

The CMRF58 antibody recognizes a subset of CD123^{hi} dendritic cells in allergen-challenged mucosa

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Abstract: CD123^{hi} CD11c⁻ dendritic cells (CD123^{hi} DC) are a distinct subset of human DC present in bone marrow, blood, lymphoid organs, and peripheral tissues. Pathogen stimulation, cytokine, or CD40 ligation induces CD123^{hi} DC maturation, involving a shift from their innate immune to cognate antigen-presenting functions. In this study, we revealed that blood CD123^{hi} DC in the presence of cytokine (granulocyte macrophage-colony stimulating factor and interleukin-3) undergo progressive, step-wise maturation through an “early” stage, delineated by expression of the antigen detected by the new monoclonal antibody CMRF58 (CD123^{hi}CMRF58⁺CD40⁻CD86⁻CD83⁻) to the “late” stage with costimulatory antigen expression (CD123^{hi}CMRF58⁺CD40⁺CD86⁺CD83^{+/+}). In this early stage, cytokine-maintained CD123^{hi} DC do not display changes in their morphology, no longer produce interferon- α (IFN- α) in response to bacteria, and develop the capacity to induce proliferation and polarization of allogeneic T cells. CD123^{hi}CMRF58⁺ DC, phenotypically similar to in vitro cytokine-maintained CD123^{hi} DC, were not detected in tonsil but are present in allergen-challenged nasal mucosa of allergic individuals. Thus, CD123^{hi} DC in certain tissue environments such as allergen-challenged nasal mucosa share a common CD123^{hi}CMRF58⁺ phenotype with in vitro cytokine-maintained blood CD123^{hi} DC characterized by lack of IFN- α production. *J. Leukoc. Biol.* 77: 000–000; 2005.

Key Words: mAb · nasal allergy

INTRODUCTION

Human dendritic cells (DC) include phenotypically and functionally different types of antigen-presenting cells [1, 2]. Amongst them, the CD123^{hi}CD11c⁻ DC [CD123^{hi} DC or blood DC antigen (BDCA)-4⁺] are defined by plasma cell-like morphology, high-density cell-surface expression of CD123, CD4, and neuropilin-1 (BDCA-4) [3] antigen and by lack of expression of myeloid CD13, CD33, and CD11c antigens [4, 5]. CD123^{hi} DC are present in bone marrow, blood, and around

and within high endothelial venules (HEV) in tonsil, lymph nodes, and nasal mucosa of allergic individuals [4, 6, 7].

CD123^{hi} DC are identical to the originally described, natural interferon (IFN)- α / β -producing cells, which in response to gram-positive [e.g., *Staphylococcus aureus* Cowan I (SAC)] and gram-negative bacteria (e.g., *Escherichia coli*), produce IFN- α / β [8, 9]. Later studies showed that CD123^{hi} DC react to pathogen-associated molecular patterns (PAMP) via their Toll-like receptors (TLRs), namely TLR7 and TLR9 [10, 11]. The expression of TLR9 on CD123^{hi} DC correlates with their responsiveness to bacterial DNA, which requires TLR9 [11, 12]. Unmethylated CG dinucleotides within a particular sequence context of bacterial DNA (CpG motifs) are responsible for different CD123^{hi} DC function, the IFN- α / β production and maturation [13]. In response to CpG, CD123^{hi} DC transiently (during 24 h) produce large amounts of IFN- α / β [8, 10, 14], which acts as an autocrine survival factor and rescues about half of the initial number of CD123^{hi} DC viable after 3 days of culture [14].

In response to PAMP, CD123^{hi} DC concomitantly differentiate, driven by endogenous tumor necrosis factor α (TNF- α), into fully mature DC with costimulatory antigen expression [14]. Also, exogenous TNF- α could induce maturation of CD123^{hi} DC, and antibody to TNF- α diminishes this process [5, 15]. The cytokine interleukin (IL)-3, alone or combined with CD40L, has also been implicated in generation of fully mature CD123^{hi} DC [14, 16]. Mature CD123^{hi} DC deliver costimulatory signals to T cells along with information about the nature of the maturation stimuli, which polarize emerging T cell responses toward T helper cell type 1 (Th1) or Th2 effectors. For example, after stimulation with virus or with CD40L, mature CD123^{hi} DC polarize T cells toward Th1 or Th2 effectors, respectively [6, 14, 17].

However, little is known about CD123^{hi} DC maturation (and function) in vivo. A recent study showed that CD123^{hi} DC can migrate to nasal mucosa during experimentally induced allergic reaction, suggesting the interesting possibility that they could contribute to local inflamed reaction [7]. CD123^{hi} DC, which accumulate in nasal inflammatory lesions exposed to the

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local cytokines IL-3 (produced by activated mast cells [18]) and granulocyte macrophage-colony stimulating factor (GM-CSF; produced by activated T cells, macrophages, or endothelial cells [19, 20]), may undergo maturation similar to that observed in vitro in the presence of these cytokines. Therefore, we compared the phenotype and function of in vitro cytokine-maintained blood CD123^{hi} DC with the CD123^{hi} DC found in nasal mucosa of allergic individuals. We also applied a new monoclonal antibody (mAb) CMRF58 as an additional tool to define the phenotype of these two types of CD123^{hi} DC.

MATERIALS AND METHODS

Tissue

Blood (300–400 ml) was obtained from healthy volunteers. Samples of palatine tonsils were obtained from patients operated on for recurrent tonsillitis. Nasal biopsy specimens were obtained from allergic rhinitis patients challenged before and after daily challenge with grass pollen allergen. A hand-driven spray was used to deliver 50 μ l allergen (Aquagen Timothy, ALK, Horsholm, Denmark) in the nostrils, as described in detail elsewhere [7]. The Mater Adult Hospital Ethics Committee and the National Ethics Committee of Norway approved the study.

Cell lines

Epstein-Barr virus-transformed B cell line, Mann and Burkitt's lymphoma line, and Raji were grown in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (FCS; Life Technologies, Melbourne, Victoria, Australia). The Hodgkin cell lines L428 and HDLM-2 were grown in 20% FCS.

