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Two Phenotypically Distinct Subsets of Spleen Dendritic Cells in Rats Exhibit Different Cytokine Production and T Cell Stimulatory Activity

Cécile Voisine, François-Xavier Hubert, Benjamin Trinité, Michèle Heslan, and Régis Josien

We recently reported that splenic dendritic cells (DC) in rats can be separated into CD4+ and CD4− subsets and that the CD4− subset exhibited a natural cytotoxic activity in vitro against tumor cells. Moreover, a recent report suggests that CD4− DC could have tolerogenic properties in vivo. In this study, we have analyzed the phenotype and in vitro T cell stimulatory activity of freshly isolated splenic DC subsets. Unlike the CD4− subset, CD4+ splenic DC expressed CD5, CD90, and signal regulatory protein α molecules. Both fresh CD4+ and CD4+ DC displayed an immature phenotype, although CD4+ cells constitutively expressed moderate levels of CD80. The half-life of the CD4+, but not CD4− DC in vitro was extremely short but cells could be rescued from death by CD40 ligand, IL-3, or GM-CSF. The CD4+ DC produced large amounts of the proinflammatory cytokines IL-12 and TNF-α and induced Th1 responses in allogeneic CD4+ T cells, whereas the CD4+ DC produced low amounts of IL-12 and no TNF-α, but induced Th1 and Th2 responses. As compared with the CD4− DC that strongly stimulated the proliferation of purified CD8+ T cells, the CD4+ DC exhibited a poor CD8+ T cell stimulatory capacity that was substantially increased by CD40 stimulation. Therefore, as previously shown in mice and humans, we have identified the existence of a high IL-12-producing DC subset in the rat that induces Th1 responses. The fact that both the CD4+ and CD4− DC subsets produced low amounts of IFN-α upon viral infection suggests that they are not related to plasmacytoid DC. The Journal of Immunology, 2002, 169: 2284–2291.

Dendritic cells (DC) are a heterogeneous population of professional APC (1). Human blood contains a subset of CD11c+ DC, also called DC1, thought to be of myeloid origin and a subset of CD11c− DC, also called DC2, thought to be of lymphoid origin (2). Beyond their distinct cell surface phenotype, these DC subsets exhibit different functions. DC1 can produce large amounts of IL-12 upon exposure to specific pathogens or activated T cells and drive the differentiation of Th1 cells (2). DC2 produce large amounts of type I IFNs upon exposure to virus (3) and drive the differentiation of Th2 cells (2). However, the Th cell differentiation capacity of both DC1 (4) and DC2 (2, 5) seems to depend on the nature of the stimulus. Murine DC subsets have also been described in lymphoid organs (1). CD8− CD4− DC produce IL-12 and induce Th1 cell differentiation, whereas CD8+ CD4+ DC do not produce IL-12 and promote Th2 differentiation (6, 7). The precise origin of these subsets in vivo is not clear as both common myeloid and lymphoid progenitors can give rise to CD8− and CD8+ DC in vivo (8, 9).

Different subsets of DC have also been described in rats. MacPherson and colleagues (10) first described that a CD4+ and a CD4− subset of DC can be purified fromafferent lymph and more recently, we described similar subsets in the spleen (11). The fact that CD4+ DC isolated from both the afferent lymph and spleen coexpress signal regulatory protein (SIRP)α (CD172a), which was recently found to be the target of the OX41 mAb (12), and CD90 suggest that they represent the same subset of DC. Interestingly, both subsets were also found in lymph nodes but in different proportions (11). Previous studies have shown that CD4+ DC were better stimulators of CD4+ and CD8+ T cells than the CD4− cells in allogeneic MLR or in soluble Ag presentation assays (10). However, it is not known whether this difference is related to different costimulatory molecule expression or cytokine production by these DC subsets. More recently, the CD4− subset of rat DC has been shown to exhibit two new and unusual functions. Huang et al. (13) have shown that CD4− SIRP− DC constitutively migrated through afferent lymph and, more importantly, transported phagocytosed fragments of apoptotic gut epithelial cells to the T cell area of mesenteric lymph nodes. It has therefore been suggested that this pathway of self-Ag transport could be involved in the maintenance of self-tolerance (14). In addition, we found that CD4− SIRP− DC in spleen (11) but also in lymph nodes (B. Trinité and R. Josien, unpublished observations) exhibited a potent cytotoxic activity in vitro against selected tumor cell lines such as YAC-1 and Jurkat cells. Several reports have now confirmed that myeloid human DC also exhibited such a cytolytic function (15–17). Whether this function plays a role in vivo in unknown; however, so far we have been unable to detect any cytotoxic activity of DC against normal cells in general or in particular against T cells.

