni,**† Maria Cristina Mingari,**† Lorenzo Moretta,** and Guido Ferlazzo†‡

Dendritic cells (DCs) migrate from peripheral tissues to secondary lymphoid organs (SLOs) through the afferent lymph. Owing to limitations in investigating human lymph, DCs flowing in afferent lymph have not been properly characterized in humans until now. In this study, DCs present in seroma, an accrual of human afferent lymph occurring after lymph node surgical dissection, were isolated and analyzed in detail. Two main DC subsets were identified in seroma that corresponded to the migratory DC subsets present in lymph nodes, that is, CD14+ and CD1a+. The latter also included CD1a bright Langerhans cells. The two DC subsets appeared to share the same monocytic precursor and to be developmentally related; both of them spontaneously released high levels of TGF-β and displayed similar T cell-activating and –polarizing properties. In contrast, they differed in the expression of surface molecules, including TLRs; in their phagocytic activity; and in the expression of proteins involved in Ag processing and presentation. It is worth noting that although both subsets were detected in seroma in the postsurgical inflammatory phase, only CD1a+ DCs migrated via afferent lymph under steady-state conditions. In conclusion, the high numbers of DCs contained in seroma fluids allowed a proper characterization of human DCs migrating via afferent lymph, revealing a continuous stream of DCs from peripheral regions toward SLOs under normal conditions. Moreover, we showed that, in inflammatory conditions, distinct subsets of DCs can migrate to SLOs via afferent lymph.

Lymph is the fluid formed when interstitial fluid is collected through lymph capillaries. It can be thus defined as the extracellular fluid produced continuously by filtration from the blood—enriched with peripheral tissue catabolites, cells, and debris—collected in afferent lymphatic vessels, and conveyed into lymph nodes. It then leaves the lymph nodes via efferent vessels before emptying ultimately into the right or left subclavian vein, where it mixes back with blood. The composition of lymph fluid differs according to anatomical origin and the pathophysiology of the draining tissue.

Dendritic cells (DCs) are critical for initiating immune responses. At an immature stage, they act as sentinels in peripheral tissues, continuously sampling the environment, sensing the presence of pathogens, and secreting chemokines and cytokines to amplify the immune response. Upon activation by danger signals, they upregulate chemokine receptors and costimulatory molecules, which allow them, respectively, to migrate into lymph nodes via afferent lymph and to efficiently induce T cell responses. In many experimental animal models, DCs have been shown to be able to continuously migrate from either intestine to mesenteric lymph nodes or from skin to associated secondary lymphoid organs (SLOs) (1, 2). These studies support the assumption that DCs traveling from peripheral tissues might be crucial in balancing immunity and tolerance of the drained tissues (3, 4). However, similar information on the continuous migration of DCs in steady state is still lacking in the human system. Human DCs had indeed been observed in afferent lymph as “veiled” cells (5, 6), but owing to limitations in investigating this fluid in humans, they were simply defined on the basis of their morphology.

In contrast, in the past few years, different subsets of DCs have been identified in human blood (BDCA1+ and BDCA3+) (7–10) and skin (1, 11–15). In addition, we have lately gained access to a comprehensive depiction of DCs harbored in human lymph nodes (16–18), obtaining valuable information on migratory DC subtypes.

Recently, we have reported that seroma, an accrual of fluid reported after surgical procedures such as axillary lymph node dissection (19, 20), is associated with an accumulation of afferent lymph drained from upstream tissues during the interval of time needed for lymphatic vessels to reanastomose with the efferent ducts after removal of lymph nodes (21). Further supporting this hypothesis, we reported the presence, in seroma fluids, of large mononuclear cells partially expressing CD14 and reminiscent of veiled cells detectable in afferent lymph (5, 21).

In this study, we analyzed in detail the cellular content of seroma fluids. This approach offers the unique possibility of characterizing...
DCs flowing in human afferent lymph, a biological fluid extremely difficult to investigate in humans.

Materials and Methods

Samples and cell isolation

Seroma samples were collected as previously described (21), in accordance with institutional ethical guidelines. In general, needle aspiration was performed once a week and repeated as necessary. More than 100 seromas were collected for this study. DCs and T lymphocytes were isolated from seroma fluids by flow cytometry cell sorting (FACS/Aria II, Becton Dickinson).