Generation of the mAb CMRF58

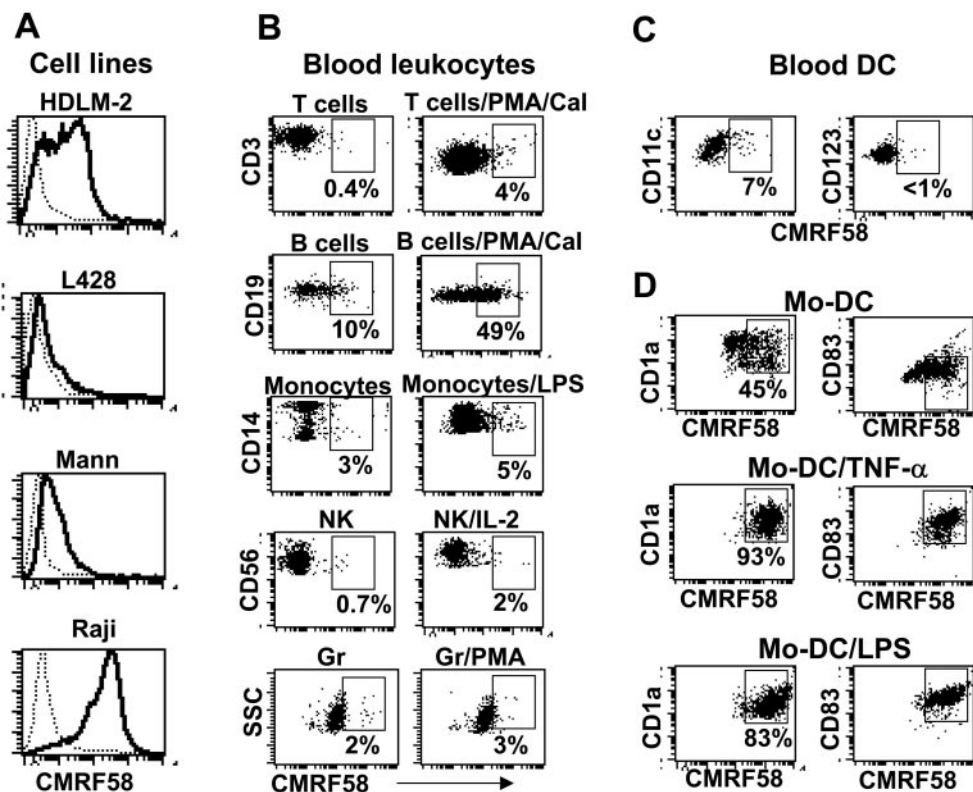
The immunogen used to generate the CMRF58 hybridoma (European Patent No. 00981083.9-2402-AU0001486) was the Raji cell line. The CMRF58

hybridoma reacts with Raji and HDLM-2 in moderate level with Mann but not with L428 cell lines (see Fig. 1A). The antigen recognized by the mAb CMRF58 cannot be immunoprecipitated, and it is not affected by pronase, trypsin, or neuraminidase treatment.

mAb

mAb CMRF58 [immunoglobulin M (IgM)], negative-control CMRF50 (IgM), mAb specific to CD3 (OKT3, IgG_{2a}), CD8 (OKT8, IgG_{2a}), CD45RO (UCHL-1, IgG_{2a}), human leukocyte antigen (HLA)-DR (L243, IgG_{2a}), CD19 (FMC63, IgG₁), CD14 (CMRF31, IgG_{2a}), and CD16 (HuNK2, IgG_{2a}) were produced in-house. The following mAb were purchased: pure mAb specific to CD235a (Glycophorin A, HIR2, IgG_{2b}); phycoerythrin (PE)-conjugated mAb specific to CD34 (My10, IgG₁), CD14 (M0P9, IgG_{2b}), CD3 (SK7, IgG₁), CD19 (4G7, IgG₁), CD56 (B159, IgG₁), CD4 (SK3, IgG₁), CD25 (M-A251, IgG₁), CD69 (FN50, IgG₁), and HLA-DR (L243, IgG_{2a}); allophycocyanin (APC)-conjugated mAb specific to CD14 (M0P9, IgG_{2b}), CD11c (S-HCL-3, IgG_{2b}), and CD33 (P67.6, IgG₁); peridinin chlorophyll protein (PerCP)-conjugated mAb specific to HLA-DR, negative-control IgG₁-fluorescein isothiocyanate (FITC), IgG_{2b}-PE, IgG_{2b}-APC, and IgG₁-PerCP (BDIS, Sydney, New South Wales, Australia); BDCA-4-APC mAb (Miltenyi Biotec, Sydney, New South Wales, Australia); FITC-conjugated mAb specific to CD86 (IT2.2, IgG_{2b}), CD83 (HB15, IgG_{2b}), and CD40 (MAB89, IgG₁) or PE-conjugated mAb specific to CD40 (MAB89, IgG₁) and CD83 (HB15a, IgG_{2b}; Coulter-Immunotech, Sydney, New South Wales, Australia); PE-conjugated mAb specific to CD86 (FUN-1, IgG₁), CD123 (7G3, IgG_{2a}), and negative-control IgG_{2a}-PE (PharMingen, Sydney, New South Wales, Australia); PE-conjugated mAb specific to CD1a (Dako, Cytometron, Sydney, New South Wales, Australia); biotinylated goat anti-mouse IgM and cyanine (Cy3)-conjugated goat anti-mouse IgG_{2a} (Southern Biotechnology Associates, Birmingham, AL); Cy2-conjugated streptavidin (Amersham, Galesbury, UK); FITC- or PE-conjugated sheep anti-mouse Ig (SAM; AMRAD, Melbourne, Victoria, Australia); FITC-conjugated mAb specific to IFN- γ (4S.B3, IgG₁); PE-conjugated mAb specific to IL-4 (8D4-8, IgG₁), negative-control IgG₁-FITC, and IgG₁-PE (PharMingen); mAb specific to IFN- α (C10F5, IgG₁; Biodesign, Saco, ME). PE-conjugated rat anti-mouse Ig κ light-chain (R8-140, PharMingen) was used as secondary antibody for intracellular detection of IFN- α .