Several arguments therefore suggest that the CD4− subset of DC in rats might be involved in T cell tolerance. The aim of this study was to analyze in detail the phenotype of splenic DC subsets and to assess their proinflammatory cytokine production and their
T cell stimulatory activity in terms of Th and CD8+ T cell differentiation. We found that both CD4+ and CD8+ splenic subsets exhibited an immature phenotype in spleen but induced a strong proliferative response in resting CD4+ allogeneic T cells. Freshly isolated CD4+ DC were the main producers of IL-12p40 and drove potent Th1 cell differentiation, whereas CD4+ DC induced non-polarized Th cell differentiation. In contrast, as compared with CD4+ DC, the CD4− subset was a poor stimulator of allogeneic CD8+ T cells.

Materials and Methods

Animals

Sprague Dawley and Lewis rats were obtained from the Centre d’Elevage Janvier (Le Genest Saint-Isle, France) and were used when 6–10 wk old.

Reagents

The CD40 ligand (CD40L)-human CD8 fusion molecule (supernatant of transfected Si9 insect cells) was kindly provided by Dr. Y. Choi (Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA). Poly(I:C) and LPS were obtained from Sigma-Aldrich, (St. Louis, MO). Recombinant rat IFN-α was obtained from R&D Systems (Minneapolis, MN). Recombinant rat IFN-γ and TNF-α were obtained from Serotec (Oxford, U.K.), recombinant rat IL-3β was from PeproTech (Rocky Hill, NJ), and Staphylococcus aureus Cowan I (SAC) strain Pansorbin cells were purchased from Calbiochem (San Diego, CA). GM-CSF (supernatant of COS cells transfected with murine GM-CSF) was used at a dilution of 1/1000.

Monocalon Abs

The following mouse anti-rat mAbs obtained from the European Collection of Cell Culture (Salisbury, U.K.) were used in cytofluorometric studies and cell sorting after coupling to FITC, biotin, or PE (BioAtlantic, Nantes, France; Oxoid Biosciences, Mountain View, CA). The following mAbs were obtained from Sigma-Aldrich, (St. Louis, MO). Recombinant rat IFN-α was obtained from R&D Systems (Minneapolis, MN). Recombinant rat IFN-γ and TNF-α were obtained from Serotec (Oxford, U.K.), recombinant rat IL-3β was from PeproTech (Rocky Hill, NJ), and Staphylococcus aureus Cowan I (SAC) strain Pansorbin cells were purchased from Calbiochem (San Diego, CA). GM-CSF (supernatant of COS cells transfected with murine GM-CSF) was used at a dilution of 1/1000.

Cell preparation

DC. Splenocytes were mixed and digested in 2 mg/ml collagenase D (Roche Diagnostics, Meylan, France) in RPMI 1640/1% FCS for 30 min at 37°C. EDTA at 10 mM was added during the last 5 min and the cell suspension was then pipetted up and down several times and filtered. Cells were washed once in PBS/2 mM EDTA/1% FCS and low-density cells were obtained after centrifugation on a 14.5% Nycodenz (Nycomed, Oslo, Norway) gradient as previously described (18). Cells were then washed once and incubated with a saturating concentration of biotinylated OX62 mAb at 4°C for 20 min. After two washes, cells were mixed with streptavidin-conjugated MACS microbeads following the manufacturer’s instructions (Miltenyi Biotec). Positive selection was performed on MiniMACS type-positive selection columns (Miltenyi Biotec).

T cells. CD4+ and CD8+ T cells were prepared from lymph node cells by negative selection of class II+, Ig+, NKR-P1A+, and CD8+ or CD4+ cells, respectively, with specific mAbs followed by antimonouse IgG-coated magnetic beads (Dynal, Oslo, Norway). Purity was routinely ≥98% and ≥90% for CD4+ and CD8+ T cells, respectively.

Flow cytometry and cell sorting

For cyttofluorometric analyses, 5 × 106 cells were incubated with PE-conjugated OX6 or W3/25 mAb along with another FITC-conjugated mAb for 20 min at 4°C. Cells were washed twice and analyzed on a FACS Calibur (BD Biosciences, Mountain View, CA). For cell sorting of CD4+ and CD8+ subsets of splenic DC, OX62+ cells were incubated with OX6-PE and W3/25-FITC-conjugated mAbs and sorted using a FACSVantage (BD Biosciences).

May-Grünwald-Giemsa coloration

Sorted DC (2 × 105) were cytospun onto a glass slide (800 rpm, 4 min) and air dried for 1–2 h. Cells were then stained using a classic Giemsa coloration.

Mixed leucocyte reaction

Increasing numbers of allogeneic DC were cultured with 5 × 106 purified CD4+ or CD8+ T cells in round-bottom 96-well plates in a final volume of 200 μl of IMDM (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 1 mM HEPES, 5 × 10−5 M 2-ME, and 1% normal rat serum for 4 days at 37°C in 5% CO2. After 4 h of incubation, nonadherent cells were removed by gentle shaking until analysis at ~70°C. Stimuli were used at the following concentrations: CD40L, 1/300 dilution of the supernatant; LPS, 0.5 μg/ml; poly(I:C), 50 μg/ml; SAC, 20 μg/ml; IFN-γ, 20 U/ml; and inactivated influenza virus (kindly provided by F. Brière, Dardilly, France).

