oratories (Bar Harbor, ME). All explants were cultured on 8-μm polycarbonate filters in Transwell plates (Costar) at the interface of medium (15% fetal bovine serum in Dulbecco's modified Eagle's medium, L-glutamine, penicillin, and streptomycin). For ectoderm separation, tissues were incubated with 6% pancreatin (Gibco-BRL) for 30 min on ice, washed in 10% serum for 20 min, and peeled (*13*). NIH 3T3 cell lines were aggregated by culture in hanging drops (*20*). Explant cultures shown are representative of at least four independent experiments. Rat *NT-3* and human γ-*actin* cDNAs were used as probes for the Northern blot, which was quantified with a multiimager (Fuji).

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Reciprocal Control of T Helper Cell and Dendritic Cell Differentiation

Marie-Clotilde Rissoan,[1]* Vassili Soumelis,[1]* Norimitsu Kadowaki,[2]* Geraldine Grouard,[1] Francine Briere,[1] René de Waal Malefyt,[2] Yong-Jun Liu[1,2]†

It is not known whether subsets of dendritic cells provide different cytokine microenvironments that determine the differentiation of either type-1 T helper (T_H1) or T_H2 cells. Human monocyte (pDC1)–derived dendritic cells (DC1) were found to induce T_H1 differentiation, whereas dendritic cells (DC2) derived from $CD4^+CD3^-CD11c^-$ plasmacytoid cells (pDC2) induced T_H2 differentiation by use of a mechanism unaffected by interleukin-4 (IL-4) or IL-12. The T_H2 cytokine IL-4 enhanced DC1 maturation and killed pDC2, an effect potentiated by IL-10 but blocked by CD40 ligand and interferon-γ. Thus, a negative feedback loop from the mature T helper cells may selectively inhibit prolonged T_H1 or T_H2 responses by regulating survival of the appropriate dendritic cell subset.

The cytokine microenvironment plays a key role in T helper cell differentiation toward the T_H1 or T_H2 cell type during immune responses (*1–6*). IL-12 induces T_H1 differentiation, whereas IL-4 drives T_H2 differentiation. Because T helper cell differentiation requires the presence of different cytokines at an initial stage of the T cell–dendritic cell (DC) interaction (*1–7*), we investigated whether distinct DC lineages or subsets may produce different cytokines and directly induce T_H1 or T_H2 differentiation.

Humans have two distinct types of DC precursors. Peripheral blood monocytes (designated pDC1) give rise to immature myeloid DCs after culturing with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (*8–10*) or after transmigration through endothelial cells and phagocytosis (*11*). These immature cells become mature myeloid DCs (designated DC1) after stimulation with CD40 ligand (CD40L) or endotoxin (*12, 13*). The $CD4^+CD3^-CD11c^-$ plasmacytoid cells (designated pDC2) from blood or tonsils give rise to a distinct type of immature DC after culture with IL-3 (*14–16*). These cells differentiate into mature DCs (designated DC2) after CD40L stimulation (*17*). However, unlike pDC1 and DC1, pDC2 and DC2 display features of the lymphoid lineage: (i) pDC2 and DC2 express few myeloid antigens, such as CD11b, CD11c, CD13, and CD33 (*14*); (ii) pDC2 cells do not differentiate into macrophages, following culture with GM-CSF and M-CSF; (iii) pDC2 and DC2 have little capacity to phagocytose or macropinocytose antigens at all stages of their maturation (*14*) (iv) like the putative mouse lymphoid DCs (*18*), pDC2 cells depend on IL-3, but not GM-CSF for their survival and maturation (*14*) [this can be explained by high GM-CSF receptor and low IL-3 receptor expression in pDC1 cells and low GM-CSF receptor and high IL-3 receptor expression in pDC2 cells (Fig. 1)]; and (v) pDC2 cells have high levels of pre–T cell receptor α-chain expression (*19*).