Pleural effusion was obtained from patients with primary or metastatic tumors of different origin (age range: 49–96 y, 22 males and 3 females). Pleural effusions obtained from thoracentesis were maintained at 4˚C and just after were centrifugated at 400 g for 10 min. Cells were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ).

Monocyte-derived DCs were generated in vitro, as previously described (22). For DC activation, on day 6, half of the medium was replaced with fresh medium containing 1 μg/ml LPS or 1000 IU/ml IFN-α (Sigma-Aldrich) for 2 d.

Abs and flow cytometry

The following mouse anti-human mAbs were used in our study: anti-HLA-DR Pe-Cy7, -CD1b PE, -CD3 FITC, -CD4 PE-APC, -CD11c PE and APC, -CD16 PE, -CD40 PE, -CD48 PE, -CD62L PE, -CD68 PE, -DEC205, -DC-SIGN, all from BD Biosciences; anti–BDCa-1, -BDCa-2, -BDCa-3, -BDCa-4 PE, anti-CD11c PE and APC, anti-CD14 APC, anti-CD64 PE, -Foxp3 APC, CLEC9A FITC from Miltenyi Biotec; anti-CD4 PeCy7, -CD25 FITC, -CD3 FITC, -CD83 PE, -CD86 PE, -ILT3 FITC, -langerin PE (CD207), from Instrumentation Laboratories; anti-CD1a Pacific Blue, -CD32 PE, -FceRI PE, -CD15 PE, -CD135 PE, -sirp-α PE, -ILT2 PE, -ILT4 PE, -Foxp3 PE from BioLegend; anti-CCR1, -CCR5, -CCR7, -CXC-R3 from R&D; anti–PD-1 PE, -CD11b PE, -CD64 PE, -Foxp3 PE (AbD Serotec); and anti-NKp46 (BAB281) and anti-B7H3, kindly provided by Prof. Alessandro Moretta, Genoa, Italy.

The mAbs specific for proteins of the Ag presentation machinery—namely, anti-α (SY-5), anti–MB-1(SJ-3), anti-low-molecular-mass polypeptide (LMP)-2 (SY-1), anti–LMP-7 (HB-2), anti–LMP-10 (TO-7), anti–TAP-1 (NOB-1), anti–TAP-2 (NOB-2), anti-calnexin (TO-5), anti-calreticulin (TO-11), anti–Eps57 (TO-2), and anti-Tapasin (TO-3)—were developed and characterized as described (23–25). For the use of these mAbs, cells were first stained for HLA-DR and CD14; then, after three washings with PBS containing 1% BSA, cells were fixed in 2% paraformaldehyde (Sigma-Aldrich) at room temperature for 20 min. Following three washes, cells were treated in a microwave oven at 200 W for 45 s. At the end of treatment, cells were cooled on ice for 10 min. The cell membrane was permeabilized by incubating cells for 30 min at room temperature in PBS containing 1% BSA and 0.1% saponin (Sigma-Aldrich). Cells (5 × 10^5) were incubated for 30 min at room temperature with an appropriate amount of primary mAb or isotype control. Following three washings with PBS containing 1% BSA and 0.1% saponin, cells were incubated for 30 min at room temperature in the dark with an optimal amount of R-PE-labeled goat Abs specific to mouse IgG Fc fragments. All these Abs were purified from ascitic fluid by sepharose 4B column.

The subsets of T lymphocytes contained in seroma fluids were considered significant.

Statistical analysis

Statistical analyses were performed using Prism 5.0 (GraphPad Software). Results were analyzed by a parametric Student t test. The p values < 0.05 were considered significant.

