Human thymus contains 2 distinct dendritic cell populations
Stéphane Vandenabeele, Hubertus Hochrein, Nasim Mavaddat, Ken Winkel, and Ken Shortman

In this study, 2 distinct populations of mature dendritic cells (DCs) were identified in the human thymus. The major population is CD11b\(^+\), CD11c\(^+\), and CD45RO\(^{low}\) and does not express myeloid-related markers. It displays all the characteristics of mature DCs with a typical dendritic morphology, high surface levels of HLA-DR, CD40, CD83, and CD86, and expression of DC–lysosome-associated membrane glycoprotein messenger RNA (mRNA). In addition, CD11b\(^+\) thymic DCs do not express macrophage inflammatory protein-1\(\alpha\) (MIP-1\(\alpha\)) mRNA, but express thymus-expressed chemokine (TECK) mRNA and are able to secrete bioactive interleukin 12 (IL-12) upon stimulation. In contrast, the minor and variable thymic DC population is CD11b\(^-\), CD11c\(^{high}\), and CD45RO\(^{high}\) and comprises CD83\(^-\)CD14\(^-\) mature and CD83\(^-\)CD14\(^+\) immature DCs. It expresses macrophage-colony stimulating factor receptor, MIP-1\(\alpha\) mRNA and high amounts of decysin mRNA after CD40 activation, but does not express TECK and is a weak bioactive IL-12 producer. Also identified were the IL-3R\(\alpha\)^{high} plasmacytoid cells, which are present in the thymic cortex and medulla. Upon culture with IL-3, granulocyte/macrophage–colony stimulating factor, and CD40 ligand, the plasmacytoid cells can adopt a phenotype resembling that of freshly isolated CD11b\(^-\) thymic DCs. However, these plasmacytoid-derived DCs fail to secrete bioactive IL-12; therefore, conclusions cannot be made about a direct relation between thymic plasmacytoid cells and CD11b\(^-\) DCs. Whereas CD11b\(^+\) thymic DCs appear to be related to tonsillar germinal-center DCs, the major CD11b\(^-\) IL-12–secreting human thymus DC population has similarities to mouse CD11b\(^-\)CD8\(^+\) DCs. (Blood. 2001;97:1733-1741)

Introduction

Dendritic cells (DCs) are professional antigen presenting cells\(^1\) that form a dynamic network throughout most tissues and organs and that are crucial for the immune surveillance of the body.\(^2\)\(^-\)\(^4\) The current model is that Langerhans cells, immature DCs found in epithelia, are the precursors of mature “interdigitating” DCs, found in the T-cell zones of lymphoid organs.\(^5\) Immature interstitial DCs in various tissues, such as the dermis, move into germinal centers as germinal center DCs (GCDCs). In the lymph nodes, tonsils, and spleen, the mature DCs present the antigen captured in the periphery to naive T cells and induce immunity. The DCs of the thymus have a somewhat different role, namely, to present self-antigens and induce negative selection of potential auto-reactive T-cell clones.\(^6\) Until recently, the interdigitating DCs were considered a single population of mature DCs. However, this laboratory and others showed that distinct DC subsets of different lineage derivation and different functions\(^5\)\(^-\)\(^13\) could be distinguished in mouse lymphoid organs. On the one hand, CD8\(^+\) DCs, lacking myeloid markers such as CD11b and apparently arising from a lymphoid-committed progenitor,\(^14\) are typically potent interleukin 12 (IL-12)–secreting mature DCs.\(^10\),\(^13\),\(^15\) CD8\(^+\) DCs are present at various levels in all mouse lymphoid organs and constitute the major population in the thymus.\(^15\) On the other hand, CD8\(^-\) DCs, which comprise CD4\(^+\) and CD4\(^-\) subsets,\(^16\) express CD11b, appear to be myeloid related, and are weak IL-12 producers.\(^10\),\(^13\)