Both myeloid DC1 and "lymphoid" DC2 induce strong proliferation of allogeneic naïve $CD4^+$ T cells (*14*). First, we examined the profile of cytokine production from DC1 and DC2 after CD40L activation. DC1 produced large amounts of IL-12 within 24 hours after CD40L activation (Fig. 2) as reported (*12, 13*), whereas DC2 did not (Fig. 2). In addition, unlike CD40L-activated DC1, CD40L-activated DC2 produced little IL-1α , IL-1β, IL-6, and IL-10, but produced comparable amounts of chemokine IL-8 (Fig. 2) (*20*). Neither CD40L-activated DC1 nor DC2 produced detectable amounts of IL-4 and IL-13. Quantitative polymerase chain reaction (PCR) analyses showed that CD40L activation up-regulated the expression of mRNA for IL-12p40, IL-1α, and IL-1β in DC1, but not in DC2 (Table 1) (*21*). Neither DC1 nor DC2 transcribed detectable amounts of IL-4 mRNA, either before or after CD40L activation (Table 1).

We next examined the nature of primary allogeneic T cell responses induced by DC1 or DC2. Naïve $CD4^+CD45RA^+$ T cells isolated from human peripheral blood or umbilical cord blood were cocultured for 7 days with CD40L-activated DC1, CD40L-activated DC2, or antibodies to CD3 and CD28 (*22*). The cultured cells were counted and restimulated with anti-CD3 and anti-CD28 for either 4 hours for single-cell cytokine analyses by flow cytometry (Fig. 3B) or 24 hours for cytokine secretion analyses by enzyme-linked immunosorbent assay (ELISA) (Fig. 3A). T cells originally cul-

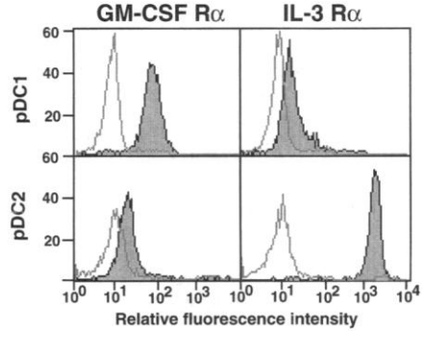


Fig. 1. Expression of GM-CSF receptor α chain (GM-CSF Rα) and IL-3 receptor α chain (IL-3 Rα) on pDC1 and pDC2 (open curve, isotype control; shaded curve, specific staining).

[1]Schering-Plough, Laboratory for Immunological Research, 27 chemin des Peupliers, Boite Postale 11, 69571, Dardilly, France. [2]DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304–1104, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: yliu@dnax.org

tured with DC1 secreted large amounts of interferon-γ (IFN-γ) (34 to 37 ng/ml, from three independent experiments), but little IL-4, IL-5, and IL-10. T cells originally cultured with DC2 secreted little IFN-γ (2 to 4 ng/ml), but large amounts of IL-4 (230 to 1500 pg/ml), IL-5 (300 to 900 pg/ml), and IL-10 (4 to 10 ng/ml). T cells originally cultured with anti-CD3 and anti-CD28 secreted mainly IL-2 (Fig. 3A). These polarized cytokine production profiles were confirmed by single-cell cytokine analysis using in situ immunocytology (*23*) and by immunofluorescence flow cytometry (Fig. 3B) (*24*). Thus, myeloid DC1 and "lymphoid" DC2, respectively, induce T_H1 versus T_H2 differentiation in vitro.