Results

Two distinct subsets of DCs are present in human afferent lymph from seroma fluids

We have previously reported that seroma fluids represent an accumulation of afferent lymph consequent to a surgical interruption of lymphatic vessels draining lymph from interstitial spaces. To confirm the nature of this fluid and to evaluate a possible contamination by surgery-induced leukocyte–derived cells, we analyzed the subsets of T lymphocytes contained in seroma fluids. T cells recirculating to SLOs via afferent lymph are solely represented by naive and central memory T cells, both expressing CCR7, a chemokine receptor known to be relevant for the migration of leukocytes toward SLOs (31). Conversely, blood and exudates should contain a certain number of effector memory T cells, which do not express CCR7. By comparing these different human biological fluids, we found that seroma contained only CCR7^+ T cells, whereas a variable number of both CCR7^- and CCR7^+ T cells were detectable in peripheral blood and exudates such as neoplastic pleural effusions and endometrial fluids (Fig. 1A, 1B).

Having confirmed that lymph-derived seromas were in general minimally contaminated by cells of peripheral blood or concomitant inflammatory exudates, a combination of informative phenotypic
markers were used to investigate the presence of DCs in seroma fluids. By gating on cells negative for CD3, CD19, NKp46, and BDCA2, two main HLA-DR\textsuperscript{dim} large cell subsets could be identified: one characterized by high levels of HLA-DR and CD1a (HLA-DR\textsuperscript{bright}) and the other expressing lower levels of HLA-DR and CD14 (HLA-DR\textsuperscript{dim}) (Fig. 1C). Notably, the HLA-DR\textsuperscript{bright} subset also contained CD1\textsuperscript{bright} Langerhans cells. HLA-DR\textsuperscript{bright} cells, compared with the HLA-DR\textsuperscript{dim} counterpart, presented a classical DC morphology with longer surface dendrites. Nevertheless, both subsets were generally firmly associated with T cells (Fig. 1D, 1E). Among all leukocytes contained in the seroma fluids, the median value of lineage\textsuperscript{−} (CD3, CD19, NKp46, BDCA2) HLA-DR\textsuperscript{+} cells was 8.5%, ranging between 3.5 and 22%. The mean value of lineage\textsuperscript{−} mononuclear cells (MNCs) contained in each seroma was 71 ± 108 × 10\textsuperscript{6} in a mean volume of 360 ± 147 ml.

The two main subsets of afferent lymph DCs differed in a variety of other relevant markers (Fig. 2A). In general, HLA-DR\textsuperscript{bright} CD1a\textsuperscript{+} DCs displayed a mature phenotype, as they expressed significantly higher levels of costimulatory molecules and DC hallmark markers such as CD83 and DEC205. They also displayed higher levels of inhibitory molecules of both programmed death ligands, but not of Ig-like transcript families. As expected in DCs present in human afferent lymph, CCR7 was expressed by both subsets, whereas CD62L expression was restricted to HLA-DR\textsuperscript{bright} DCs. The inflammatory chemokine receptors CCR1 and CCR5 were also exclusively detectable in the HLA-DR\textsuperscript{dim} subset. Myeloid DC markers BDCA1 and BDCA3 were mostly expressed on both subsets of afferent lymph DCs, although a fraction of HLA-DR\textsuperscript{bright} DCs expressed BDCA3 at lower levels (Fig. 2B, Supplemental Fig. 1). A specific subset of BDCA3\textsuperscript{bright}/CLEC9a\textsuperscript{+}/Sirp\alpha\textsuperscript{+} DCs has recently been described in different human tissues (10–12, 32). These DCs resemble murine CD8\textsuperscript{α}+ DCs in their ability to cross-present exogenous Ags. Despite Sirp\alpha\textsuperscript{+} DCs having been described in different human tissues, they were not detectable in seroma fluids. Remarkably, although CLEC9a has been previously reported to be expressed only on Sirp\alpha\textsuperscript{−} DCs (10), we found that HLA-DR\textsuperscript{bright} contained a small subset of BDCA3\textsuperscript{bright}/Sirp\alpha\textsuperscript{+} DCs expressing CLEC9a (Fig. 2C).

Because the HLA-DR\textsuperscript{dim} subset expresses CD14, we further analyzed the expression of CD68, a marker that is widely employed for the monocyte/macrophage lineage. However, neither DC subsets analyzed displayed CD68 expression. As previously reported, CD48 expression was partially downregulated on the most activated HLA-DR\textsuperscript{bright} DCs (22).

