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*J Immunol* published online 27 September 2013
http://www.jimmunol.org/content/early/2013/09/27/jimmunol.1300760

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2013/09/27/jimmunol.1300760.DC1

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Characterization of Human Afferent Lymph Dendritic Cells from Seroma Fluids

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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 CD1α+. The latter also included CD1αbright 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 displayed similar T cell–activating and –polarizing properties. Upon activation by danger signals, they upregulated 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 (FACSAria II, Becton Dickinson). Pleural effusion was obtained from patients with primary or metastatic tumors of different origin (age range: 40–96 y, 22 males and 3 females). Pleural effusions obtained from thoracentesis were maintained at 4˚C and just after were centrifuged at 400 × g × 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, -CD4 APC, -CD11c PE and APC, -CD16 PE, -CD40 FITC, -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, -CD80 FITC, -CD83 PE, -CD86 PE, -ILT3 FITC, -langerin PE (CD207), from Instrumentation Laboratories; anti-CD1a Pacific Blue, -CD32 PE, -FceRI PE, -CD115 PE, -CD135 PE, -sirp-α PE, -ILT2 PE, -ILT4 PE, -Foxp3 PE from BioLegend; anti–CCR1, –CCR5, –CCR7, –CXCR3 from R&D; -anti–PDL-1 PE, –PDL-2 PE, -ILT2 PE, -ILT4 PE, -CD14 PE, -CD1a PE, -CD1b PE, -CD3 FITC, -CD4 PE, -CD11b APC, -CD32 PE, -CD14 PE, -CD64 PE, -CD19 FITC, -CD40 PE, -CD48 PE, -CD11c PE and APC, -CD16 PE, -CD19 FITC, -CD4 PE, -CD4 APC, -CD11b APC, -GATA3, -RORγt PE, -RORαt APC from eBiosciences; anti-CD14 Alexa700 (AbD Serotec); and anti-Nkp46 (BAB281) and anti-B7-H3, kindly provided by Prof. Alessandro Moretta, Genoa, Italy.

The mAbs specific for proteins of the Ag presentation machinery—namely, anti-Δ (SY-5), anti-MB-1(SJJ-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–Erp57 (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 washings, cells were treated in a microwave oven at 200 W for 45 s. At the end of treatment, cells were washed and chilled 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 PE-labeled goat Abs specific to mouse IgG Fc fragments. All these Abs were purified from ascitic fluid by sequential precipitation with ammonium sulfate and caprylic acid (26). The purity and activity of mAbs preparations were assessed by SDS-PAGE analysis and by binding assays, respectively.

Intracellular staining was performed using the Fix/Perm Buffer Set (BioLegend) according to the manufacturer’s instructions.

Immunophenotypic analysis of cells was performed using FACSCanto II (BD Biosciences), Gallios (Beckman Coulter), or MACSQuant (Miltenyi Biotec, Bergisch Gladbach, Germany) flow cytometers. Data were analyzed using FlowJo software (TreeStar).

Microscopy

To determine DC morphology, HLA-DR<sup>mean</sup> and HLA-DR<sup>dim</sup> DCs were sorted by flow cytometry and incubated with T lymphocytes sorted from the same seroma; after 3 h they were immobilized on poly-L-lysine-coated coverslips (BioCoat; BD). Images were acquired with an Apatome Optical Section Microscope (Zeiss).

RT-PCR

RNA was extracted from lysed cells using an RNaseasy Micro Kit (QIAGEN, Hilden, Germany). cDNA was generated using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Reverse transcription was performed at 25˚C for 10 min, 50˚C for 60 min, and 85˚C for 5 min, using 0.5 mg total RNA and random primers. PCR amplifications were performed for 30 cycles using Platinum Taq (Invitrogen). PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. The TLR primers used in this study (27–29) are summarized in Supplemental Table I.

Functional studies

To evaluate cytokine secretion by CD14<sup>+</sup> DCs and CD1a<sup>+</sup> DCs, cells were isolated by positive selection using anti-CD14 and anti-CD1a microbeads and a magnetic separator (Miltenyi Biotec). Sorted cells were then cultured for 48 h with or without 50 ng/ml M-CSF, GM-CSF, or Flt3-L (Miltenyi Biotec).