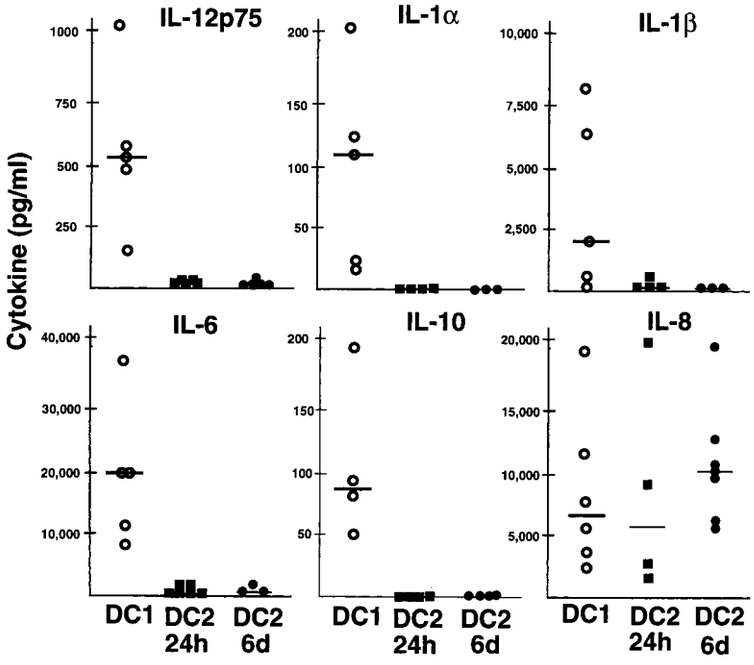
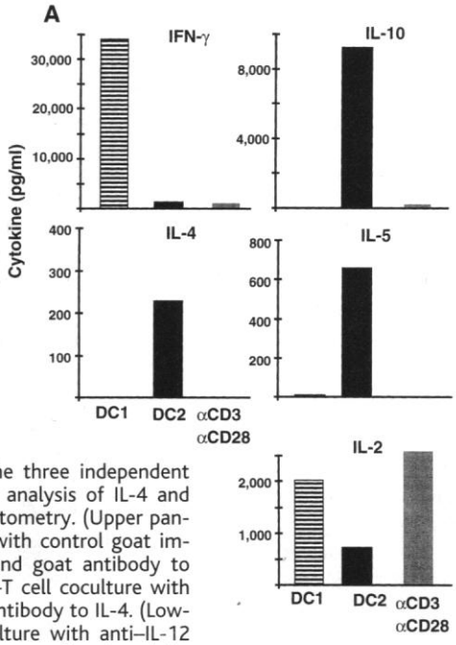


Fig. 2. Cytokine and chemokine secretion by DC 1 after 24 hours of activation with CD40L (open circles) and DC2 after CD40L activation for 24 hours (solid squares) or 6 days (solid circles). Each symbol represents an independent experiment.

Because DC2 cells do not produce detectable amounts of IL-4, as determined by both mRNA (to a sensitivity of 10^{-12} gram) and protein analysis, it suggests that the DC2-induced T_H2 differentiation is IL-4–independent. T_H2 development was not blocked by adding either polyclonal antibody to IL-4 BDA11 (15 μg/ml) or monoclonal antibody (mAb) to IL-4 MP4-25D2 (5 μg/ml) at the beginning of DC–T cell coculture (*22*). These two antibodies to IL-4 completely inhibited the IL-4–dependent proliferation of phytohemagglutinin-stimulated human T cells or CD40- and IL-4–dependent human B cell proliferation and immunoglobulin E synthesis. Although addition of antibody to IL-4 increased the number of IFN-γ–producing cells, it did not block the generation of IL-4–producing cells (Fig. 3B). The DC2-induced T_H2 differentiation was not a default mechanism due to an inability of DC2s to produce IL-12, because polyclonal activation with antibodies to CD3 and CD28 in the absence of