Whereas in mice our concepts of DC life history rely mainly on in vivo studies, investigations of mature human DCs are based mainly on in vitro models of DC generation. Several human DC precursors have been identified, including peripheral blood monocytes\(^17\)\(^-\)\(^20\) and earlier CD34\(^+\) hematopoietic progenitors.\(^21\),\(^22\) All were shown to differentiate into DCs exhibiting high levels of major histocompatibility complex class II and costimulatory molecules upon culture with granulocyte/macrophage–colony stimulating factor (GM-CSF) and IL-4 or tumor necrosis factor alpha (TNF-\(\alpha\)). Recently, the CD4\(^+\)CD3\(^-\)CD11c\(^-\) plasmacytoid cells, whose origin and function had been an enigma, were shown to differentiate into DCs upon culture, albeit with an atypical surface phenotype and without a capacity to secrete IL-12.\(^23\)\(^-\)\(^25\) Such plasmacytoid cells are found in tonsils,\(^23\)\(^-\)\(^25\) blood,\(^24\),\(^26\) cord blood,\(^27\) and thymus.\(^28\) They were subsequently considered to embody the “lymphoid-related” DC lineage in humans as, first, they express the pre–T-cell receptor alpha chain\(^28\)\(^,\)^{28} and, second, in contrast to monocyte- or CD34\(^+\) progenitor–derived DCs, plasmacytoid-derived DCs do not express the “lymphoid-related” markers CD11b, CD11c, and CD33 and strikingly retain CD45RA expression.\(^23\),\(^25\),\(^28\) Moreover, as in the lymphoid-restricted DC precursor in the mouse,\(^26\) growth of plasmacytoid cells requires IL-3 but not GM-CSF.\(^23\) However, there remains one problem in considering the plasmacytoid cells to be a normal step in DC development. Although they differentiate into DCs in vitro, their mature dendritic form is still to be identified in vivo.

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1733
Studies of mature DCs in humans ex vivo\textsuperscript{31-36} have been hampered by the difficulty of isolating these cells. Often, short-term culture steps were required to overcome this problem. In the present study, we use freshly isolated DCs to give an instant picture of a human DC network, adapting a purification procedure used in the mouse\textsuperscript{16} to human tissue. We have studied human thymus, which represents a good source of mature DCs in terms of the yield and the consistency between donors. We have attempted to compare the mature DC forms found in vivo with those generated in vitro and to compare human with mouse thymus DCs. We provide an extensive phenotype and characterization of these DCs and show that 2 distinct populations of mature DCs, segregated on the basis of CD11b expression, can be isolated from the human thymus. The major population does not express CD11b and shares similarities with thymic plasmacytoid-derived DCs grown in the presence of IL-3, CD40 ligand (CD40L) plus GM-CSF, whereas the minor DC population is CD11b\textsuperscript{+}, arises from immature myeloid DCs, and is related to tonsillar GCDCs.

## Materials and methods

### Monoclonal antibodies

The following mouse anti–human monoclonal antibodies (mAbs) were purified and conjugated in this laboratory from supernatants of hybridomas obtained from American Type Culture Collection (Manassas, VA); anti-CD3 (OKT3), anti-CD8 (OKT8), anti-CD7 (3A1), anti-CD15 (WEMG1), anti-CD19 (FMC-63), anti-CD20 (B1), anti-glycoporphinA (10F7MN), anti-CD40 (28.5), anti-CD45RO (UCHL-1), anti-CD45RA (SHL-3) from Becton Dickinson (San Jose, CA); FITC-conjugated anti-CD20 (B1), anti–HLA-DR (2.06), phycoerythrin-conjugated (PE-conjugated) anti-CD20 (B1), Cy5-conjugated anti–HLA-DR (2.06), and biotin-conjugated anti-CD11b (OKM1).

The following mAbs were purchased commercially: purified anti-CD86 (BT63) from Ancell (Bayport, MN); FITC-conjugated anti-CD1a (HI149), anti-CD14 (M5E2), anti-CD16 (3G8), anti-CD33 (HIM3-4), purified and conjugated in this laboratory from supernatants of hybridomas for 10 minutes. The first cycle was at a 3:1 and the second at a 6:1 bead-to-cell ratio. The cells were then kept overnight at 4°C in EDTA-SS containing 10\(^{-6}\) M EGTA and 10\(^{-5}\) M Ca\(^{2+}\).