Fig. 1. Reactivity of mAb CMRF58 with cell lines, blood leukocytes, and DC. (A) Histograms show cell lines HDLM-2, L428, Mann, and Raji stained with mAb CMRF58 (solid line) or isotype control (dotted line). (B) Dot plots show expression of CMRF58 on freshly isolated or activated CD3⁺ T cells, CD19⁺ B cells, CD14⁺ monocytes, CD56⁺ NK cells, and SSC^{hi} granulocytes. Gr, . (C) Dot plots show expression of CMRF58 on freshly isolated blood CD11c⁺ DC and CD123^{hi} DC, gated on HLA-DR⁺ cells. (D) Dot plots show expression of CMRF58 on immature Mo-DC and mature Mo-DC/TNF- α and Mo-DC/LPS. In all dot plots, the gates delineating positive staining shown were set on the basis of isotype-matched, negative-control staining. Blood leukocytes and DC stained with mAb CMRF58 are indicated (percentages, squares). Data are representative of three to five experiments performed.



Media, cytokine, and reagents

Except where specifically noted, cells were cultured in medium RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% FCS or 10% pooled human AB serum (Red Cross, Brisbane, Queensland, Australia). The following cytokines and reagents were purchased: recombinant human cytokine GM-CSF (Sandoz-Pharma, Sydney, New South Wales, Australia); IL-3 and TNF-α (Gibco, Life Technologies, Melbourne, Victoria, Australia); Ficoll/Hypaque gradient (Pharmacia, Uppsala, Sweden); GolgiPlug (PharMingen); Fix & Perm kit (Caltag Laboratories, Burlingame, CA); magnetic cell sorter MicroBeads (Miltenyi Biotec); fluorescein-activated cell sorter lysing solution (BDIS); phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 (CaI), and lipopolysaccharide (LPS; Sigma, Sydney, New South Wales, Australia); and dihydrorhodamine 123 (Molecular Probes, BioScientific, Sydney, New South Wales, Australia). SAC was purchased from American Type Culture Collection (Manassas, VA, Number 25923).

Cell preparation

Mononuclear cells (MNC) from peripheral blood and tonsil were prepared by Ficoll/Hypaque gradient separation, and MNC negative for Lin markers (CD3, -19, -20, -14, -56, -16, -34; Lin⁻ cells) were obtained by negative selection (AutoMACS, Miltenyi Biotec) and cell-sorting (FACS Vantage). Sorted Lin⁻ cells included ~70% HLA-DR⁺ cells (contained CD123^{hi} DC and CD11c⁺ DC) and 30% HLA-DR⁻ cells. Freshly isolated blood CD123^{hi} DC were sorted as Lin⁻CD4⁺CD11c⁻ cells, and freshly isolated blood CD11c⁺ DC were sorted as Lin⁻CD11c^{hi}CD4^{low/-} cells, as described in detail elsewhere [16]. To avoid contamination with cytokine-maintained CD11c⁺ DC, which up-regulate CD123 antigen and react with mAb CMRF58, cytokine-maintained CD123^{hi} DC were sorted as CD11c⁻CMRF58⁺ cells. Cytokine-maintained CD11c⁺ DC were sorted as CD11c^{hi}CMRF58⁺ cells. All sorted populations were checked for HLA-DR expression, and they always included HLA-DR⁺ cells exclusively, confirming that this sorting strategy excluded cells other than DC (e.g., CD123^{hi}HLA-DR⁻ basophils). CD14⁺ monocytes were purified by AutoMACS positive selection. CD3⁺ T cells were purified by AutoMACS negative selection using a mixture of mAb specific to CD19, CD20, CD14, CD16, CD56, CD34, CD235a, CD11c, HLA-DR antigen, and magnetic bead. Allogeneic, naïve CD4⁺CD45RA⁺ cells were purified by AutoMACS negative selection using a mixture of mAb specific to CD19, CD20, CD14, CD16, CD56, CD34, CD235a, CD11c, HLA-DR, CD8, and CD45RO antigen and magnetic bead. Purity of sorted cells was generally greater than 90%.

Cell culture

Activated cells were obtained: T cells by culture of purified CD3⁺ T cells (2×10⁶ cells/ml) in RPMI/10% FCS supplemented with PMA (10 ng/ml) plus CaI (1 µg/ml) for 72 h; B cells by culture of peripheral blood MNC (PBMC; 2×10⁶ cells/ml) in RPMI/10% FCS supplemented with LPS (1 µg/ml) or with PMA (10 ng/ml) plus CaI (1 µg/ml) for 24 h; monocytes by culture of PBMC (2×10⁶ cells/ml) in RPMI/10% FCS supplemented with LPS (1 µg/ml) for 24 h; natural killer (NK) cells by culture of PBMC (2×10⁶ cells/ml) in RPMI/10% FCS supplemented with IL-2 (100 IU/ml) for 3–7 days; granulocytes by culture of 100 µl whole blood with 500 ng PMA at 37°C for 10 min. Immature monocyte-derived DC (Mo-DC) or mature Mo-DC (Mo-DC/LPS and Mo-DC/TNF-α) were generated by culture of monocytes for 7 days in RPMI 1640/10% FCS with GM-CSF (800 U/ml) and IL-4 (1000 U/ml), with or without the addition of LPS (1 µg/ml) or TNF-α (20 ng/ml) during the last 2 days of culture, as published elsewhere [21, 22]. Sorted blood Lin⁻ cells or Lin⁻CD4⁺CD11c⁻ cells were cultured at a density of 0.5–1 × 10⁶ cells/ml for 12–36 h in RPMI/10% FCS with GM-CSF (200 U/ml) and IL-3 (10 ng/ml), with or without addition of grass allergen (Aquagen Timothy, 10–50 µl/ml culture). In some experiments, sorted blood Lin⁻ cells were cultured at a density of 0.5–1 × 10⁶ cells/ml for 12 h in RPMI supplemented with 2 mM L-glutamine and 10% FCS (without penicillin and streptomycin) in the presence of ~5 × 10⁶ heat-killed SAC. Blood Lin⁻ cells were survived with a viability of >90% when cultured with GM-CSF and IL-3 or with a viability of <60% when cultured in the presence of grass allergen or SAC.