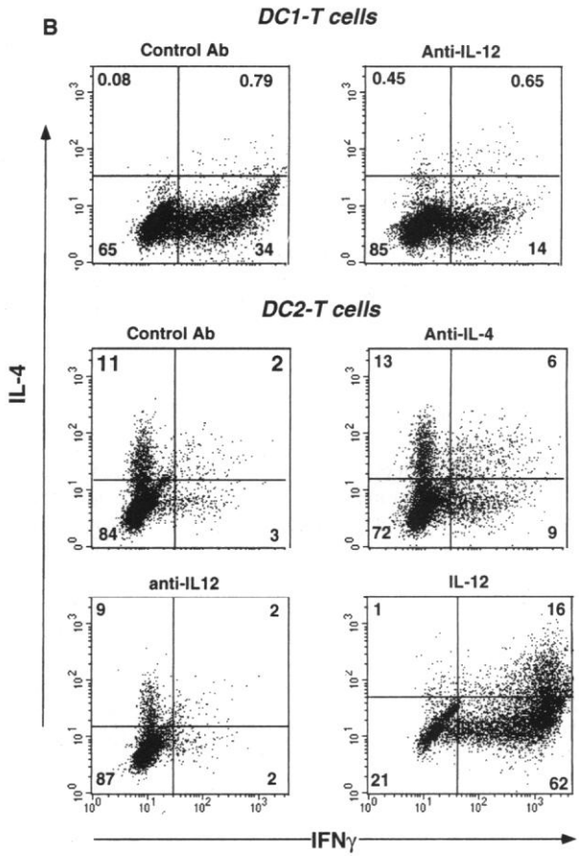


Fig. 3. DC1 and DC2 induce T_H1 versus T_H2 cytokine production, respectively. (**A**) Quantitation of T_H1 and T_H2 cytokines by ELISA. Human $CD4^+$ $CD45RO^-$ naïve T cells were cultured for 6 days with allogeneic CD40L-activated DC1 or DC2, or anti-CD3 plus anti-CD28αCD3/αCD28). Cells were counted and restimulated with anti-CD3 and anti-CD28 for 24 hours. Amounts of IFN-γ, IL-4, IL-5, IL-10, and IL-2 within culture supernatants were collected after 24 hours and measured by ELISA. Results represent one of the three independent experiments. (**B**) Two-color analysis of IL-4 and IFN-γ expression by flow cytometry. (Upper panels) DC1–T cell cocultures with control goat immunoglobin G antibodies and goat antibody to IL-12. (Middle panels) DC2–T cell coculture with control antibody and goat antibody to IL-4. (Lower panels) DC2–T cell coculture with anti–IL-12 and IL-12. Some 10^4 cells were analyzed, and the percentages of each T cell population are indicated in the plots. Figure 4 represents the results from one of the four independent experiments performed.

IL-12 did not induce T_H2 differentiation (Fig. 3A). To support this conclusion, we performed two experiments. First, neutralizing antibody to IL-12 (AB-219-NA, 25 μg/ml) was added at the beginning of the DC–T cell cocultures to see whether anti–IL-12 could induce IL-4–producing T cells in DC1–T cell culture or increase the number of IL-4–producing cells in the DC2–T cell cocultures. Although addition of antibody to IL-12 decreased the percentage of IFN-γ–producing cells in both DC1–T cell and DC2–T cell cultures (Fig. 3B), it did not induce IL-4–producing cells nor did it significantly increase IL-4–producing cell number in DC1–T cell or DC2–T cell cultures (Fig. 3B). Second, IL-12 (5 ng/ml) was added at the beginning of DC2–T cell cocultures, to see if it could block the generation of IL-4–producing T cells. Although the addition of IL-12 increased the percentages of IFN-γ–producing T cells, it did not inhibit the number of IL-4–producing cells. However, IL-12 induced the IL-4–producing cells to produce IFN-γ (Fig. 3B). Thus, DC2 may produce one or more positive T_H2 differentiation factors distinct from IL-4, and its activity can neither be blocked by IL-12 nor enhanced by anti–IL-12.

A common feature of T cell cytokine-mediated T helper cell differentiation is the positive autocrine effect. IL-2 promotes the IL-2–producing T_H0 cells, IL-4 promotes the IL-4–producing T_H2 cells, IFN-γ promotes the IFN-γ–producing T_H1 cells, IL-10 promotes the IL-10–producing regulatory $CD4^+$ T cells (*25*), and TGF-β promotes the TGF-β–producing T_H3 cells (*26*). Because negative feedback regulation represents a general mechanism used by living organisms to maintain homeostasis of physiological processes, the immune system may need a negative feedback mechanism to control the balance between T_H1 and T_H2 responses in order to prevent T_H-mediated autoimmune inflammatory responses or T_H2-mediated allergic responses. The studies on the regulation of DC1 and DC2 maturation allowed us to identify a potential negative feedback loop in which IL-4 and IFN-γ may negatively regulate T_H1 and T_H2 development, respectively, by interfering with the survival and maturation of pDC1 and pDC2.