### Plasmacytoid cell purification

For plasmacytoid cell isolation, the undepleted thymic cell preparation was stained with PE-conjugated anti–IL-3R\(\alpha\) (nonblocking) and Cy5-conjugated anti–HLA-DR. Plasmacytoid cells were sorted on the basis of high CD11c level of staining with anti–molecule immunoglobulin–coupled magnetic beads (Dynabeads, Dynal, Oslo, Norway). The first cycle was at a 3:1 and the second at a 6:1 bead-to-cell ratio. The cells were then kept overnight at 4°C in EDTA-SS containing 10\(^{-6}\) M EGTA and 10\(^{-5}\) M Ca\(^{2+}\). For IL-12 production, isolated DCs (5 \times 10\(^4\)) were cultured in 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ), in 150 \(\mu\)L of complete RPMI (RPMI-1640 supplemented with 10% heat-inactivated FCS, Hepes buffer, pH 7.2; 2 \(\mu\)M glutamine; and 10\(^{-4}\) M 2-mercaptoethanol and antibiotics) containing 7.5 \(\mu\)g/mL of agonistic anti-CD40 (2G8.5). After 1 to 3 days of activation, DCs were either recovered, washed, and incubated with SS-EhTDA for further cell-surface staining, or recovered, washed, and lysed. Measurement of CD11b and CD14 expression after sorting and after culture was performed by means of a FACScan (Becton Dickinson). A different clone of anti-CD11b antibody recognizing a different epitope was used to assess CD11b expression after sorting.

### Short-term culture of isolated DCs

Both thymic DC populations (10\(^3\)) were cultured in 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ), in 150 \(\mu\)L of complete RPMI (RPMI-1640 supplemented with 10% heat-inactivated FCS, Hepes buffer, pH 7.2; 2 \(\mu\)M glutamine; and 10\(^{-4}\) M 2-mercaptoethanol and antibiotics) containing 7.5 \(\mu\)g/mL of agonistic anti-CD40 (2G8.5). After 1 to 3 days of activation, DCs were either recovered, washed, and incubated with SS-EhTDA for further cell-surface staining, or recovered, washed, and lysed. Measurement of CD11b and CD14 expression after sorting and after culture was performed by means of a FACScan (Becton Dickinson). A different clone of anti-CD11b antibody recognizing a different epitope was used to assess CD11b expression after sorting.

For IL-12 production, isolated DCs (5 \times 10\(^4\)) were cultured in 96 round-bottom plates (Becton Dickinson Labware) in a final volume of 200 \(\mu\)L complete RPMI containing recombinant human rGM-CSF (50
Culture of isolated plasmacytoid cells

Plasmacytoid cells (5 × 10⁴) were cultured in flat-bottom 96-well plates (Becton Dickinson Labware) in 200 μL complete RPMI containing 25 ng/mL rhIL-4 (Peprotech) and 1 μg/mL of soluble trimeric CD40L, with or without 100 ng/mL rhGM-CSF. On day 2 of culture, 100 μL supernatant was recovered for IL-12 enzyme-linked immunosorbent assay (ELISA), and fresh medium was added. On day 5, plasmacytoid-derived DCs were restimulated with either fresh soluble CD40L (1 μg/mL) alone or CD40L (1 μg/mL), rhGM-CSF (100 ng/mL), rhIL-4 (20 ng/mL), and rhIFN-γ (20 ng/mL). Supernatants were then recovered on day 7 for IL-12 ELISA.

Reverse transcriptase–polymerase chain reaction

Total cytoplasmic RNA was prepared from freshly sorted or activated DCs by means of RNaseasy Mini kit (Qiagen, Hilden, Germany). First-strand complementary DNA (cDNA) was synthesized by means of random hexamers, deoxynucleotidetriphosphate (dNTP) mixture, rTNASin ribonucleoside inhibitor, and Moloney murine leukemia virus reverse transcriptase (all reagents from Promega, Madison, WI). The polymerase chain reaction (PCR) was carried out in a final volume of 50 μL containing 1 μL cDNA, 1.5 mM MgCl₂, thermo-reaction buffer (Promega), dNTP mixture (0.2 mM each dNTP) (Promega), 0.5 mM each oligonucleotide primer (Geneworks Pty, Adelaide, Australia), and 2 U Taq DNA polymerase (Boehringer Mannheim). Each set of 25 to 35 cycles was performed by means of a PerkinElmer thermal cycler (PerkinElmer/Cetus, Norwalk, CT). After 3 minutes of inactivation at 94°C, each cycle consisted of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 30 seconds of extension at 72°C. A final extension at 72°C was carried out for 5 minutes. For analysis, 10 μL of each reaction was electrophoresed through a 2% agarose gel. Monocyte-derived DCs (MoDCs) used as controls were produced from peripheral blood monocytes selected by adherence followed by depletion of contaminants by means of anti-CD3, anti-CD8, anti-CD20, anti-CD15, and anti-glycophorin A mAbs. Monocytes were then cultured for 6 days with rhGM-CSF (100 ng/mL) and rhIL-4 (20 ng/mL). MoDCs were activated by means of an agonistic anti-CD40 (7.5 μg/mL) (Peprotech); and soluble trimeric CD40L (1 μg/mL) (kind gift from Dr. D. Lynch, Immunex). Cytokine binding was then detected with a biotinylated detection mAb (anti-human IL-12 p40; C8.6) (Pharmingen). The readout was then obtained by using streptavidin-horseradish peroxidase conjugate (Amersham Life Science) and a substrate solution containing 548 mg/mL ABTS (2,2′-Azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) and 0.01% hydrogen peroxide (Ajax Chemicals, Auburn, Australia) in 0.1 M citric acid, pH 4.2, followed by scanning the optical density at 405 to 490 nm.