Cytokine production assays

Stimulation of isolated DC for cytokine production. Sorted splenic rat DC (1.25 × 105 cells) were cultured in 1 ml RPMI 1640 medium (Sigma-Aldrich), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 5 × 10−5 M 2-ME, and 1% rat serum in well plates. After 24 h, supernatants were collected and stored until analysis at ~70°C. Stimuli were used at the following concentrations: CD40L, 1/300 dilution of the supernatant; LPS, 0.5 μg/ml; poly(I:C), 50 μg/ml; SAC, 20 μg/ml; IFN-γ, 20 U/ml; and inactivated influenza virus (kindly provided by F. Brière, Dardilly, France).

T cell cytokine production. A total of 2 × 104 allogeneic sorted DC were cultured with 1 × 105 purified CD4+ or CD8+ T cells in a final volume of 200 μl of IMDM with 1% rat serum. After 4 days of stimulation, the supernatants were collected and stored for cytokine assays. Cells were then washed and viable T cells were immediately restimulated in an anti-CD3 (5 μg/ml)-coated plate in the presence of anti-CD28 mAb (2.5 μg/ml) at a concentration of 2 × 105 cells/ml. Twenty-four hours after restimulation, supernatants were collected and frozen until further use.

ELISA test. The amount of IL-2, IFN-γ, IL-10, and TNF-α in the supernatant was measured using an ELISA kit (OptiELA set; BD PharMingen) according to the manufacturer’s instructions. Rat IL-13 and IL-12p40 were detected using a BioSource International (Fleurus, Belgium) ELISA kit according to the manufacturer’s instructions. IL-4 measurement was performed using a two-site sandwich ELISA. Anti-IL-4 hybridoma OX81 was obtained from the European Collection of Cell Culture, biotinylated anti-IL-4 mAb (clone B11-3) and recombinant rat IL-4 were purchased from BD Pharmingen. IFN-α was detected using an ELISA kit specific for both mouse and rat IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ).

RT-PCR

Total RNA was extracted from 5 × 106 resting or stimulated FACS-sorted DC using Trizol (Life Technologies, Paisley, U.K.) and reverse transcribed into cDNA as described previously (19).

Nonquantitative PCR. PCR primer sequences used were: rCD4 forward, CTTCCTCCAGGGACAGCCTA; rCD4 reverse, TGGCTCTTCATCTCACA CTCCTC, resulting in a 218-bp PCR product; rCSF forward, GTGCGTCTGCTACTACAG, rCSF reverse, GGCTGTCCTGTA CTTAC, resulting in a 120-bp PCR product; rSIRPα forward, GGCA GCCAAGACAGAGGAT; rSIRPα reverse, GCAGCCATCG CAGAGACT, resulting in a 273-bp PCR product. The hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was also amplified as a control. PCR amplification consisted of an initial denaturation step at 94°C for 1 min, followed by 27 cycles at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s in each cycle. PCR products were electrophoresed on an ethidium bromide-stained 1% agarose gel.

Quantitative RT-PCR. Real-time quantitative PCR was performed using an Applied Biosystems GenAmp 7700 Sequence Detection System with the SYBR Green PCR Core Reagent (Applied Biosystems, Foster City, CA). Following cDNA synthesis, the following oligonucleotides were used: rIL-2 forward, GACAAACGTAAGCTG CAG; rIL-2 reverse, GGAGTCCTGCA AAGT; rIL-12p35 forward, TGATGATGACCCTGTGCCTT; rIL-12p35 reverse, TGATGATGACCCTGTGCCTT; rIL-12p40 forward, TGATGATGACCCTGTGCCTT; rIL-12p40 reverse, TGATGATGACCCTGTGCCTT; and rIL-12p70 forward, TGATGATGACCCTGTGCCTT. The PCR products were then electrophoresed on an ethidium bromide-stained 1% agarose gel.
55°C to allow the uracil-N-glycosylase to eliminate putative PCR contaminants, followed by 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase, and then 40 cycles each consisting of 15 s at 95°C and 1 min at 60°C. The real-time PCR data were plotted as the ΔΔCt fluorescent signal vs the cycle number. The Applied Biosystems 7700 sequence detection software calculates the ΔΔCt using the formula: ΔΔCt = (CtTarget - Ctβ-actin) - (CtTarget - Ctβ-actin)baseline, where Rn is the fluorescent signal of the product at any given time and Rnbaseline is the mean fluorescent signal during cycles 3–3 and referred to as the baseline. The Ct value is defined as the cycle number at which the ΔΔCt crosses a threshold. The threshold is set above the background fluorescence to intersect the exponential portion of the amplification curve of a positive reaction. The Ct is inversely proportional to the log amount of template in the PCR. HPRT was used as an endogenous control gene to normalize for RNA amounts. The transcript accumulation index (TAI) is the cation curve of Rnfl/HTPRfl/HTPRbaseline.

Statistical analyses

Statistical analyses were performed using Student’s t test.