Flow cytometric analysis

Cell lines were processed for single-color labeling with the mAb CMRF58 followed by SAM-FITC. Freshly isolated or activated T cells, B cells, mono-

cytes, and NK cells were processed for two-color labeling with mAb specific to CD3-PE, CD19-PE, CD14-PE, or CD56-PE in combination with CMRF58 followed by SAM-FITC. Freshly isolated or activated granulocytes were analyzed in whole lysed blood as side-scatter (SSC)^{hi} cells. Cell activation was assessed by monitoring changes in the expression of CD25, CD69 (for T cells), HLA-DR antigen (for B cells, monocytes), and rhodamine 123 (for granulocytes). NK activation was assessed in ⁵¹Cr release assay against K562 target [23].

Freshly isolated (blood and tonsil Lin⁻ cells) and cytokine-maintained blood Lin⁻ cells were processed for four-color labeling with mAb specific to HLA-DR-PerCP, CD11c-APC, and CD123-PE in combination with CMRF58 followed by SAM-FITC, CD40-FITC, CD86-FITC, or CD83-FITC (see Fig. 2, A and B). To address phenotype of CD123^{hi}CMRF58⁺ DC, cytokine-maintained blood Lin⁻ cells were processed for four-color labeling with mAb CMRF58 followed by SAM-FITC, HLA-DR-PerCP, and BDCA-4-APC in combination with CD40-PE, CD86-PE, or CD83-PE (Fig. 2C). Mo-DC, Mo-DC/LPS, and Mo-DC/TNF-α were processed for four-color labeling with mAb CMRF58 followed by SAM-FITC, CD14-APC, HLA-DR-PerCP in combination with CD1a-PE or CD83-PE. Sample acquisition was performed on a FACS-Calibur equipped with a 48-nm argon ion laser, and data were analyzed using Cellquest 3.1 software.

Transmission electron microscopy

Sorted, freshly isolated, and cytokine-maintained blood CD123^{hi} DC and CD11c⁺ DC were fixed in 3% glutaraldehyde plus 4% paraformaldehyde, 0.8% calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.4, post-fixed in 1% aqueous OsO₄, stained and blocked with 5% aqueous uranyl acetate, dehydrated in graded ethanol solution, and embedded in Epon/Araldite epoxy resin. Ultrathin sections were observed in a JEOL 1200EXII TEM (University of Queensland, Brisbane, Australia).

IFN-α detection and mixed leukocyte reaction (MLR) assays

Sorted, freshly isolated, and cytokine-maintained blood CD123^{hi} DC and CD11c⁺ DC (5000–10,000 DC; total vol, 0.2 ml) were cocultured with ~5 × 10⁶ heat-killed SAC. After 12 h of culture, cells were collected and analyzed for IFN-α mRNA in parallel with intracellular detection of IFN-α, as described elsewhere [13, 24]. Allogeneic MLR assays were performed by culturing increasing numbers of sorted, freshly isolated, and cytokine-maintained blood CD123^{hi} DC and CD11c⁺ DC with allogeneic, naïve CD4⁺CD45RA⁺ T cells (10⁵ T cells/well) in RPMI/10% AB serum for 5 days. Proliferation was measured by adding 1 µCi [³H]thymidine for the last 16 h of culture. Intracellular production of IFN-γ or IL-4 was assessed in T cells expanded with PMA (10 ng/ml) and CaI (1 µg/ml) for 8 h, and GolgiPlug was added during the last 2 h of culture. Cells were fixed/permeabilized and then labeled with mAb specific to cytokine IFN-γ or IL-4.

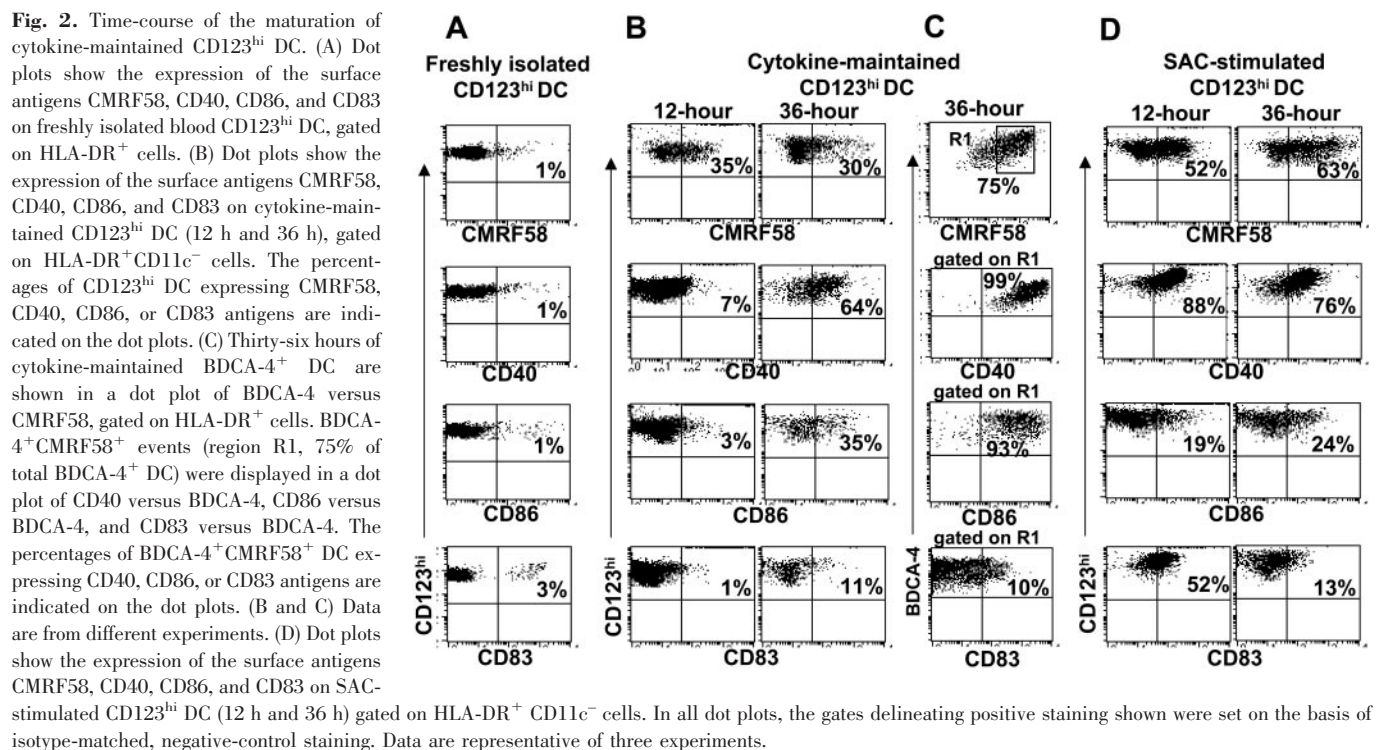
Tissue immunostaining

Acetone-fixed cryosections were stained as described elsewhere [7]. Briefly, mAb to CD123 was mixed with mAb CMRF58 and applied for 1.5 h, followed by biotinylated goat anti-mouse IgM, and finally, by a mixture of Cy3-conjugated goat anti-mouse IgG2a (red) and Cy2-conjugated streptavidin (green).