Results

In order to study mature thymic DCs, which represent only a small fraction of thymic cells (0.02%), we first enriched the light-density cell population (3%) after collagenase digestion and EDTA treatment of the thymic tissue. All detectable DCs were in this fraction. We then carefully depleted non-DC contaminants and in particular CD4–CD19–CD20– B-cell populations, which expressed surprisingly high levels of costimulatory molecules (data not shown). Finally, we stained the preparation for immuno-fluorescence using 4 colors, analyzed the preparation by flow cytometry, and focused attention on cells expressing high levels of HLA-DR as our primary criteria for mature DCs. Other markers were then used to confirm a mature DC phenotype. As shown in Figure 1, thymic DCs gated on CD83 and CD11b were then collected by gently washing the flasks. The naive CD4 T cells were depleted of contaminating cells by incubation with a mixture of mAbs including anti-CD19, anti-CD20, anti-CD45R0, anti-CD8, anti-CD11b, anti-CD15, anti-glycophorin A, and anti-CD56, followed by 2 rounds of Dynabeads (Dynal). The purity of CD4⁺CD3⁺CD45RA⁺ T cells was greater than 90%. Sorted DCs were used as stimulator cells for naive allogeneic CD4 T cells. DCs (10 to 3000) were cocultured with 1.5 × 10⁵ T cells in round-bottom 96-well plates (Becton-Dickinson Labware) by means of complete RPMI. After 5 days of coculture, cells were pulsed for 9 hours with 1 μCi 3H-TdR per well and then harvested, and thymidine incorporation was measured by liquid scintillation counting. Assays were performed in triplicate, and results were expressed as mean counts per minute (SD).

IL-12 p70 quantitation by ELISA

Aliquots of DCs and plasmacytoid cell culture supernatants were assayed by 2-site ELISAs. Briefly, 96-well polystyrene plates (Dynatech Laboratories, Chantilly, VA) were coated with purified capture mAb (anti-human IL-12 p70; 20C2) (Pharmingen). Cytokine binding was then detected with a biotinylated detection mAb (anti-human IL-12 p40; C8.6) (Pharmingen). The readout was then obtained by using streptavidin-horseradish peroxidase conjugate (Amersham Life Science) and a substrate solution containing 548 mg/mL ABTS (2,2′-Azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) and 0.01% hydrogen peroxide (Ajax Chemicals, Auburn, Australia) in 0.1 M citric acid, pH 4.2, followed by scanning the optical density at 405 to 490 nm.

Figure 1. CD11b⁺ and CD11b⁻ mature thymic DCs. Mature thymic DCs comprise 2 populations segregated by CD11b expression. DCs were released from a thymus tissue sample, light-density cells selected, and non-DC lineages including T and B cells depleted. The enriched DCs were then immunofluorescent stained and analyzed. (A) DCs were gated on the basis of high HLA-DR (Cy5) and positive CD86 (Texas Red) fluorescence. (B) HLA-DR⁺CD86⁺ DCs express CD40 (FITC) and CD83 (PE). All express CD11c (PE); however, a major population (65%) lacks CD11b (FITC) expression and a minor population (35%) is CD11b⁺. This figure is representative of 4 separate thymuses.
In contrast to the phenotype of plasmacytoid cells, CD11b and CD64 and expressed only a low level of CD33. Interestingly, in mouse and CD4. These cells did not express the myeloid markers CD14 and CD4. These cells did not express the myeloid markers CD14 and CD68. CD11b expression revealed 2 populations of mature DCs: the major population was CD11b+CD68+CD14−CD68− cells as well as less mature CD40lowCD86lowCD83lowCD14+CD64+ DCs. All CD11b+ DCs expressed CD4 and the GM-CSF receptors, but did not express either the IL-3Ra or CD45RA. Importantly, in contrast to CD11b- DCs, all CD11b+ DCs expressed high levels of CD11c, CD33, and CD45RO, regardless of their state of maturation.