Results

Purification procedure

DC were isolated from rat spleens by positive selection using the OX62 mAb that was previously shown to recognize the rat CD2 integrin chain or CD103 (20, 21). Of note, CD103 was recently found that CD4+ T cells were negative for TCR and are therefore DC (21). This positive selection method avoided the classical step of overnight culture that allows DC to mature in vitro and therefore dramatically modify their cell surface phenotype and function. The yield was 3–5 × 10⁶ OX62-positive cells per spleen with a purity of 96%, as determined by the slope of the curve Ct = f(log(target DNA)).

Phenotype of freshly isolated splenic DC subsets

As we have previously shown, two subsets of splenic DC can be identified in rats based on their expression of the CD4 molecule. (11). Similar subsets have been previously described by others in afferent lymph (10). The phenotype of these subsets is described in Table I. We found that, unlike in mice, no splenic DC in rats express CD8α. The CD4+ subset of splenic DC coexpressed CD5 and SIRPα and also exhibited high levels of CD90 (Thy1.1), whereas CD4- cells were negative for CD5 and SIRPα but expressed low levels of CD90. CD4+ DC expressed slightly higher levels of NKR-P1A (CD161A), CD11c, class I, and lower levels of the OX62 integrin than CD4- DC, whereas both subsets expressed similar levels of CD11a and CD11b. Both subsets were negative for the macrophage markers ED2 and sialoadhesin (ED3). We found that CD4- DC expressed high levels of the OX2 (CD200) molecule whereas CD4+ stained weakly for this Ag. This is, to our knowledge, the first description of a potentially useful marker for rat CD4+ DC. Finally, we did not observe significant changes in the expression of CD4, CD5, and SIRP on splenic DC subsets after 3 days of in vitro culture (data not shown).

Table I. Phenotype of freshly isolated splenic DC subsets

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<tr>
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<th>CD4⁺⁺</th>
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* Freshly isolated OX62⁺⁺ splenic DC were double stained with FITC- or PE-conjugated W3/25 (CD4) and FITC- or PE-conjugated mAbs listed in the left column. – , –/+, ++, ++++. The relative staining intensity on gated CD4⁺⁺⁺ or CD4⁺⁺ DC as compared with the isotype control (arbitrary units).
FIGURE 2. Freshly isolated splenic DC subsets exhibit an immature phenotype and mature spontaneously in vitro. Freshly isolated and overnight cultured OX62+ cells were labeled with directly conjugated MHC class II-, B7-1-, B7-2-, CD40-, Thy-1, or CD25- and CD4-specific mAbs and analyzed by FACS. Histograms represent the relative expression of indicated Ags on gated CD4+ (upper) and CD4− (lower) splenic DC.

very low levels of B7-1 were expressed on CD4+ and CD4− DC, respectively (Fig. 2). As the expression of those costimulatory molecules as well as class II strongly increased upon overnight culture (Fig. 2), the phenotype of freshly isolated CD4+ cells can be considered as immature, whereas CD4+ DC have an intermediate maturity stage. However, following the spontaneous maturation that occurred upon overnight in vitro culture of DC, CD4+ DC expressed significantly higher levels of CD80 and CD86 than CD4− DC (Fig. 2). When maturation stimuli such as soluble CD40L (Fig. 2) or GM-CSF (data not shown) were added to the culture, this resulted in increased CD80 expression on CD4− DC but not CD4+ DC.

Morphology of freshly isolated splenic DC subsets

FACS analysis revealed that CD4+ DC were larger than CD4− cells (data not shown). This was confirmed when cytospun, freshly isolated splenic DC subsets were examined after Giemsa staining (Fig. 3). CD4+ DC exhibited a relative homogenous myeloid-related morphology with a large and irregularly shaped nucleus and a large cytoplasm containing inclusions (Fig. 3A). Morphological features of CD4+ DC were much less homogenous and very different from the CD4− subset (Fig. 3B). Most CD4+ DC had a small and regular nucleus and a small cytoplasm that did not contain large inclusions. Fine and long dendrites could be observed on

some of the CD4+ DC. A small percentage of CD4+ splenic DC exhibited a morphology similar to CD4− cells and, finally, a few cells had an intermediate morphology.

CD4, CD5, and SIRPα mRNA are expressed in CD4+ splenic DC

As previously shown in mice, several surface markers that are found on the surface of DC are actually not expressed by DC but rather acquired from surrounding cells (23, 24). We therefore analyzed the expression of CD4, CD5, and SIRPα mRNA in CD4+ splenic DC by RT-PCR. As shown in Fig. 4, mRNA for CD4, CD5, and SIRP was expressed in CD4+ DC. As expected, CD4 and SIRPα mRNA were not detected in CD4− DC; however, a weak signal for CD5 mRNA was observed.