RESULTS

Reactivity of mAb CMRF58 with blood leukocytes

The mAb CMRF58 did not react with freshly isolated T cells, NK cells, granulocytes, and monocytes (1–3% positive cells, Fig. 1B). Similarly, the mAb CMRF58 did not react with activated T cells, monocytes, NK cells, or granulocytes (2–5% positive cells, Fig. 1B). It bound to a subset of freshly isolated or PMA/CaI-activated B cells (~10% of fresh B cells; 40% of activated B cells, Fig. 1B). The mAb CMRF58 did not react with freshly isolated blood CD123^{hi} DC (<1% of CD123^{hi}



cells, Fig. 1C). It bound to a subset of freshly isolated blood CD11c⁺ DC (<10% of CD11c⁺ cells). The mAb CMRF58 labeled the majority of Mo-DC, almost all mature Mo-DC/TNF- α or Mo-DC/LPS (>45% of Mo-DC, >90% of Mo-DC/TNF- α , >80% Mo-DC/LPS, Fig. 1C).

Reactivity of mAb CMRF58 with cytokine-maintained CD123^{hi} DC: step-wise maturation from the "early" CMRF58⁺CD40⁻CD86⁻CD83⁻ stage to the "late" CMRF58⁺CD40⁺CD86⁺CD83⁺ stage

Initial screening of different types of DC indicated reactivity of mAb CMRF58 with different forms of cytokine-maintained Mo-DC and limited or no reactivity with fresh blood DC subsets. Therefore, we were interested in analyzing the reactivity of mAb CMRF58 with other types of cytokine-maintained DC, and in particular, we delineated its reactivity with cytokine-maintained blood CD123^{hi} DC. Initially, we maintained blood CD123^{hi} DC within sorted blood Lin⁻CD4⁺CD11c⁻ cells in culture with GM-CSF and IL-3, which are known to maintain their survival and induce maturation [14]. Under these culture conditions, sorted blood Lin⁻CD4⁺CD11c⁻ cells, unlike their tonsil counterparts [16], remained negative for myeloid CD33 and CD11c antigen but acquired CMRF58 antigen (data not shown). However, as a result of the low numbers of sorted blood CD123^{hi} DC available, we continued to maintain blood CD123^{hi} DC within sorted blood Lin⁻ cells in culture supported by the cytokines GM-CSF and IL-3 for 12–36 h. The time-course analysis revealed that the 12-h cytokine-maintained CD123^{hi} DC generated from sorted blood Lin⁻ cells acquired CMRF58 antigens (Fig. 2B). Compared with freshly isolated CD123^{hi} DC, there was a slight increase in the number of CD123^{hi}CD40⁺ DC (from 1% to 7%) and in the number of CD123^{hi}CD86⁺ DC (from 1% to 3%), and CD123^{hi}CD83⁺ DC

were largely absent (<1%; Fig. 2, A and B). The induction of CMRF58 antigen on 12 h cytokine-maintained CD123^{hi} DC occurred with high reproducibility, although large, interindividual variations were seen amongst randomly screened, normal donors (30–80% of cytokine-maintained CD123^{hi} DC, n=20). It is worth noticing that expression of the CMRF58 antigen was not restricted to 12 h cytokine-maintained CD123^{hi} DC, as it was also induced on cytokine-maintained CD11c⁺ DC from the same culture. In contrast to the 12-h cytokine-derived CD123^{hi}CMRF58⁺ DC, CD11c⁺CMRF58⁺ DC have a phenotype of fully mature DC with coexpression of CD40, CD86, and CD83 antigen (data not shown).

Compared with freshly isolated blood CD123⁺ DC, 12 h cytokine-maintained CD123^{hi}CMRF58⁺ DC lose the capacity to produce IFN- α in response to heat-killed SAC (data not shown). In contradistinction to freshly isolated blood CD123^{hi} DC, 12 h cytokine-maintained CD123^{hi} DC induced proliferation of allogeneic T cells but still to a lesser extent, compared with 12 h cytokine-maintained CD11c⁺ DC (at 10,000 DC/well; DC/T cell ratio 1:10; mean $\pm$ SD cpm: CD11c⁺ DC, 108,649 $\pm$ 6465; CD123^{hi} DC, 39,155 $\pm$ 4584). The 12-h cytokine-maintained CD123^{hi} DC were capable of polarizing proliferating T cells toward Th1 effector cells, as did the freshly isolated CD11c⁺ DC or 12 h cytokine-maintained CD11c⁺ DC (IFN- γ ⁺ CD4⁺ T cells: 13%, 15%, and 18%, respectively).

After 36 h culture, the proportions of CD123^{hi}CMRF58⁺ DC remained the same, whereas CD123^{hi}CD40⁺ DC, CD123^{hi}CD86⁺ DC, or CD123^{hi}CD83⁺ DC emerged compared with 12 h cytokine-maintained CD123^{hi} DC (Fig. 2B). Further phenotype analysis revealed that virtually all CD123^{hi}CMRF58⁺ DC (defined as BDCA-4⁺CMRF58⁺ DC, region R1, Fig. 2C) coexpressed the CD40 and CD86

antigens, but a minority of them expressed the CD83 antigen (Fig. 2C).