For the proliferation assay, CD4<sup>+</sup> and CD8<sup>+</sup> naive T cells were separated from a healthy donor’s PBMCs by the Naive T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s instructions. Naive T cells (5 × 10<sup>5</sup> cells per well) were labeled with 5 μM CFSE, as previously described (30), and incubated with allogeneic CD14<sup>+</sup>HLA-DR<sup>mean</sup> DCs, HLA-DR<sup>dim</sup> DCs, and CD1a<sup>+</sup>HLA-DR<sup>mean</sup>langerin<sup>+</sup> Langerhans. The three DC subsets used in the T cell stimulation assay were previously sorted by flow cytometry according to the expression of HLA-DR, CD14, CD1a, and langerin. After 6 d, CFSE dilution was evaluated on both CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD4<sup>+</sup> cells by flow cytometry.

To study Th cell polarization induced by DCs, naive CD4<sup>+</sup> T cells were separated from a healthy donor’s PBMCs by the Naive CD4<sup>+</sup> T Cell Isolation Kit (Miltenyi Biotec) and cultured (10<sup>5</sup> cells per well) with HLA-DR<sup>dim</sup> or HLA-DR<sup>mean</sup> DCs (10<sup>5</sup> cells per well for 6 d. After washing, cells were incubated with anti-CD3/CD28 beads (T Cell Activation/Expansion Kit, Miltenyi Biotec) for 24 h and then analyzed for the expression of T-bet, GATA3, RORα, and Foxp3.

For phagocytosis experiments, 60 mg Lactobacillus reuteri bacteria were labeled with amine-reactive pHodo SE dye according to the manufacturer’s instructions (Molecular Probes, Invitrogen). Briefly, cells were incubated with bacteria at 37˚C or, as control, in ice. After 6 h, cells were labeled with anti–HLA-DR PE-CY7 (BD Biosciences) and anti–CD14 APC (Miltenyi Biotec), and phagocytosis was evaluated by flow cytometry.

To analyze the conversion of CD14<sup>+</sup> DCs to CD1a<sup>+</sup> DCs, cells were labeled with anti–HLA-DR FITC or PE-conjugated (BD Biosciences), anti–CD14 Alexa 700 (AbD Serotec) or anti–CD14 FITC (BD Biosciences) and anti–CD1A Pacific Blue (BioLegend) and sorted by FACSaria II. T lymphocytes were sorted according to their physical parameters and CD3 expression and cocultured with DCs (ratio of T:DC 10:1) in the presence of GM-CSF (20 ng/ml) for 72 h. Surface expression of CD1a, CD14, and HLA-DR was monitored each day, following labeling with anti–CD14 APC, anti–CD1A PE, and anti–HLA-DR PE-CY7.

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 tissues. To confirm the nature of this fluid and to evaluate a possible contamination by surgery-induced leaky blood–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<sup>+</sup> T cells, whereas a variable number of both CCR7<sup>+</sup> and CCR7<sup>−</sup> 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 was 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$^+$ large cell subsets could be identified: one characterized by high levels of HLA-DR and CD1a (HLA-DR$^{bright}$) and the other expressing lower levels of HLA-DR and CD14 (HLA-DR$^{dim}$) (Fig. 1C). Notably, the HLA-DR$^{bright}$ subset also contained CD14$^{bright}$ Langerhans cells. HLA-DR$^{bright}$ cells, compared with the HLA-DR$^{dim}$ 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$^-$ (CD3, CD19, Nkp46, BDCA2) HLA-DR$^+$ cells was 8.5%, ranging between 3.5 and 22%. The mean value of mononuclear cells (MNCs) contained in each seroma was $71 \pm 108 \times 10^6$ in a mean volume of 360 $\pm$ 147 ml.