We observed that IL-4, IL-10, and CD40L inhibited the IL-3–dependent proliferation of pDC2 (Fig. 4A, a) (*27*). In contrast to CD40L, which enhanced the survival and maturation of pDC2, IL-4 and IL-10 decreased pDC2 numbers during a 6-day culture period in the presence IL-3 in a concentration-dependent fashion (Fig. 4A, b and c) (*27*). IL-4 and IL-10 have an additive effect in killing pDC2 (Fig. 4B). The ability of IL-4 and IL-10 to kill pDC2 by apoptosis was confirmed by direct culture morphology, Giemsa staining of cytospin preparations, and double-staining with annexin–fluorescein isothiocyanate (FITC) and propidiumiodide.

Because IL-4 and IL-10 are T_H2 cytokines, we investigated whether CD40L and IFN-γ could block the negative effect of IL-4 and IL-10 on the survival of pDC2 maintained by IL-3. CD40L blocked the killing effects of IL-4 or IL-10 on pDC2 during a 6-day culture period with IL-3 (Fig. 5A). CD40L partially rescued pDC2 in the presence of both IL-4 and IL-10. IFN-γ also blocked the negative effect of IL-4 or IL-10

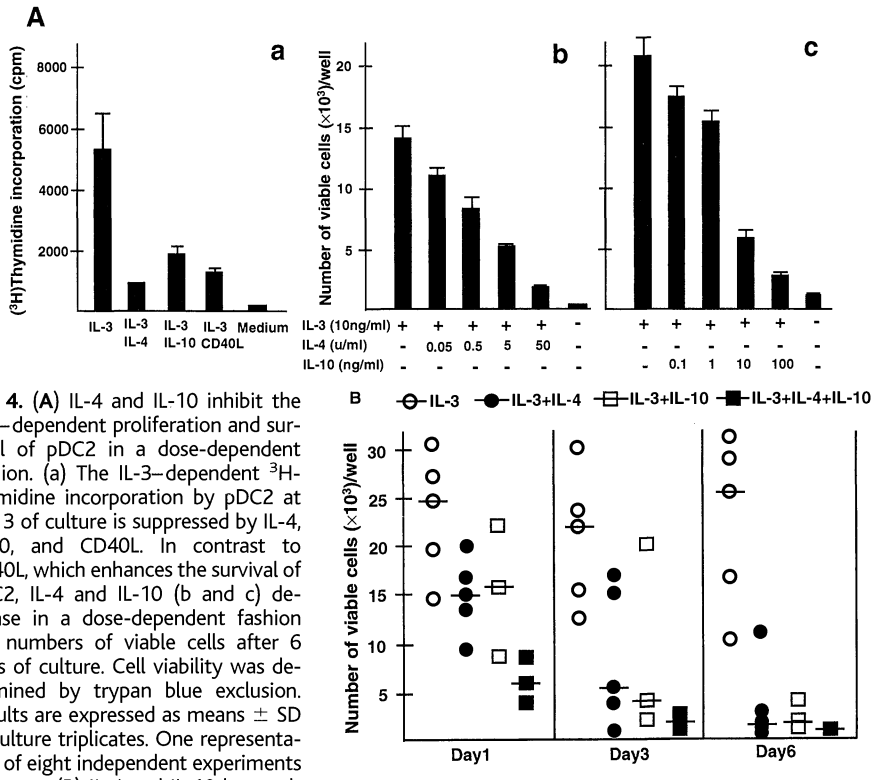


Fig. 4. (**A**) IL-4 and IL-10 inhibit the IL-3–dependent proliferation and survival of pDC2 in a dose-dependent fashion. (a) The IL-3–dependent ^3H-thymidine incorporation by pDC2 at day 3 of culture is suppressed by IL-4, IL-10, and CD40L. In contrast to CD40L, which enhances the survival of pDC2, IL-4 and IL-10 (b and c) decrease in a dose-dependent fashion the numbers of viable cells after 6 days of culture. Cell viability was determined by trypan blue exclusion. Results are expressed as means ± SD of culture triplicates. One representative of eight independent