CD4− but not CD4+ splenic DC have a short half-life in vitro

It is now well established that the duration of DC-T cell interaction and therefore of DC half-life is an important parameter of the T cell activation process (25). Therefore, specific functions of DC subsets might be related to their survival. We thus examined the in vitro survival of CD4+ and CD4− splenic DC subsets. After 3 days of culture, >80% of CD4− DC were dead, suggesting a very short spontaneous half-life (Fig. 5). In three independent experiments, a substantial percentage of CD4+ DC could be rescued from programmed cell death by CD40 cross-linking, IL-3 or GM-CSF ($p < 0.05$ as compared with untreated cells) but not by LPS, poly(I:C), IFN-α, or TNF-α. In contrast, roughly two-thirds of CD4+ splenic DC were still alive after 3 days of culture ($p < 0.01$ as compared with CD4− DC) and we could not observe any consistent effect of soluble CD40L, LPS, poly(I:C), IL-3, GM-CSF, IFN-α, or TNF-α on the survival of this DC subset (Fig. 5).

Differential production of IL-12 p40 and TNF-α by splenic DC subsets

Previous reports have shown that human and mouse DC subsets differentially influence the nature of the Th cell response (1). This was in part related to the capacity of DC subsets to produce IL-12 (26). We therefore examined the production of the IL-12 p40 subunit by rat splenic DC subsets. High levels of IL-12 p40 were detected in the supernatant of CD40L (range, 54–270 pg/ml in four independent experiments) and to a lesser extent LPS-stimulated

FIGURE 3. Morphology of splenic DC subsets. Freshly isolated OX62+ cells were stained with W3/25-FITC and OX6-PE. CD4+ class II+ and CD4− class II− cells were then sorted immediately by FACS. Cells were then cytospun onto a glass slide, air dried, and stained using Giemsa coloration (original magnification, ×400).

FIGURE 4. CD4, CD5, and SIRPα mRNA expression in CD4+ and CD4− splenic DC subsets. PCR for CD4, CD5, and SIRPα was performed on cDNA from FACS-sorted CD4+ and CD4− splenic DC as described in Materials and Methods. Similar results were obtained in three independent experiments.
(range, 44–167 pg/ml) CD4− DC, whereas poly(I:C) did not induce IL-12p40 production (Fig. 6). In contrast, CD4+ DC did not produce detectable IL-12p40 in response to CD40 cross-linking, LPS, or poly(I:C) stimulation in the same experiments. However, stimulation of DC with SAC plus IFN-γ induced variable but statistically comparable amounts of IL-12p40 production in both CD4+ and CD4− spleen DC (CD4+ DC, range, 46–235 pg/ml; CD4− DC, range, 18–482 pg/ml, \( p = 0.4 \)). The expression of IL-12p40 and p35 chain mRNA was also analyzed in fresh and stimulated DC subsets by real-time quantitative RT-PCR (Table II). Both chains were strongly up-regulated in SAC plus IFN-γ- and to a lesser extent LPS- or CD40L-stimulated CD4− DC, indicating that CD4− DC have the capacity to produce bioactive IL-12p70. In CD4+ DC, IL-12p40 mRNA was up-regulated by SAC plus IFN-γ but not reproducibly by CD40L, a finding consistent with IL-12p40 ELISA. However, unlike in CD4− DC, IL-12p35 mRNA was not significantly up-regulated upon stimulation of CD4+ DC.

CD4− but not CD4+ splenic DC produced TNF-α upon stimulation by poly(I:C) but not LPS, CD40L, or SAC plus IFN-γ (Fig. 6). Both subsets of splenic DC produced little (10 pg/ml) if any IL-10 after LPS, CD40L, or poly(I:C) stimulation, whereas SAC plus IFN-γ stimulation induced variable amounts of IL-10 in both CD4+ (range, 0–93 pg/ml) and CD4− DC (range, 0–108 pg/ml) (Fig. 6). Very low (10 pg/ml) if any IFN-γ was detected in the supernatants of poly(I:C)-stimulated CD4+ but not CD4− DC (Fig. 6). Finally, in three independent experiments, CD4+ and CD4− DC produced low amounts of IFN-α (CD4+ DC, range, 4–16 pg/ml; CD4− DC, range, 23–76 pg/ml, \( p = 0.12 \)), respectively, upon infection by influenza virus, suggesting that CD4+ and CD4− DC are not related to the plasmacytoid DC previously described in humans and mice.

Differential T cell stimulatory activity of fresh and mature splenic DC subsets in MLR

The T cell stimulatory activity of splenic DC subsets was analyzed in allogeneic MLR. We previously reported that, at high DC:T cell ratios, CD4+ DC were slightly less potent APC in MLR than CD4− DC (11). Similar experiments were repeated using more physiological conditions, i.e., using normal rat serum- instead of FCS-containing culture medium. In these conditions, CD4− DC subsets induced a similar or slightly higher proliferation of allogeneic naive CD4+ T cells than CD4− DC (Fig. 7). However, a major difference between CD4+ and CD4− DC subsets was the extremely low capacity of CD4− DC to induce allogeneic naive CD8+ T cell proliferation as compared with CD4+ DC (Fig. 7).