Because the HLA-DR\textsuperscript{bright} DC population is heterogeneous and contains CD1a\textsuperscript{+} and CD1a\textsuperscript{bright} Langerhans cells, we comparatively analyzed these two DC subsets. As shown in Supplemental Fig. 1, no significant phenotypic differences were detectable between the two subsets, except for the expression of BDCA3, which occurred at a lower level in only a fraction of CD1a\textsuperscript{+} Langerin\textsuperscript{−} DCs.

**Human afferent lymph DCs from seroma fluids display a phenotype similar to that of migratory lymph node DCs**

The phenotype of DC subsets isolated from seroma fluids appears to identify DCs that are different from other myeloid human APCs either isolated ex vivo from blood or derived in vitro from precursors (Table I, Fig. 2B). They also differ from APCs identified in other human biological fluids, such inflammatory CD14\textsuperscript{+} cells detectable in exudates (e.g., neoplastic effusions) (Fig. 2B). In contrast, HLA-DR\textsuperscript{bright} seroma DCs resemble the recently described migratory DCs harbored in human lymph nodes (17, 18). However, in addition to the CD11c\textsuperscript{low}/CD1a\textsuperscript{bright} DC subset (Fig. 3A, left panel) (18) and the CD14\textsuperscript{+} CD1a\textsuperscript{bright} DC subset (Fig. 3A, right panel) (17) (both contained in seroma as HLA-DR\textsuperscript{bright} DCs), we could also detect CD14\textsuperscript{+} cells partially expressing CD1a. We observed that, during in vitro culture, CD14 and CD1a were respectively down- and upregulated on cultured DCs (Fig. 3B). In these experiments, seroma DCs were sorted according to the expression of CD14 and CD1a, and the three resulting DC subsets—namely, CD14\textsuperscript{+}CD1a\textsuperscript{+}, CD14\textsuperscript{−}CD1a\textsuperscript{+}, and CD14\textsuperscript{−}CD1a\textsuperscript{−}—were cultured for 72 h. In this interval, CD14\textsuperscript{+}CD1a\textsuperscript{−} lost CD14 expression, whereas they progressively acquired CD1a. During the acquisition of CD1a, CD14\textsuperscript{+} cells in parallel upregulated HLA-DR molecules on their surface (not shown). A similar phenotypic modulation was observed for CD14\textsuperscript{+}CD1a\textsuperscript{+} “transitional” DCs, whereas CD14\textsuperscript{−}CD1a\textsuperscript{+} DCs maintained a stable phenotype. Although we

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Identification of DCs in human afferent lymph from seroma fluids. (A and B) CD3\textsuperscript{+} T cells isolated from seroma, peripheral blood, and the indicated exudates were analyzed for the expression of CCR7, known to be expressed only on naive and central memory T cells. Dot plots shown in (A) are representative of the comparative stainings summarized in (B). (C) MNCs isolated from seroma fluids were stained for CD3, CD19, NKp46, BDCA2 (lineage) and HLA-DR, CD14, CD1a, and CD207. HLA-DR\textsuperscript{bright}/CD1a\textsuperscript{+}, HLA-DR\textsuperscript{dim}/CD14\textsuperscript{−}, and CD207\textsuperscript{−} Langerhans cells were gated as depicted. (D) HLA-DR\textsuperscript{bright} and HLA-DR\textsuperscript{dim} DCs were sorted by flow cytometry and analyzed by optical microscopy. (E) Afferent lymph DCs form strong aggregates with CD3\textsuperscript{+} T cells. MNCs from seroma fluids were stained for CD3, HLA-DR, and BDCA1. Large and scattered BDCA1\textsuperscript{+}CD3\textsuperscript{+} events represent DC/T cell aggregates.
identified a subset of transitional DCs expressing both CD14 and CD1a (HLA-DR<sup>dim</sup>), we also found that CD1a acquisition occurred after CD14 downregulation in CD14<sup>+</sup>CD1a<sup>−</sup> DCs during the 72 h of culture. However, we could not detect a CD14<sup>−</sup>CD1a<sup>+</sup> population in seroma DCs. It is possible that this finding may reflect culture conditions, which are likely to differ from the in vivo microenvironment.