**Both CD11b+ and CD11b− thymic DC populations display functional and morphological characteristics of DCs**

Hallmarks of DCs are, first, their unique ability to present antigens and stimulate naive T cells and, second, the exhibition of dendrites. As shown in Figure 3, in an allogeneic–mixed-leukocyte reaction (MLR) assay, both CD11b+ and CD11b− DCs, sorted as shown in Figure 2A, proved to be potent stimulators of naive CD4 T cells. As shown in Figure 4A and B, CD11b− DCs just after sorting displayed a stellate morphology, typical of fully mature DCs. The CD11b− fraction (Figure 4D-E) comprised cells of dendritic morphology as well as “hairy” cells, which were not adherent to glass or plastic. We observed a rapid clustering (1 to 2 hours) of CD11b− DCs in culture, and after CD40 activation for 24 hours, we observed large clusters of DCs exhibiting long dendrites (Figure 4C). Freshly sorted CD11b+ DCs clustered slowly (3 to 5 hours) and, after CD40 activation, formed small clusters of cells displaying long dendrites (Figure 4E). The morphological features before and after activation, coupled with the fundamental property of activating naive T cells, indicated that both CD11b+ and CD11b− fractions were DCs, albeit with some differences in form.

**CD11b+ and CD11b− thymic DC populations are distinct**

CD11b expression by DCs has been described in both humans and mice as having the ability to be transient and as depending on the state of activation. In mice, CD8+ DCs were shown to acquire CD11b expression after activation, and in humans “fraction 1” DCs in blood up-regulated CD11b following maturation. In order to determine if the discrepancies in CD11b expression that we observed among human thymic DCs reflected different activation states of the same DC population, we activated both thymic DC populations and measured CD11b expression. As shown in Figure 5, CD11b- DCs did not express CD11b or CD14, either when

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**Figure 2. The surface phenotype of thymic DCs.** (A) By means of 4-color analysis, DCs were gated as HLA-DRhighCD83low/CD11b+ (Texas Red) and HLA-DRhighCD11b−. (B) Both fractions were then analyzed by means of FITC and PE channels. Particular cases: CD86 and IL-3Ra expressions were revealed in T exas-Red after the gating carried out with FITC-conjugated anti-CD11b antibody. This analysis is representative of 6 separate thymus samples.

HLA-DRhighCD83high were also CD40high and CD83high. Thus, these thymic DCs displayed a surface phenotype typical of mature DCs. Furthermore, HLA-DRhighCD83high DCs were all CD11c+. The broad CD11c profile suggested the existence of different DC populations but did not allow clear segregation for further sorting. In contrast, CD11b expression revealed 2 populations of mature DCs: the major population was CD11b+, and the minor one CD11b-. The proportion of the respective populations varied among donors, but the proportion of the CD11b− DC population was always above 65% of the HLA-DRhighCD83high mature DCs.

On the basis of this analysis, we adopted the sorting strategy shown in Figure 2A to collect sufficient numbers of cells to study both CD11b+ and CD11b− DC populations. Both populations were simultaneously sorted on the basis of high HLA-DR and negative or positive CD11b fluorescence, together with characteristic high forward and side scatter. In order to recover a sufficient number of CD11b+ cells, we chose to include among the CD11b+ cells some expressing slightly lower levels of HLA-DR, although these would then be expected to include some less mature DCs.

As revealed by means of 4-color staining (Figure 2B), CD11b− DCs were CD40high, CD86high, and CD83high and expressed CD11c and CD4. These cells did not express the myeloid markers CD14 and CD68 and expressed only a low level of CD33. Interestingly, in contrast to the phenotype of plasmacytoid cells, CD11b− DCs expressed the GM-CSFRs but not the IL-3Ra; did not express the marker of immature leucocytes, CD45RA; and expressed only low levels of the marker of mature leucocytes, CD45RO. On the other hand, the CD11b+ DC fraction comprised mature CD40highCD86highCD83highCD14−CD64+ cells as well as less mature CD40lowCD86lowCD83lowCD14+CD64+ DCs. All CD11b+ DCs expressed CD4 and the GM-CSF receptors, but did not express either the IL-3Ra or CD45RA. Importantly, in contrast to CD11b− DCs, all CD11b+ DCs expressed high levels of CD11c, CD33, and CD45RO, regardless of their state of maturation.