Compared with cytokine-maintained CD123^{hi} DC, SAC-stimulated CD123^{hi} DC underwent more rapid maturation. After 12 h culture, SAC-stimulated CD123^{hi} DC expressing CMRF58, CD40, CD86, and CD83 antigens were detected and remained present at similar percentages after 36 h culture (Fig. 2D). The above results indicate that the cytokines (GM-CSF and IL-3) differ from SAC by inducing progressive, step-wise maturation of CD123^{hi} DC from the early stage, characterized by the CMRF58⁺CD40⁻CD86⁻CD83⁻ phenotype, to the late stage, characterized by the CMRF58⁺CD40⁺CD86⁺CD83⁺ phenotype.

No obvious changes in cell morphology were noted in the 12-h cytokine-maintained CD123^{hi} DC compared with their freshly isolated counterpart. Freshly isolated CD123^{hi} DC and 12 h cytokine-maintained CD123^{hi} DC were 5–6 μ in diameter and possessed irregular nuclei with marginal heterochromatin and a prominent nucleolus (Fig. 3, A and C). Parallel arrays of RER margined toward the plasma membrane were also observed in both. In contrast, numerous short dendritic processes occurred on the plasma membrane of freshly isolated blood CD11c⁺ DC, and these processes were elongated on 12 h cytokine-maintained CD11c⁺ DC (Fig. 3, B and D).

Phenotypic characterization of CD123^{hi} DC in tonsils

To test whether CD123^{hi} DC undergo maturation in vivo similar to that induced by cytokines in vitro, we analyzed the phenotype of CD123^{hi} DC, residing in the tonsil and nasal mucosa of allergic individuals [4, 7, 25]. Immunofluorescence staining of tonsil sections showed that most of the CD123^{hi} DC were located around vessels (Fig. 4A, red cells, arrow), as described previously [25]. All of these cells had the

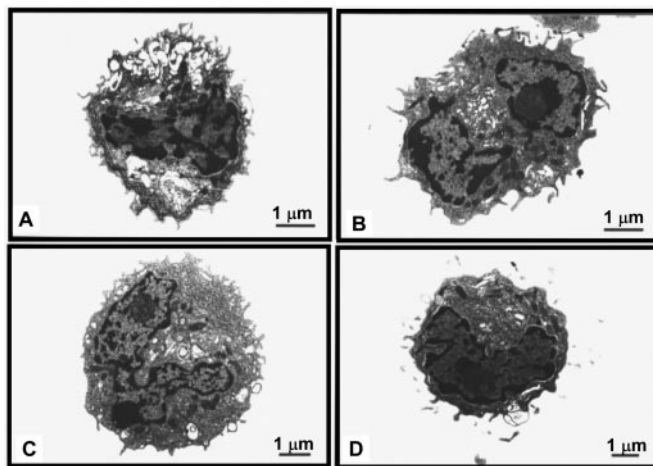


Fig. 3. Transmission electron micrographs of freshly isolated and cytokine-maintained DC. (A) Freshly isolated CD123^{hi} DC (sorted Lin⁻CD4⁺CD11c⁻ cells) and (C) 12 h cytokine-maintained CD123^{hi} DC (sorted CD11c⁻CMRF58⁺ cells) have parallel arrays of rough endoplasmic reticulum (RER). (B) Freshly isolated CD11c⁺ DC (sorted Lin⁻CD11c^{hi}CD4^{low/-} cells) have numerous short dendritic processes, and they are elongated on (D) 12 h cytokine-maintained CD11c⁺ DC (sorted CD11c^{hi}CMRF58⁺ cells; all $\times 8000$ original magnification).

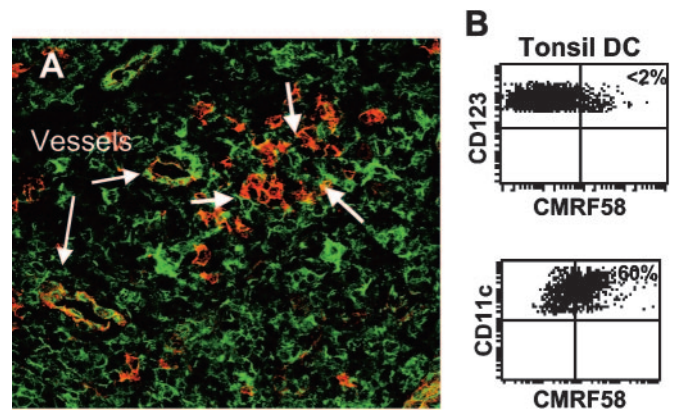


Fig. 4. CD123^{hi}CMRF58⁺ DC are not present in tonsil. (A) Immunofluorescence staining for CD123 (red) and CMRF58 (green) in cryosections from tonsils. CD123^{hi} DC clustered around a HEV do not stain with CMRF58 mAb (red cells, arrow, $\times 400$ original magnification). Weak CD123⁺ vessels are indicated (arrow). (B) Dot plots show expression of CMRF58 on freshly isolated CD123^{hi} DC or CD11c⁺ DC from tonsil, gated on Lin⁻HLA-DR⁺ cells. The percentages of CD123^{hi}CMRF58⁺ DC and CD11c⁺CMRF58⁺ DC are indicated on the dot plots. The gates delineating positive staining shown were set on the basis of isotype-matched, negative-control staining.