These results indicate that HLA-DR<sup>dim</sup>CD1a<sup>−</sup>CD14<sup>+</sup> DCs can convert, at least in vitro, into HLA-DR<sup>bright</sup>CD1a<sup>−</sup>CD14<sup>+</sup> DCs. HLA-DR<sup>bright</sup>CD1a<sup>+</sup> DCs continuously migrate from peripheral tissues to SLOs in steady state.

Seroma fluids were collected at different time intervals in patients operated on for axillary lymph node removal. In many patients,
seroma fluids were still accumulating several weeks after axillary lymph node dissection, that is, when the surgical wound had completely healed and no sign of local inflammation was noticeable. These lines of evidence support the idea that seroma fluid is not associated with an inflammatory exudate secondary to the surgical procedure. Of note, whereas the proportions of the other leukocytes present in seromas (mainly T cells) were similar in fluids collected at earlier or later time intervals from surgery, only

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a Mono DCs, DCs derived in vitro from monocytes in the presence of the indicated soluble factors. n.d., Not determined; -, negative; +, positive; +/-, partially positive.

**FIGURE 3.** Developmental relationship between DC subsets of afferent lymph. (A) HLA-DR* DCs of afferent lymph resemble CD11clowCD1a+/bright migratory DCs recently identified in lymph nodes (19, 25). One representative experiment of 25 is shown. (B) CD14* cells and CD14*CD1a* cells (both composing the HLA-DR** DC subset) represent transitional stages of differentiation from CD14* precursors to CD1a*DC14* DCs (HLA-DR*). CD14*CD1a*, CD14*CD1a*, and CD14*CD1a* were sorted by flow cytometry and cultured up to 72 h in the presence of autologous T cells and GM-CSF. Although the CD14*CD1a* DC population (HLA-DR*) maintained a stable phenotype in vitro, the other two populations progressively lost CD14 and acquired CD1a. Data shown are representative of results from five independent experiments. (C) Seroma fluids were collected once a week until clinically required. In many instances, a single patient required up to seven seroma aspirations. After 7 wk, the surgical wound had completely healed and no sign of inflammation was present. As shown, the composition of DCs changed between early (postsurgical) and late time points of lymph collection, because after 30 d from surgery only HLA-DR* DCs were almost exclusively detectable in seroma fluids. Dot plots shown are representative of seroma samples collected from 10 patients.
HLA-DR\textsuperscript{bright} DCs, including both CD1a\textsuperscript{a} and CD1a\textsuperscript{bright} langerin\textsuperscript{+} DCs, were detectable in seroma fluids collected at later time intervals (i.e., after 7 wk from surgical procedure) (Fig. 3C). Thus, it is possible to speculate that during postsurgical inflammatory processes a high number of HLA-DR\textsuperscript{dim}CD14\textsuperscript{+} DCs is drained from inflamed tissues to SLOs via afferent lymph. In contrast, several weeks after surgery, only HLA-DR\textsuperscript{bright}CD14\textsuperscript{+} DCs were abundant in afferent lymph (Fig. 3C). These data suggest that, even in the absence of inflammation, HLA-DR\textsuperscript{bright}CD14\textsuperscript{−} DCs are inclined to continuously migrate from peripheral tissues to SLOs via afferent lymph.

The two subsets of DCs contained in afferent lymph present similar T cell–stimulating abilities but differ in their pathogen recognition, phagocytosis, and Ag-processing properties

To gain insight into afferent lymph DC functions, we first analyzed DCs for their pattern recognition receptor profile. TLR repertoire differed between the two subsets, as TLR1, 2, 4, 5, 6, and 7 appeared to show higher expression in HLA-DR\textsuperscript{dim} DCs. TLR3 was weakly expressed in both subsets, and TLR8 and 9 were detectable on neither (Fig. 4A).