**Figure 3. Stimulation of naive CD4+ T cells by CD11b+ and CD11b− DCs.** Both CD11b− and CD11b+ DCs are potent stimulators of naive CD4+ T cells. Freshly isolated DCs (10 to 3 × 106) were cultured for 5 days with 15 × 105 allogeneic CD45RA−CD4− T cells. As few as 10 CD11b− or CD11b+ DCs were able to induce naive T-cell proliferation. This figure is representative of 3 experiments.
freshly isolated or after 2 days of CD40 activation by means of an agonistic anti-CD40 antibody. In parallel, CD11b<sup>+</sup> DCs slowly down-regulated CD11b after 2 days of CD40 activation, but they still retained low levels of CD11b even after 3 days. Importantly, CD14 and CD64 expression was lost early in culture (less than 24 hours) by the immature CD11b<sup>+</sup> fraction, suggesting that these immature DCs needed only CD40 signaling to mature.

**Differences in messenger RNA expression between CD11b<sup>+</sup> and CD11b<sup>−</sup> DCs**

As another test for basic differences, we searched by reverse transcriptase PCR (RT-PCR) for potential markers that would segregate DC populations (Figure 6). We analyzed freshly sorted or CD40-activated CD11b<sup>+</sup> and CD11b<sup>−</sup> thymic DCs. To ensure we were working with similar amounts of cDNA from each population, we made dilutions of cDNA samples to obtain similar expression of the housekeeping gene β-actin. We then compared expression of the maturation marker DC-LAMP<sup>a</sup>, the disintegrin proteinase decysin (which was identified from CD40-activated GCDCs)<sup>40</sup>, the TECK (which was initially described in mouse proteinase decysin (which was identified from CD40-activated CD11b<sup>+</sup> DCs) display a typical dendritic morphology. (C) After CD40 activation, CD11b<sup>−</sup> DCs form large, tight clusters of cells displaying long dendrites. (D) (E) Freshly isolated CD11b<sup>−</sup> DCs do not adhere to glass or plastic and display a "fairy" morphology. (F) After CD40 activation, CD11b<sup>−</sup> DCs form small clusters of cells exhibiting dendrites. Original magnification: 20 × (panels A, D, C, and F); 40 × (panels B and E).

as shown in Figure 6, the thymic DC populations corresponded to the mature dendritic form of plasmacytoid cells. As a first approach, we rechecked the localization of mature DCs and plasmacytoid cells on thymic tissue sections. As shown in Figure 7A, mature DCs expressing high levels of CD40 in blue were found close to Hassall corpuscles, which identify the thymic medulla. Round plasmacytoid cells, identified by high expression of the IL-3Rα in red, were found in both the cortex and the medulla, near blue CD40<sup>high</sup> DCs displaying typical dendrites (Figure 7B). In contrast to medullary B cells and epithelial cells, which can express CD40, only DCs expressed detectable levels of CD86 in blue. As shown in Figure 7C, blue CD86<sup>high</sup> DCs were surrounded by round red IL-3Rα<sup>high</sup> plasmacytoid cells in the medulla. Consistent with our observations by means of flow cytometry, no blue CD86<sup>high</sup> DC was found to coexpress the IL-3Rα.

**Thymic plasmacytoid cells can, upon culture, adopt a phenotype similar to CD11b<sup>−</sup> DCs**

As a second approach to assess the relationship between plasmacytoid cells and our mature thymic DCs, we analyzed the phenotype of plasmacytoid cells using 4-color immunofluorescence staining. Plasmacytoid cells represented 1.5% to 2% of the thymic light-density cell fraction as gated on the basis of IL-3Rα<sup>high</sup> and CD11b<sup>−</sup> DCs were stained for CD11b and CD14 expression immediately after sorting and after 2 days of CD40 activation. Whereas CD11b and CD14 expression is negative before and after CD40 activation of CD11b<sup>−</sup> DCs, all CD11b<sup>−</sup> DCs lose CD14 expression and slightly down-regulate but do not lose CD11b expression following CD40 activation. This figure is representative of 3 experiments.
from a putative CD45RO 
1
suggested that, in contrast to original descriptions, 23 GM-CSF 

\[ \text{a} \]
or CD83. Thus, IL-3R 

\[ \text{a} \]
high thymic cells corresponded in phenotype 

2
activation; CD11b 

\[ \text{a} \]
cells derived from thymus and tonsils expressed the GM-CSFR 

to either of our freshly isolated mature DC populations. Thus these IL-3–derived culture-matured DCs did not correspond 

they then expressed high levels of HLA-DR and CD86 and still 
matured and acquired CD83 expression (Figure 9A). Moreover, 