The low capacity of fresh CD4− DC, as compared with the CD4+ subset, to stimulate CD8+ T cells in vitro could be related to their short half-life in vitro (see Fig. 5) and/or their lower levels of TNF-α and IL-10 (Fig. 6).

Table II. IL-12p35 and p40 mRNA expression in splenic DC subsetsa

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<th>Expt.</th>
<th>Fresh Cells</th>
<th>LPS</th>
<th>CD40L</th>
<th>SAC + IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IL-12p35</td>
<td>0.66</td>
<td>0.47</td>
<td>0.25</td>
</tr>
<tr>
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<td>CD4+</td>
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</tr>
<tr>
<td>3</td>
<td>CD4−</td>
<td>ND</td>
<td>1.89</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>CD4+</td>
<td>2.32</td>
<td>1.80</td>
<td>20.53</td>
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</table>

a IL-12p35, IL-12p40, and HPRT mRNA levels were measured in freshly isolated or LPS-, CD40L-, or SAC plus IFN-γ-stimulated sorted splenic CD4+ or CD4− DC subsets by quantitative RT-PCR as described in Materials and Methods. Results are expressed as TAI corresponding to the fold change between a sample and a calibrator. The calibrator represents the 1-fold expression of each gene in fresh cells (−1). Results of two independent experiments are shown.
that is likely to be rapidly expressed on CD4+H11001/H11001 cell proliferation was assessed by [3 H]TdR incorporation. SD were washed, and viable cells were counted. As shown in Fig. 8, CD4-

Following overnight culture, DC were harvested, extensively washed and viable cells were counted before mixing with T

FIGURE 8. Allostimulatory activity of mature CD4+ and CD4- splenic DC subsets in MLR. FACS-sorted CD4+ and CD4- splenic DC (2 × 10^6) were used either fresh or after overnight culture in the absence or presence of soluble CD40L as stimulator cells in MLR with purified allogeneic CD4+ (A) or CD8+ (B) T cells (1 × 10^5) as responders. Cultured DC were extensively washed and viable cells were counted before mixing with T cells. Cultures were performed in complete IMDM supplemented with 1% normal rat serum in triplicate in round-bottom 96-well plates for 4 days. T cell proliferation was assessed by [3 H]TdR incorporation. Similar results were obtained in three independent experiments.

of CD80 and CD86 costimulatory molecules following spontaneous maturation (see Fig. 2). However, both in vitro survival (Fig. 5) and maturation (Fig. 2) of CD4- DC can be enhanced by CD40L that is likely to be rapidly expressed on CD4+ T cells during MLR. We therefore analyzed the capacity of CD4+ and CD4- DC to stimulate CD4+ and CD8- T cells after the DC have matured in vitro in the absence or in the presence of CD40L or GM-CSF. Following overnight culture, DC were harvested, extensively washed, and viable cells were counted. As shown in Fig. 8, CD4- splenic DC matured in medium alone promoted potent proliferation of allogeneic CD4+ T cells but remained poor stimulators of CD8- T cell proliferation as compared with CD4+ DC. However, overnight exposure of CD4- DC to soluble CD40L (Fig. 8B) or GM-CSF (data not shown) increased their capacity to induce CD8- T cell proliferation by 231 ± 27% (mean ± SD of three independent experiments) which nevertheless remained 26 ± 15% of that obtained with overnight cultured CD4+ DC. In contrast, CD4+ DC matured overnight in the absence or presence of CD40L retained their ability to induce strong proliferation in both CD4+ and CD8- T cells (Fig. 8A).

CD4+ and CD4- splenic DC subsets promote different Th cell differentiation in vitro

We next analyzed the capacity of splenic DC subsets to induce CD4+ and CD8- T cell differentiation in vitro. Freshly isolated and unstimulated CD4+ and CD4- DC were cultured with allogeneic CD4+ or CD8+ lymph node T cells at a DC:T ratio of 1:5. Four days later, T cells were restimulated with anti-CD3 and anti-CD28 mAbs and supernatants were harvested after 24 h for cytokine analyses. As shown in Fig. 9A, both CD4- and CD4+ DC promoted the differentiation of CD4+ T cells consistently producing large amounts of IFN-γ (mean ± SD of five independent experiments: CD4- DC, 70.1 ± 19.5 ng/ml; CD4+ DC, 58.2 ± 19.3, NS) and IL-2 (CD4+ DC, 8.9 ± 5.5 ng/ml; CD4- DC, 9.7 ± 6.1, NS) (Th1). The amounts of IL-10 produced by CD4+ T cells upon restimulation were quite variable (ranges, 47–15,000 pg/ml for CD4+ DC, 54–10,700 for CD4- DC in five independent experiments) but not significantly different between supernatants of CD4+ or CD4- DC-stimulated T cells. Although IL-4 was never detected, substantial amounts of IL-13 (250 ± 167 pg/ml), another typical Th2 cytokine, were produced by CD4+ T cells that had been stimulated by CD4+ DC. In contrast, CD4- DC were unable to promote the differentiation of IL-13-producing Th2 cells (Th2). CD4- splenic DC promoted the differentiation of CD8- T cells producing large amounts of IFN-γ (49 ± 35 ng/ml, Fig. 9B). In contrast and according to the inability of CD4- DC to induce strong proliferation of allogeneic CD8- T cells (Fig. 7), these CD8- T cells produced very low amounts of IFN-γ upon in vitro restimulation as compared with CD4+ DC-stimulated CD8- T cells (4.2 ± 8 ng/ml, p = 0.026 as compared with CD4+ DC). In