The two main subsets of afferent lymph DCs differed in a variety of other relevant markers (Fig. 2A). In general, HLA-DR$^{bright}$ CD1a$^+$ DCs displayed a mature phenotype, as they expressed significantly higher levels of costimulatory molecules and DC hallmarks 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$^{bright}$ DCs. The inflammatory chemokine receptors CCR1 and CCR5 were also exclusively detectable in the HLA-DR$^{bright}$ subset. Myeloid DC markers BDCA1 and BDCA3 were mostly expressed on both subsets of afferent lymph DCs, although a fraction of HLA-DR$^{bright}$ DCs expressed BDCA3 at lower levels (Fig. 2B, Supplemental Fig. 1). Among all leukocytes contained in the seroma fluids, the median value of lineage$^-$ (CD3, CD19, Nkp46, BDCA2) HLA-DR$^+$ cells was 8.5%, ranging between 3.5 and 22%. The mean value of mononuclear cells (MNCs) contained in each seroma was $71 \pm 108 \times 10^6$ in a mean volume of 360 $\pm$ 147 ml.

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A specific subset of BDCA3$^{bright}$/CLEC9a$^-$/Sirp$^-$ DCs has recently been described in different human tissues (10–12, 32), These DCs resemble murine CD8$^+$ DCs (10), we found that HLA-DR$^{bright}$ contained a small subset of BDCA3$^{bright}$/Sirp$^-$ DCs expressing CLEC9a (Fig. 2C).

Because the HLA-DR$^{dim}$ 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$^{bright}$ DCs (22).

Because the HLA-DR$^{bright}$ DC population is heterogeneous and contains CD1a$^+$ and CD1a$^{bright}$ 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$^{langerin^-}$ 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$^+$ cells detectable in exudates (e.g., neoplastic effusions) (Fig. 2B). In contrast, HLA-DR$^{bright}$ seroma DCs resemble the recently described migratory DCs harbored in human lymph nodes (17, 18). However, in addition to the CD11c$^{low}$/CD1a$^{bright}$ DC subset (Fig. 3A, left panel) (18) and the CD14$^+$ CD1a$^{bright}$ DC subset (Fig. 3A, right panel) (17) (both contained in seroma as HLA-DR$^{bright}$ DCs), we could also detect CD14$^+$ 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$^+$CD1a$^-$, CD14$^-$CD1a$^-$, and CD14$^-$CD1a$^+$—were cultured for 72 h. In this interval, CD14$^+$CD1a$^-$ lost CD14 expression, whereas they progressively acquired CD1a. During the acquisition of CD1a, CD14$^-$ cells in parallel upregulated HLA-DR molecules on their surface (not shown). A similar phenotypic modulation was observed for CD14$^+$CD1a$^+$ “transitional” DCs, whereas CD14$^-$CD1a$^+$ DCs maintained a stable phenotype. Although we...
identified a subset of transitional DCs expressing both CD14 and CD1a (HLA-DR$^{\text{dim}}$), we also found that CD1a acquisition occurred after CD14 downregulation in CD14$^{+}$CD1a$^{-}$ DCs during the 72 h of culture. However, we could not detect a CD14$^{+}$CD1a$^{-}$ 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$^{\text{dim}}$CD1a$^{-}$CD14$^{+}$ DCs can convert, at least in vitro, into HLA-DR$^{\text{bright}}$CD1a$^{-}$CD14$^{+}$ DCs.

**HLA-DR$^{\text{bright}}$CD1a$^{+}$ 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

### Table I. Comparative analysis of the expression of relevant surface molecules in different human myeloid APCs

<table>
<thead>
<tr>
<th>Seroma DCs HLA-DR^{bright}</th>
<th>Seroma DCs HLA-DR^{dim}</th>
<th>Blood DCs</th>
<th>Monocytes</th>
<th>Mono DCs^{GM-CSF/IL-4}</th>
<th>Mono DCs^{GM-CSF/IL-4 + LPS}</th>
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*Mono DCs, DCs derived in vitro from monocytes in the presence of the indicated soluble factors. n.d., Not determined; −, negative; +, positive; +/−, partially positive.