was only after 6 days of culture that plasmacytoid-derived DCs had 

amounts of IL-12 p70 after 2 days of stimulation, whereas CD11b 

DCs were very weak producers regardless of the time length of 
culture. Moreover, we tested the ability of thymic plasmacytoid 
cells and plasmacytoid-derived DCs to secrete IL-12 p70. We were 

not able, in our system, to detect any bioactive IL-12 after CD40 ligation. 25 We used this 

functional characteristic as a tool to help determine if plasmacytoid 
cells would differentiate into thymic DCs in vivo. Using a set of 
cytokines described as being the most powerful inducer of bioac-
tive IL-12 p70 secretion (including GM-CSF, IFN-\( \gamma \), IL-4, and 
soluble CD40L), 43 we stimulated freshly isolated CD11b 

anti–IL-3R 

a

We then sorted thymic plasmacytoid cells using a nonblocking anti–IL-3R 

antibody and assessed their further development in culture with 

IL-3 and soluble CD40L, plasmacytoid cells clustered after 6 to 8 hours and, after 2 
to 3 days, displayed long dendrites (data not shown). However, it 

was only after 6 days of culture that plasmacytoid-derived DCs had 
matured and acquired CD83 expression (Figure 9A). Moreover, 

they then expressed high levels of HLA-DR and CD86 and still 

expressed CD45RA, but did not express CD45RO and CD11c. Thus these IL-3–derived culture-matured DCs did not correspond 
to either of our freshly isolated mature DC populations.

However, in preliminary studies, we found that plasmacytoid 
cells derived from thymus and tonsils expressed the GM-CSFRa 
(data not shown), as did both CD11b+ and CD11b+ DCs. This 
suggested that, in contrast to original descriptions, 23 GM-CSF 
could be of importance for the fate of plasmacytoid cells. Accord-
ingly, we introduced GM-CSF in our culture system and analyzed 
the phenotype of plasmacytoid-derived DCs after 6 days (Figure 9B). Plasmacytoid cells grown with GM-CSF, IL-3, and soluble 
CD40L expressed higher levels of CD83 than those grown with 
IL-3 and CD40L alone. More importantly, there was a marked 
down-regulation of CD45RA expression and up-regulation of 
CD11c by a fraction of plasmacytoid-derived DCs. These 
CD11c+CD86CD83CD56CD14CD11b+CD45RA+ DCs did not seem to arise from a putative CD45RO+ monocyte contaminant, as no CD45RO 
expression was detected. However, we cannot exclude the possibility 
that this phenotype results from the selected outgrowth of a 
particular plasmacytoid cell subset and not from the homogeneous 
differentiation of these cells. Thus, with the help of GM-CSF, 
which appeared to facilitate maturation, a proportion of plasmacytoid-
derived DCs had acquired a phenotype resembling that of freshly 
isolated CD11b+ thymic DCs. We could detect by RT-PCR the 
expression of DC-LAMP but not decysin mRNA by these plasma-
cytoid-derived DCs (data not shown).

CD11b+ DCs, but not CD11b+ or plasmacytoid-derived DCs, 
secrete bioactive IL-12 following CD40 ligation

One major difference recorded between monocyte-derived and 
plasmacytoid-derived DCs was that the latter appear unable to 
produce any bioactive IL-12 after CD40 ligation. 25 We used this 
functional characteristic as a tool to help determine if plasmacytoid 
cells would differentiate into thymic DCs in vivo. Using a set of 
cytokines described as being the most powerful inducer of bioac-
tive IL-12 p70 secretion (including GM-CSF, IFN-\( \gamma \), IL-4, and 
soluble CD40L), 43 we stimulated freshly isolated CD11b+ and 
CD11b+ DCs for 1 to 7 days and measured IL-12 p70 secretion by 
ELISA. As shown in Table 1, CD11b+ DCs secreted substantial 
amounts of IL-12 p70 after 2 days of stimulation, whereas CD11b+ 
DCs were very weak producers regardless of the time length of 
culture. Moreover, we tested the ability of thymic plasmacytoid 
cells and plasmacytoid-derived DCs to secrete IL-12 p70. We were 
not able, in our system, to detect any bioactive IL-12 from the 
supernatant of plasmacytoid cells cultured for 2 days with IL-3, 
GM-CSF, and CD40L or from the supernatant of plasmacytoid-
derived DCs restimulated with GM-CSF, IFN-\( \gamma \), IL-4, and CD40L 
on day 5 of culture.