FIGURE 9. The influence of splenic DC subsets on allogeneic Th and CD8+ T cells differentiation in vitro. Freshly isolated CD4+ (A) or CD4- (B) splenic DC were cultured with purified allogeneic CD4+ (A) or CD8+ (B) T cells at a ratio of 5:1 for 4 days in complete RPMI 1640 supplemented with 1% normal rat serum in 24-well plates. Viable T cells were then harvested and immediately restimulated on anti-CD3-coated plates in the presence of anti-CD28 mAb at 1 × 10^5/ml for 24 h. The production of IL-2, IFN-γ, IL-10, and IL-13 was assessed by ELISA. Similar results were obtained in five independent experiments except for IL-10 whose production was highly variable from one experiment to another.
pressed by rat splenic DC but two subsets were identi-

lymphoid dichotomy of splenic DC subsets. CD4

studied; however, our results do not suggest a clear myeloid and

receptors (32) as recently reported for human DC subsets (33).

and Tc1 differentiation in CD4

be a homogenous population of cells with myeloid-like morpho-

over, the Toll-like receptor 3 ligand poly(I:C) (31) induced TNF-

strongly up-regulated in stimulated CD4

upon SAC exposure. However, the fact that IL-12p35 mRNA was

CD40L or LPS exposure although both subsets produce IL-12p40

from the spleen (11) and lymph nodes (our unpublished observa-

coexpress SIRP

and CD90 (Thy1.1). Although freshly isolated CD4

tions) exhibited an in vitro cytotoxic activity against tumor cells. It

is also likely that CD4

to lymph nodes or the spleen or might be temporally regulated on

procedure, occurs upon culture. Therefore, results obtained from in

phenotype, a maturation, probably due to the cell extraction pro-

methodologies underlying this different CD8

between CD4

whether IL-13, IL-2, and IFN-

independent Th1-priming capacity. However, it is not known

whether CD4

T cell differentiation is in

control experiments, we could not detect IL-2, IFN-γ, IL-10, or

IL-13 production in the supernatants of naive CD4+ or CD8−

Tcells that had been stimulated for 24 h by anti-CD3 and anti-

CD28 mAbs (data not shown).

Discussion

Several subsets of DC have been described in lymphoid organs and

close to T cell areas of mesenteric lymph nodes (13). Several data suggest that CD4+ SIRPα− DC in lymph

nodes and spleen represent the same subset. CD4− DC isolated from both lymph (10) and spleen (in this report) exhibit a very

short half-life in vitro. Moreover, CD4+ /SIRPα− DC isolated from the spleen (11) and lymph nodes (our unpublished observations)

exhibited an in vitro cytotoxic activity against tumor cells. It is also likely that CD4+ DC in the afferent lymph and in the spleen

represent the same subset of DC because in both tissues these cells coexpress SIRPα and Thy1 molecules (this report and Ref. 10).

However, CD4 and SIRPα markers are not always coexpressed on

rat DC as, for instance, Langerhans cells express SIRPα but not CD4 (30). Taken together, these data suggest that CD4− DC in the spleen, lymph node, and afferent lymph represent the same subset of DC in different locations, whereas CD4 expression might be restricted to a specific subset of DC that traffic from certain tissues to lymph nodes or the spleen or might be temporally regulated on

rat DC. However, we did not observe CD4, CD5, or SIRPα expression on CD4− DC upon in vitro culture (data not shown).

We found that CD4− but not CD4+ DC produce IL-12 upon

CD40L or LPS exposure although both subsets produce IL-12p40

upon SAC exposure. However, the fact that IL-12p35 mRNA was

strongly up-regulated in stimulated CD4− but not CD4+ DC sug-

gest that CD4− DC are the main source of bioactive IL-12. More-

over, the Toll-like receptor 3 ligand poly(I:C) (31) induced TNF-α

but not IL-12 production in CD4− DC. These results suggest that

spleen DC subsets differentially respond to a pathogen-associated molecular pattern and therefore express a different set of Toll-like

receptors (32) as recently reported for human DC subsets (33). Monocytes and myeloid-derived DC express numerous Toll-like receptors whereas lymphoid DC and immediate precursors (preDC2) appear to express a limited set of receptors (33). To our

knowledge, the ontogeny of DC subsets in rats has not been clearly studied; however, our results do not suggest a clear myeloid and

lymphoid dichotomy of splenic DC subsets. CD4− DC appear to

be a homogenous population of cells with myeloid-like morpho-

logical features and do not express lymphoid-related Ags. Splenic

CD4+ DC appeared much more heterogeneous regarding their

morphology and to a lesser extent their phenotype. Moreover, both typical lymphoid-related markers such as CD5 and myeloid-re-

lated markers such as SIRPα are expressed by CD4+ DC. In the

murine spleen, two subsets of DC were initially described by

Shortman’s group (34), one CD8+ and one CD8−. More recently,

the CD8− subset was shown to contain CD4+ and CD8− DC (23).