experiments is shown. (**B**) IL-4 and IL-10 have additive effects in killing pDC2 over a 6-day time course. Kinetics of cell survival at days 1, 3, and 6 of culture with IL-3 alone, IL-3 + IL-4, IL-3 + IL-10, and IL-3 + IL-4 + IL-10. The initial input of cells was 45,000 or 25,000 per well. Each symbol represents one independent experiment.

Table 1. Quantitation of cytokine mRNA by PCR (expressed as femtograms mRNA per 50 ng cDNA) (*21*). Results before (–) and after (+) CD40L activation are shown after experiment number.

Expt. number (CD40L)	IL-12p40	IL-1α	IL-1β	IL-4
		DC1		
1 (–)	38	34	41	0
1 (+)	12,835	573	2,175	0
2 (–)	7	45	60	2
2 (+)	12,460	346	4,165	0
3 (–)	5	44	45	0
3 (+)	40,194	441	763	0
		DC2		
1 (–)	0	12	20	0
1 (+)	605	9	16	0
2 (–)	0	37	102	0
2 (+)	714	21	50	0
3 (–)	0	7	14	0
3 (+)	0	12	45	0

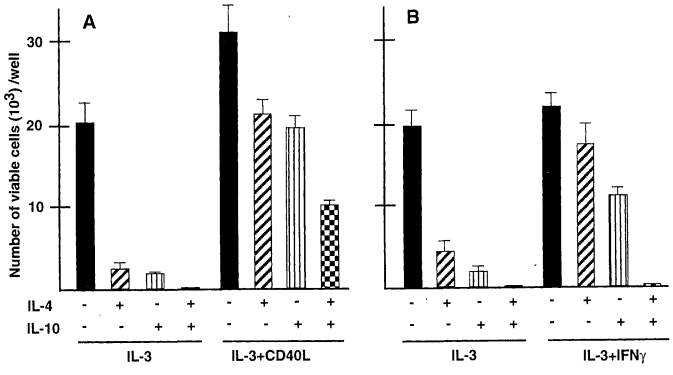


Fig. 5. CD40L and IFN-γ rescue pDC2 from cell death induced by IL-4 and IL-10. (**A**) CD40L rescues a large proportion of DC2 precursors after 6 days of culture with combinations of IL-3, IL-4, and IL-10. (**B**) IFN-γ rescues a large fraction of DC2 precursors after 6 days of culture with IL-3 + IL-4 and IL-3 + IL-10. It did not rescue DC2 precursors when cultured with IL-3 + IL-4 + IL-10. Results are representative of three independent experiments.

on pDC2 (Fig. 5B). However, IFN-γ did not rescue the cells when IL-4 and IL-10 were both added to the culture. Cells rescued by either CD40L or IFN-γ expressed high levels of major histocompatibility complex (MHC class II DR) and costimulatory molecules (B7.1/CD80 and B7.2/CD86), and stimulated the proliferation of allogeneic $CD4^+$ T cells.