An important task of DCs is the ability to acquire exogenous, potentially pathogenic, materials in peripheral tissues before migrating to SLOs. We therefore investigated this by employing bacterial bodies labeled with PHrodo, a fluorogenic dye, nonfluorescent at neutral pH, which becomes bright red fluorescent in acidic environments, thus acting as a specific sensor of endo- and phagocytosis. Following a 6-h culture at 37°C of DCs in the presence of PHrodo-labeled bacteria, fluorescence was analyzed within the two subsets. In line with their different activating phenotype, HLA-DR\textsuperscript{bright} DCs were endowed with lower phagocytic ability (Fig. 4B).

We then analyzed the ability of afferent lymph DCs to induce the proliferation of allogeneic naive T cells. Despite HLA-DR\textsuperscript{bright} DCs displaying a more activated phenotype, the allostimulating ability did not show significant differences among the subsets (Fig. 4C, 4D). Conversely, some differences were present in the endogenous Ag-processing and presentation machinery between HLA-DR\textsuperscript{bright} and HLA-DR\textsuperscript{dim} DCs (Fig. 4E). Proteasome subunit \( \delta \) and TAP proteins were more represented in HLA-DR\textsuperscript{bright} DCs. Similar differences were detected for the endoplasmic reticulum chaperone calnexin. The immunoproteasome subunit LMP-2 and LMP-7 were expressed in both DC subsets, confirming that afferent lymph cells characterized in the current study possess professional Ag-presenting features typical of DCs. Higher expression of molecules, on HLA-DR\textsuperscript{bright} DCs, either aiding the Ag-processing machinery or playing a crucial role in HLA class I Ag complex assembly and Ag presentation, is in accordance with

FIGURE 4. Conventional APC properties of afferent lymph DCs. (A) The TLR repertoire was comparatively analyzed in the two subsets of DCs contained in afferent lymph. The mRNA for the indicated receptor is shown. Consistent results were obtained in eight seroma samples analyzed. (B) pHrodo-labeled bacteria were incubated with the two different subsets of DCs. After 6 h, phagocytosis of bacteria was evaluated by flow cytometry. Data shown are representative of four independent experiments. (C and D) CD4\textsuperscript{+} and CD8\textsuperscript{+} naive T cells were sorted, labeled with CFSE, and incubated with allogeneic CD1a\textsuperscript{bright} langerin\textsuperscript{+} (LC), CD1a\textsuperscript{a} langerin\textsuperscript{−} (HLA-DR\textsuperscript{bright}) DCs, or HLA-DR\textsuperscript{dim} DCs for 6 d. Proliferation was analyzed by CFSE dilution. The results obtained in four independent experiments of T cell proliferation using the indicated percentage of DCs as stimulators are summarized in (C). Data shown represent the mean values obtained in the four experiments. A representative experiment of CFSE dilution with 10% of DCs as stimulators is shown in (D). (E) Intracellular staining with mAbs specific for the indicated components of the Ag-processing machinery was performed on HLA-DR\textsuperscript{bright} and HLA-DR\textsuperscript{dim} DC subsets. Data represent flow cytometry mean fluorescence intensity mean values and standard deviations of analyses performed in seroma samples from four patients. *\( p < 0.05 \), **\( p < 0.01 \). n.s., Not significant.
more terminal cellular differentiation of the HLA-DR<sup>bright</sup> DC subset.

**DCs contained in afferent lymph induce Foxp3 expression on CD4<sup>+</sup> naive T cells**

In accordance with high levels of TGF<sub>B</sub> contained in seroma fluids (not shown), we found that both DC subsets were able to spontaneously release significant levels of this cytokine in vitro (Fig. 5A). IL-10 was also consistently released at low levels by the two subsets but mainly when DCs were cultured in the presence of M-CSF; in contrast, TNF-α, IL-6, IL-12, and type I IFN were not detectable in the culture supernatants of DC subsets after 48 h in the indicated culture conditions. IL-1β was released only by the HLA-DR<sup>dim</sup> subset and only in the presence of M-CSF.

We then assessed the polarization of naive CD4<sup>+</sup> T cells in the presence of afferent lymph DCs. After 7 d of culture with either HLA-DR<sup>dim</sup> DCs or HLA-DR<sup>bright</sup> DCs, a discrete percentage of CD4<sup>+</sup>CD25<sup>+</sup>T cells express Foxp3, whereas T-bet, GATA3, and RORγt (i.e., transcription factors associated respectively with Th1, Th2, and Th17 patterns) were not observed in T cells upon coculture with seroma DCs (Fig. 5B, 5C).