Because CD8 is not expressed on rat splenic DC, it is not possible
to make a direct link between mouse and rat DC subsets. However,

the poor viability and spontaneous maturation, the large size, the

high IL-12 production, and the low capacity to stimulate CD8+ T

cells of the CD4− subset of DC are properties reminiscent of

mouse splenic CD8+ DC (6, 35, 36). Finally, the fact that total

OX62+ DC as well as sorted CD4+ and CD4− DC subsets produce

low amounts of IFN-α upon viral infection suggest that rat plasmacytoid DC do not express OX62 or CD103.

Whether CD4+ T cell differentiation is influenced by specific DC subsets or by the maturation stage of DC remains controversial

(4, 26, 37). As previously reported in humans (1), our study shows the existence of a high IL-12-producing DC subset in rats

(CD4+ SIRPα−) that induce Th1 responses and exhibit a myeloid-

like morphology. Although the production of both IFN-γ and

IL-10 by CD4+ T cells stimulated with CD4+ DC is reminiscent of T regulatory type 1 cells previously described in mice (38), the

associated high production of IL-2 rather suggests a Th1 differenti-
tation, a phenotype consistent with the high production of IL-12

by CD4+ DC. In contrast to CD4− DC, CD4+ SIRPα− DC induced

CD4+ T cells to produce IL-13 but also large amounts of IFN-γ

and IL-2, despite a low production of IL-12, suggesting IL-12-

independent Th1-polarizing capacity. However, it is not known

whether IL-13, IL-2, and IFN-γ are produced by the same Th0

cells or by differentiated Th1 and Th2 cells. It is also possible that

whether CD4+ DC are actually a heterogeneous population of DC with
different Th cell-priming capacities.

A recent study by Huang et al. (13) suggested that in the steady

state, rat CD4+ DC may play a role in the maintenance of self-
tolerance by transporting apoptotic tissue cells and presenting self-

peptides in a tolerogenic fashion in draining lymph nodes (14). Although freshly isolated CD4− splenic DC exhibit an immature

phenotype, a maturation, probably due to the cell extraction pro-
cedure, occurs upon culture. Therefore, results obtained from in

vitro studies, such as ours, performed with DC extracted from

lymphoid organs, do not fully reflect the actual role of DC in their

in vivo steady state. Indeed, it has been postulated that steady-state

immature DC may tolerate and mature DC may immunize (39).

Our results indicate that both immature and mature CD4+ DC are

poor stimulators of CD8+ T cells in our experimental conditions.

However, the fact that CD40 stimulation or GM-CSF increased

their capacity to stimulate CD8+ T cells suggest that, in vivo, mature

CD4+ DC might be able to prime CD8+ T cells providing that an

additional maturation signal or help from CD4+ T cells is present.

In contrast, CD4− DC are potent stimulators of CD8+ T cells even

in the absence of maturation signal or CD4+ T cells. The mecha-
nisms underlying this different CD8+ T cell stimulatory capacity

between CD4+ and CD4− DC are currently under investigation.

Although CD4− DC exhibit a potent cytotoxic activity against

tumor cells in vitro, they are unable to induce cytolysis in resting or

activated T cells (11), suggesting that this activity is not involved

in the low capacity of CD4− DC to stimulate CD8+ T cells. It is

interesting to note that CD4+ spleen DC express high levels of

CD200, a molecule that has recently been involved in T cell

tolerance (40). SIRPα+ and SIRPα− DC subsets have been de-
scribed in bovine afferent lymph (41). Similar to rat DC, bovine

SIRPα− DC were found to be poor stimulators of CD8+ T cell
responses, as compared with SIRPα⁺ DC, and this difference was related to the lack of IL-1 secretion by SIRPα⁺ DC (41).

In conclusion, we have described the phenotype of two splenic DC subsets in rats and characterized, for the first time, their cytokine production and their capacity to induce CD4⁺ and CD8⁻ T cell differentiation. As previously described in humans and mice, we have identified a DC subset in rats that lack CD4 expression and that produce high levels of IL-12 and induce Th1 differentiation in vitro. We have recently shown that the same subset of DC also exhibit a cytotoxic activity against selected tumor cells in vitro (11). CD4⁺ DC might therefore directly bridge innate and adaptive immune responses against tumors by directly killing tumor cells and inducing Th1 responses to tumor Ags. Finally, our study will be useful to decipher the complex role of DC subsets in rat models of autoimmunity, tumor, and transplantation immunity as well as tolerance.

Acknowledgments

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References