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**FIGURE 3.** Developmental relationship between DC subsets of afferent lymph. (A) HLA-DR^{bright} DCs of afferent lymph resemble CD11c^{low}CD14^{−}CD1a^{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^{dim} DC subset) represent transitional stages of differentiation from CD14^{+} precursors to CD1a^{+}CD14^{−} DCs (HLA-DR^{bright}). 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^{bright}) 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 from 10 patients. 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 (post-surgical) and late time points of lymph collection, because after 30 d from surgery only HLA-DR^{bright} DCs were almost exclusively detectable in seroma fluids. Dot plots shown are representative of seroma samples collected from 10 patients.
HLA-DR<sup>bright</sup> DCs, including both CD1a<sup>+</sup> and CD1a<sup>bright</sup>langerin<sup>+</sup> 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<sup>dim</sup>CD14<sup>+</sup> DCs is drained from inflamed tissues to SLOs via afferent lymph. In contrast, several weeks after surgery, only HLA-DR<sup>bright</sup>CD14<sup>-</sup> DCs were abundant in afferent lymph (Fig. 3C). These data suggest that, even in the absence of inflammation, HLA-DR<sup>bright</sup>CD14<sup>-</sup> 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<sup>dim</sup> 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<sup>bright</sup> 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<sup>bright</sup> 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<sup>bright</sup> and HLA-DR<sup>dim</sup> DCs (Fig. 4E). Proteasome subunit δ and TAP proteins were more represented in HLA-DR<sup>bright</sup> 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<sup>bright</sup> 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<sup>+</sup> and CD8<sup>+</sup> naïve T cells were sorted, labeled with CFSE, and incubated with allogeneic CD1a<sup>bright</sup>langerin<sup>+</sup> (LC), CD1a<sup>-</sup>langerin<sup>-</sup> (HLA-DR<sup>bright</sup>) DCs, or HLA-DR<sup>dim</sup> 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<sup>bright</sup> and HLA-DR<sup>dim</sup> 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**bright** DC subset.

**DCs contained in afferent lymph induce Foxp3 expression on CD4**^+** naive T cells**

In accordance with high levels of TGFβ 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**dim** subset and only in the presence of M-CSF.

We then assessed the polarization of naive CD4^+ T cells in the presence of afferent lymph DCs. After 7 d of culture with either HLA-DR**dim** DCs or HLA-DR**bright** DCs, a discrete percentage of CD4^+CD25^+ 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^+ 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

![FIGURE 5. DCs from afferent lymph spontaneously release TGF-β and induce Foxp3 expression on CD4^+ T cells.](http://www.jimmunol.org/)

- (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^+ T cells were cultured with HLA-DR**dim** or HLA-DR**bright** DCs. After 7 d of coculture, the expression of GATA3, 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^+CD25^+ T cells—whereas the other transcription factors were not detected. (C) CD25 and Foxp3 staining on naive CD4^+ T cells after 7 d of culture with HLA-DR**dim** or HLA-DR**bright** DCs. Data shown are representative of four independent experiments.
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+CD25+Foxp3+ 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+CD25+ 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+ 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.

Acknowledgments
We thank the staff at Carmona Home Care for help in providing seroma samples; Dr. Fabrizio Ioppolo for technical assistance with microscopy; and Dr. Fabrizio Loiacono and the staff of Centro Cellule Staminali, A.O.U. San Martino, Genoa, Italy, for flow cytometry cell sorting.

Disclosures
The authors have no financial conflicts of interest.

References
ident CD141+ (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. J. Exp. Med. 209: 935–945.


## Supplemental Table I: Sequences of PCR Primers for TLRs

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Supplemental Figure 1
Legends to Supplemental Figures

Supplemental Figure 1:
Langherans cells and CD1a+ DCs present in seroma fluids display a similar pattern of surface molecules. (A): mononuclear cells isolated from seroma were stained for HLA-DR, CD14, CD1a and Langherin. HLA-DR bright CD1a+ (CD1a) and HLA-DR bright CD1a+ Langherin (LC) cells were gated as depicted. (B): CD1a and LC cells were comparatively analyzed for the expression of the indicated markers. Empty histograms represent control staining with isotype-matched irrelevant antibodies. Bars represent mean values and standard deviations of data obtained in 4 seroma samples comparatively analyzing LC (white bars) and CD1a+ DCs (black bars). The analysis did not reveal any significant differences.

Supplemental Figure 2:
Plasmacytoid DCs are present in seroma fluids in lower amount compared to peripheral blood. Mononuclear cells (MNC) were isolated from both seroma fluids and peripheral blood and stained for CD123 (IL-3 receptor) and BDCA2. Results obtained in 14 seromas and 7 peripheral blood are summarized in (A). Representative stainings of pDCs are shown in (B).