**Discussion**

DCs are APCs crucial for the initiation of several immune responses, such as the sensitization of MHC-restricted T cells and the formation of T-dependent Abs, but they can also play relevant regulatory roles in immunologic tolerance (3). DCs are found in many nonlymphoid tissues and can migrate via the afferent lymph or the bloodstream to the T-dependent areas of lymphoid organs. Thus, they constitute a system that occupies discrete portions of nonlymphoid and lymphoid organs and that is interconnected by defined pathways of movement. Afferent, but not efferent, lymph contains leukocytes termed veiled cells in rabbit (33), pig (34), rat (35), mouse (36), human (6), and sheep (37). Like classical DCs, veiled cells were described as leukocytes displaying a buoyant density, low FcR and phagocytic activity, and high levels of MHC class II and APC activity. The origin of veiled cells is not completely clear. They may come from the interstitium and epithelium of many organs or from a pool of cells that leaves the blood, moves through nonlymphoid tissues, and enters the lymph (38).

Our current investigation in seroma fluids sheds some light on the composition and characteristics of human veiled cells. By analyzing an accrual of human afferent lymph, we have identified leukocytes with typical DC features but that differ from myeloid APCs detectable in other human biological fluids, including circulating blood DCs and inflammatory CD14<sup>+</sup> cells detectable in exudates. In line with these findings, previous studies reported that seroma fluids differ from exudates in both molecular features and cellular content (21), thus ruling out the possibility that seroma DCs might merely represent APCs that have been recruited because of local inflammation via routes other than lymphatic vessels. In support of the assumption that seroma DCs derive from

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**FIGURE 5.** DCs from afferent lymph spontaneously release TGF-β and induce Foxp3 expression on CD4<sup>+</sup> T cells. (A) DCs were isolated from seroma fluids and cultured in the indicated culture conditions. After 48 h, cytokine content was measured in culture supernatants. Data shown represent mean values and standard deviations of four independent experiments. (B) Naive CD4<sup>+</sup> T cells were cultured with HLA-DR<sup>dim</sup> or HLA-DR<sup>bright</sup> DCs. After 7 d of coculture, the expression of GATA-3, Foxp3, T-bet, and RORγt was analyzed in T cells. Data shown are representative of 10 independent experiments with consistent results—that is, showing the expression of Foxp3 on a fraction of CD4<sup>+</sup>CD25<sup>+</sup> T cells—whereas the other transcription factors were not detected. (C) CD25 and Foxp3 staining on naive CD4<sup>+</sup> T cells after 7 d of culture with HLA-DR<sup>dim</sup> or HLA-DR<sup>bright</sup> DCs. Data shown are representative of four independent experiments.
afferent lymph, we found that HLA-DR<sup>bright</sup> seroma DCs resemble the recently described migratory DCs harbored in human lymph nodes (17, 18). CD14<sup>+</sup> DCs detectable in afferent lymph, either CD1a<sup>+</sup> or CD1a<sup>-</sup>, might instead represent a transitional stage of differentiation from CD14<sup>+</sup>HLA-DR<sup>dim</sup> precursors to CD1a<sup>+</sup>CD14<sup>+</sup>HLA-DR<sup>bright</sup> DCs. Accordingly, during the acquisition of CD1a, CD14 downregulation was paralleled by the upregulation of HLA-DR molecules on the DC surface. Thus, CD14<sup>+</sup> DCs might downregulate CD14 molecules during DC migration toward SLOs, as previously suggested for CD14<sup>+</sup> dermal DCs (17). In line with this hypothesis, despite CD14<sup>+</sup> dermal DCs being regarded as migratory DCs, CD14 molecules are expressed exclusively by macrophages in human lymph nodes (17). More recently, a subset of CD14<sup>+</sup> DCs has been found in human inflammatory fluids; these cells displayed a phenotype distinct from that of macrophages isolated in the same fluids as well as that of steady-state lymphoid organ and blood DCs (39). Remarkably, these “inflammatory” DCs have a phenotype closely resembling CD14<sup>+</sup> cells found in afferent lymph from seroma. They share the expression of BDCA1, CD1a, CD11b, FcεR1, SIRPα, and CD206, and are conversely negative for both CD16 and DC-SIGN. However, inflammatory DCs described in the study by Segura and colleagues (39) strongly polarize for Th17 and were able to spontaneously release TNF-α, IL1-β, and IL-6, and IL-23 upon stimulation. On the contrary, CD14<sup>+</sup> DCs contained in seroma fluids do not spontaneously release any of these cytokines except TGF-β and do not induce in T cells the expression of RORγt, a transcription factor associated with Th17 polarization. It is thus possible to speculate that these cells have a similar origin and that their functional properties (e.g., cytokine secretion) might differ according to the nature of the inflammatory microenvironment.