Discussion

Our results show that the human thymus contains 2 populations of 
mature DCs, expressing different lineage markers and displaying 
different capacities for IL-12 secretion. The major thymic DC 
population (more than 65%) is most readily distinguished from other DCs by 
being clearly CD11b+ and also CD33+ and CD45RO+. Other key 
markers are CD11c+ and CD4+. This population expresses markers of 
fully mature DCs, being HLA-DR+, CD40+, CD86+, and CD83+; in 
addition, DC-LAMP mRNA could be detected by RT-PCR. Besides the 
absence of B- and T-cell markers, this DC population does not express 
myeloid-associated surface markers, being CD11b+, CD14+, and 
CD64+ and expressing low levels of CD33. Moreover, M-CSFR gene 
expression could not be detected by RT-PCR before or after activation, 
and only low decysin expression was noted. This major thymic DC 
population, localized in the medulla together with the other mature
thymic CD11b\textsuperscript{+} DCs, was fully able to activate naive CD4 T cells in an MLR and was characterized by a typical dendritic morphology when freshly isolated. Overall, this major population exhibited all the characteristics of mature DCs, but lacked those particular myeloid-associated markers found on MoDCs and on tonsillar GCDCs.

In contrast, the minor thymic DC population (less than 35\%) is clearly CD11c\textsuperscript{+}, and also CD11c\textsuperscript{high}, CD33\textsuperscript{high}, and CD45RO\textsuperscript{high}. Like the major population, it is CD4\textsuperscript{+}. To collect sufficient numbers of these cells, we included during sorting not only the mature form of these cells, but also an immature form expressing intermediate to high levels of HLA-DR; this immature form was also CD14\textsuperscript{+}, CD64\textsuperscript{+}, and CD83\textsuperscript{–}. On activation with CD40, the less mature DCs rapidly lost CD14 and CD64 expression and dramatically increased both DC-LAMP and decysin gene expression. M-CSFR gene expression was readily detected by RT-PCR, both before and after CD40 signaling, in contrast to the major
cyte development. Thus, as well as a range of surface-marker differences, the 2 thymic DC populations display different functional abilities.

The origin of these 2 human thymic DC populations and their relationship to the DC populations in the mouse thymus are issues we hoped to clarify. Our results provide some pointers, but some contradictions remain. The minor (and variable) CD11b⁺ thymic DC population appears from its surface markers to be of immediate myeloid origin; such DCs may have entered the thymus directly from the bloodstream. Although the major CD11b⁻ thymic DC population might also enter the thymus from the bloodstream, an endogenous origin seems more likely. Several potential DC precursors exist in the human thymus. An early CD34⁺ precursor population with limited ability to form myeloid cells has been shown to produce DCs in culture; however, when generated in culture, such DCs express CD11b. Another possibility is that the CD11b⁻ DCs represent the mature dendritic form of the thymic plasmacytoid cells. At first, this seemed unlikely since the DCs that develop in culture with IL-3 from these precursors exhibit a unique phenotype, lacking CD11c and retaining CD45RA. However, we found in our system that when GM-CSF is also added to the culture, thymic plasmacytoid cells then acquire some CD11c expression and down-regulate CD45RA expression while differentiating into DCs. In surface phenotype they now resemble the normal thymic CD11b⁻ population. However, we cannot exclude the possibility that this observation results from the selected outgrowth of a subset of plasmacytoid cells, and not from the homogeneous differentiation of these cells.

One argument against a plasmacytoid origin for the thymic CD11b⁻ DCs is that the latter are effective producers of IL-12, whereas neither the plasmacytoid cells themselves nor the DC progeny produced in culture were effective at IL-12 secretion. However, it is possible that our culture system simply is deficient in the factors needed to mature DCs into effective IL-12 producers. Indeed, Cella et al. recently reported that plasmacytoid-derived DCs were able to secrete similar amounts of bioactive IL-12 as blood CD11c⁺. It is important to note that we used a soluble trimeric CD40L in this study, and not CD40L-transfected mouse fibroblasts that were used in other studies and might secrete other growth factors.

The ability of thymic CD11b⁻ DCs to secrete IL-12 does, however, line up well with the high IL-12-producing capacities of mouse CD11b⁻ CD8⁺ DCs, which form the major DC type in the mouse thymus. Despite the absence of the CD8 marker on any human DC subpopulation, CD11b⁻ CD8⁺ mouse DCs and CD11b⁻ human DCs share common features.

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DC NETWORK IN THE HUMAN THYMUS

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Human thymus contains 2 distinct dendritic cell populations

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