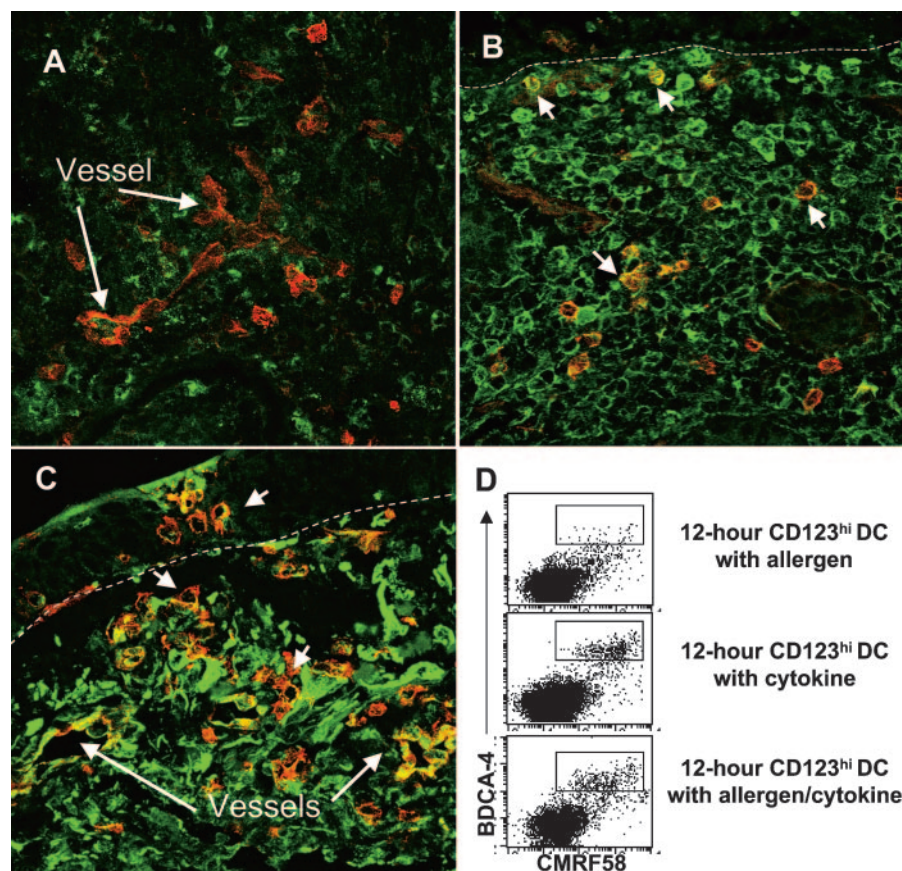
CD123^{hi}CMRF58⁻ phenotype (Fig. 4A, red cells, arrow). Numerous tonsil B cells reacted with mAb CMRF58 (green cells). Also, parallel flow cytometric analysis confirmed that mAb CMRF58 did not react with freshly isolated tonsil CD123^{hi} DC, whereas it did react with subsets of CD11c⁺ DC (<2% of CD123^{hi} DC, 50–60% of CD11c⁺ DC, Fig. 4B). These data suggested that the CD123^{hi}CMRF58⁻ phenotype corresponds to the previously described plasmacytoid monocytes [25] or immature CD123^{hi} DC in tonsil [4].

CD123^{hi}CMRF58⁺ DC were detected in allergen-challenged nasal mucosa

In specimens of nasal mucosa from the same allergic individuals used in our previous study [7], we found that CD123^{hi} DC were mainly located in lamina propria (Fig. 5A, red cells). Immunofluorescence staining showed that CD123^{hi} DC, in nasal mucosa from allergic individuals, exhibited CD123^{hi}CMRF58⁻ phenotype (Fig. 5A, red cells). However, on day 2 after allergen challenge, CD123^{hi}CMRF58⁺ DC were detected in the specimens of challenged nasal mucosa from different allergic individuals (Fig. 5, B and C, yellow cells, arrow). They are presented as 10–20% of the total CD123^{hi} DC identified in tissue sections, obtained from five different allergic individuals. CD123^{hi}CMRF58⁺ DC were located in the lamina propria, and the epithelium always contained only a few cells of this phenotype (Fig. 5, B and C). Mononuclear cells and eosinophils accumulated in allergen-challenged nasal mucosa reacted with the mAb CMRF58 (Fig. 5, B and C, green cells).

The observed presence of CD123^{hi}CMRF58⁺ DC in allergen-challenged nasal mucosa could be a result of the capacity of allergen to mediate induction of cells with this phenotype. We attempted to examine this possibility by culturing blood CD123^{hi} DC within sorted blood Lin⁻ cells with the allergen, which was used for patient challenge, with cytokines (GM-CSF

Fig. 5. CD123^{hi}CMRF58⁺ DC are present in allergen-challenged nasal mucosa. Paired immunofluorescence staining for CD123 (red) and CMRF58 (green) in cryosections from (A) allergen-unchallenged and (B, C) allergen-challenged nasal mucosa of two different allergic individuals. CD123^{hi}CMRF58⁺ DC were present in allergen-unchallenged nasal mucosa (A, red cells). CD123⁺ vessels are indicated (A, arrow). CD123^{hi}CMRF58⁺ DC are present in allergen-challenged nasal mucosa and located mainly in the lamina propria (B, C, yellow cells, arrow). The basement membrane is indicated by a dotted line (all $\times 400$ original magnification). Data are representative of samples of nasal mucosa of five allergic individuals. (D) In a dot plot of CMRF58 versus BDCA-4 gated on HLA-DR⁺ cells, squares were drawn to include all BDCA-4⁺ DC recovered after 12 h culture of blood Lin⁻ cells in the presence of grass pollen allergen (Aquagen Timothy, 50 μ l/ml), cytokines (GM-CSF and IL-3), or allergen/cytokines. Data are representative of three experiments.



and IL-3), or with cytokines/allergen. Unfortunately, culture conditions with allergen, unlike culture with cytokines or cytokine/allergen, did not support survival of blood CD123^{hi} DC (BDCA-4⁺ cells, square, Fig. 5D). It appeared that the addition of allergen to the culture with the cytokines did not affected generation of CD123^{hi}CMRF58⁺ DC (Fig. 5D). Thus, allergen challenge was followed by the appearance of CD123^{hi}CMRF58⁺ DC in nasal mucosa in allergic individuals; however, as a result of the limitation of in vitro assay, we were unable to assess the contribution of allergen in induction of cells with this phenotype.

DISCUSSION

This study shows for the first time that CD123^{hi} DC, in the presence of cytokines (GM-CSF and IL-3), undergo progressive maturation through an early stage characterized by CMRF58 antigen expression (CMRF58⁺CD40⁻CD86⁻CD83⁻) to a late stage characterized by costimulatory antigen expression (CMRF58⁺CD40⁺CD86⁺CD83⁺). The early stage in the cytokine-maintained CD123^{hi} DC reflects a major change in their innate functions, when they no longer produce IFN- α in response to bacterial stimuli and acquire the capacity to induce proliferation and polarization of allogeneic T cells. In addition, we identified CD123^{hi}CMRF58⁺ DC in allergen-challenged nasal mucosa of allergic individuals but not in tonsil, suggesting that CD123^{hi} DC in certain selective in vivo environments undergo the phenotypic and functional stages similar to those observed in vitro using cytokine-maintained CD123^{hi} DC.