An interesting feature of our study is the demonstration that DCs abundantly flow in afferent lymphatic vessels also in steady-state conditions, thus indicating a continuous stream of DCs from peripheral regions toward SLOs. Remarkably, only HLA-DR<sup>bright</sup> CD14<sup>+</sup> DCs were present in afferent lymph drained from noninflamed tissues, whereas the flux of HLA-DR<sup>dim</sup>-CD14<sup>+</sup> DCs appeared predominantly associated with the inflammatory process. The phenomenon might reflect a faster recruitment of DC precursors to sites of inflammation.

Therefore, as previously suggested in experimental animal models (1, 2), human DCs are able to continuously migrate from peripheral tissues to associated SLOs. These results raise relevant questions regarding the outcomes of Ag presentation by human DCs upon migration via afferent lymph. It is well established that afferent lymph DCs carry the Ags in a form that can be recognized by Ag-specific T cells (38). However, the nature of the consequent adaptive immune response may vary according to the microenvironmental factors of the drained tissues, which are likely to deeply affect the functional properties of migrating DCs.

The migratory properties of DCs do not preclude a role in stimulating immunity locally. This may occur in secondary responses, where DCs are found in association with T cells at delayed-type hypersensitivity sites (40, 41). Nevertheless, it is well established that in certain primary responses, like sensitization to skin transplants (42) and contact allergens (43), afferent lymphatics need to be intact, a point consistent with the idea that a flux of DCs is critical to immunogenicity.

Of interest, we also found that plasmacytoid DCs (pDCs) were present within seroma DCs, although in significant lower numbers compared with peripheral blood (Supplemental Fig. 2). Further studies, beyond the aim of the present one, are needed to confirm whether human pDCs can recirculate from peripheral tissues to SLOs, migrating via afferent lymph, as recently suggested in animal models showing a steady-state migration of pDCs in the afferent lymph (44).

Given their role in the presentation of peripheral tissue Ags and the current evidence that they can migrate continuously to SLOs, probably also from healthy tissues, it will be important to test the contribution to self-tolerance exerted by the afferent lymph DCs described in this article. Seroma DCs analyzed in the current study spontaneously release a large amount of TGF-β, whereas other proinflammatory cytokines were released at very low levels, if any. Remarkably, it has been previously demonstrated that TGF-β—producing DCs demonstrated an increased ability to generate CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells that suppress the proliferation of T lymphocytes (45, 46). Accordingly, we found that DCs from afferent lymph can induce the expression of Foxp3 on a consistent fraction of CD4<sup>+</sup>CD25<sup>+</sup> T cells.

Further studies should aim at investigating the effects of different danger signals, including TLR ligand stimulation, on the afferent lymph DC subsets characterized by the current study. In particular, it remains to be elucidated whether Foxp3<sup>+</sup> T cells induced by afferent lymph DCs might suppress T cell proliferation and whether a different polarization of T cells might occur when afferent lymph DCs are exposed to danger signals.

As a whole, these results open the way to further work on the functional role of distinct DC subsets migrating from peripheral tissues to SLOs to exert their protective or tolerogenic immune functions, most likely depending on signals received in peripheral tissues. This field of investigation might strongly contribute to the improvement of modern vaccination strategies as well as of other immune-based therapies for human diseases.

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Disclosures

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