Virus and CD40L have been reported to induce CD123^{hi} DC maturation [10, 11, 14, 16]. The cytokine IL-3, alone or combined with CD40L and TNF- α , has also been implicated in CD123^{hi} DC maturation [14–16]. Only mature CD123^{hi} DC characterized by dendritic morphology and costimulatory antigen expression have been described as having the capacity to induce Th1 or Th2 immune responses [6, 10, 14]. To date, no early stage of CD123^{hi} DC maturation has been shown with the various maturation mediators. Despite this, it is conceivable that CD123^{hi} DC maturation (at least in the presence of cytokine), like myeloid DC maturation (in the presence of GM-CSF and IL-4) [1, 21, 26, 27], would be a progressive, multi-step process. The production of the new mAb CMRF58 and its consistent reactivity with different forms of cytokine-maintained Mo-DC encouraged us to define the maturation of cytokine-maintained blood CD123^{hi} DC.

Here, we show that blood CD123^{hi} DC within 12 h culture with cytokine (IL-3 and GM-CSF) up-regulate the CMRF58 antigen as an early event in their maturation process. We found that GM-CSF is as effective as IL-3 in inducing expression of the CMRF58 antigen (data not shown), indicating the importance of GM-CSF in addition to IL-3 (and TNF- α [14, 16]) in CD123^{hi} DC maturation.

In addition, we show that the cytokines (unlike bacteria stimuli SAC) induce progressive maturation of CD123^{hi} DC through a clearly defined early stage to late stage typical of mature DC. The early stage is characterized by the expression of the CMRF58 antigen and by the slight initiation of expression of the CD40 and CD86 antigens, compared with freshly isolated blood CD123^{hi} DC. This early stage precedes obvious

changes in CD123^{hi} DC morphology. It is important to note that this progressive CD123^{hi} DC maturation is observed amongst Lin⁻ cells and may be a result of the combined action of the cytokines and other cell components present in the blood Lin⁻ fraction. Our recent data showed that certain cell component(s) in PBMC influence survival of CD123^{hi} DC during culture [28]. Occasionally, CD123^{hi}CD83⁺ events were observed among freshly isolated Lin⁻ cells (<3%, Fig. 2A), which likely represent conjugates between CD123^{hi} DC and other cells (e.g., CD3⁺ T or CD11c⁺ cells), as we previously reported [29]. To this point, the low numbers of sorted blood CD123^{hi} DC available and their poor survival in vitro prevented us from dissecting the contribution of cytokines and (or) cell component(s) in their maturation.

CpG preferentially stimulates different functions of CD123^{hi} DC, IFN- α / β production, and maturation, depending on their characteristic sequence [13]. For example, we showed that CpG (prototype 2216) strongly stimulates blood CD123^{hi} DC to produce IFN- α but fails to induce their maturation (including CMRF58 antigen expression) during 12 h of culture (unpublished data). However, we showed that in the presence of cytokines, maturation of CD123^{hi} DC (expression of the CMRF58 antigen) coincided with the down-regulation of IFN- α / β production. This indicates that in the relevant cytokine environment (e.g., GM-CSF and IL-3), maturation of CD123^{hi} DC and production of IFN- α (e.g., in response to SAC) could be regulated by a common mechanism. Whether cytokine-maintained CD123^{hi} DC retain the flexibility to respond to CpG or to induce Th1 or Th2 immune responses will have to be addressed.

We seek whether maturation of CD123^{hi} DC, demonstrated in this in vitro maturation model, correlates with a similar in vivo CD123^{hi} DC maturation process. The CD123^{hi}CMRF58⁺ DC found in allergen-challenged nasal mucosa of allergic rhinitis patients may represent a physiological counterpart to the in vitro cytokine-maintained CD123^{hi} DC. We previously reported, using biopsy specimens from the same allergic rhinitis patients, that the CD123⁺ cells recruited in nasal mucosa express CD4 and HLA-DR antigens and thus resemble the phenotype of blood CD123^{hi} DC [4, 7, 16]. They do not express CD11c, CD14, CD20, and Fc receptor for IgE-I, thus confirming that they were distinct from other CD11c⁺ DC, monocytes, B cells, basophils, or mast cells [7].

It is, for example, possible to postulate that blood CD123^{hi} DC recruited to sites of allergen challenge mature from CD123^{hi}CMRF58⁻ into CD123^{hi}CMRF58⁺ phenotype as a result of exposure to IL-3, produced by activated mast cells [18], and to GM-CSF, produced by activated T cells, macrophages, or endothelial cells [19, 20]. Allergen might also contribute to generation of CD123^{hi}CMRF58⁺ DC, but unfortunately, the poor survival of CD123^{hi} DC in vitro in the presence of allergen prevented us from addressing this possibility. Also, the addition of allergen to the culture with GM-CSF and IL-3 appeared not to affect generation of CD123^{hi}CMRF58⁺ DC. Whether during experimentally induced allergic reaction, CD123^{hi}CMRF58⁺ DC occurred in the blood or whether CD123^{hi} DC differentiate into CD123^{hi}CMRF58⁺ DC after they arrive in mucosa will have to be addressed in future studies. The accumulation of

CD123^{hi}CMRF58⁺ DC in allergen-challenged nasal mucosa provides a further phenotypic marker, which reinforces the previous description of CD123^{hi} DC lacking the capacity to produce IFN- α in this tissue [7].

The absence of CD123^{hi}CMRF58⁺ DC in tonsil suggests that blood CD123^{hi} DC, which migrate via HEV to lymphoid tissues, retain the CD123^{hi}CMRF58⁻ phenotype, and it is interesting that this correlates with their known capacity to produce IFN- α [6]. Unlike tonsil CD123^{hi} DC, the majority of tonsil CD11c⁺ DC exhibits the CD11c⁺CMRF58⁺ phenotype. To this point, the mAb CMRF58 provides a useful tool to extend our understanding of the complexity of tonsil CD11c⁺ DC [30].

Our study emphasizes the progressive maturation of cytokine-maintained CD123^{hi} DC in the absence of overt pathogen. In particular, we documented the presence of CD123^{hi}CMRF58⁺ DC in allergen-challenged nasal mucosa of allergic individuals, suggesting their contribution to nasal allergy.

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