Our study suggests that a negative feedback loop may exist in regulating the balance between T_H1 versus T_H2 responses. IL-4, a key T_H2 cytokine, kills the pDC2, a professional antigen-presenting cell subset that induces T_H2 differentiation. The ability of CD40L (a potent DC maturation factor) to prevent IL-4–induced killing suggests that IL-4 cannot kill mature DC2 during their cognate interaction with T cells in established responses. This may allow the rapid and efficient development of T_H2 responses needed for the host defense. However, overproduction of IL-4 may inhibit the development of pDC2. By contrast, IL-4 promotes DC1 maturation together with GM-CSF (*12, 13*). These opposing effects of IL-4 on DC1 versus DC2 may enhance T_H1 development, but inhibit T_H2 development at a late stage of immune response. The ability of IFN-γ to protect pDC2 from IL-4–and IL-10–induced apoptosis and promote DC2 differentiation may represent an indirect mechanism to inhibit T_H1 development at later stages of T_H1 responses. This represents another example of antagonism between IL-4 and IFN-γ (*28*).

During the last two decades, studies on the relationship between lineage and function have been a main focus of B and T lymphocyte immunology. Mouse DC cells may also have different lineages with distinct functions (*29–35*). Whereas the $CD8\alpha^-CD11c^+CD11b^+$ myeloid DC cells are immunogenic for T cells, the $CD8\alpha^+CD11c^+CD11b^-$ lymphoid DC cells may be tolerogenic (*31*). This concept is supported by two findings: (i) the lymphoid DC subset that appears to be localized within the T cell areas of mouse spleen highly express MHC class II–self peptide complexes (*32*); and (ii) the myeloid subset that appears to be localized around the marginal zone–bridging channels migrates into the T cell areas and produces IL-12 after endotoxin stimulation (*33, 34*). Our results here extend the concept regarding the functional heterogeneity of DC subsets and suggest two additional mechanisms for T_H1 and T_H2 regulation. DC1 and DC2 stimulate naïve T helper cells and directly induce their differentiation toward T_H1 or T_H2. pDC1 and pDC2 provide the potential targets for negative feedback regulation by IL-4 and IFN-γ. Two important relationships still need to be established: (i) the relationship between the mechanisms regulating immunity/tolerance versus T_H1/T_H2 and (ii) the correlation between mouse and human DC subsets. These studies may ultimately lead to the understanding of the molecular mechanism underlying DC2-induced IL-4–independent T_H2 differentiation and the distinct functions of DC subsets in normal and disease states.

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10. Generation of DC1 from monocytes in vitro. Blood mononuclear cells were isolated as described (*8, 9*). To generate DC1, monocytes were cultured with GM-CSF (200 ng/ml) and IL-4 (5 ng/ml; Schering-Plough, Kenilworth, NJ) for 5 days in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 μM L-glutamine, and 0.08 mg/ml gentamycin. The resulting immature DC1 were cultured with CD40L-transfected L cells (one L cell per four DC cells) for 24 hours.
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17. Generation of DC2 from the $CD4^+CD11c^-$lineage$^-$ pDC2 precursors. $CD4^+CD11c^-$lineage$^-$ plasmacytoid cells were isolated from human tonsils or peripheral blood as detailed (*13*). In two experiments, pDC1 and pDC2 were isolated from the same blood donor. These cells were cultured with IL-3 in the presence or absence of CD40L-transfected L cells for 6 days or with IL-3 alone for 5 days, followed by culture with CD40L-transfected L cells for 24 hours.
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20. Quantitation of cytokine secretion by ELISA. The presence of cytokines in culture supernatants of DC1 and DC2 after 24 hours of CD40L activation or in T cell cultures for 24 hours with anti-CD3 and anti-CD28 was determined by ELISA. ELISA kits for IL-1α, IL-1β, IL-5, IL-6, IL-10, IL-12, IL-13, and IFN-γ were obtained from R&D Systems (Minneapolis, MN); for IL-2 and IL-4 from Cayman Chemical (Ann Arbor, MI).
21. Quantitation of mRNA expression. RNA isolation was according to P. Chomczynski and N. Sacchi [*Anal. Biochem.* **162**, 156 (1987)]. The reverse transcription was performed with SuperScriptII (Gibco-BRL, Rockville, MD). We analyzed 50 ng of cDNA for the expression of cytokine genes by the Fluorogenic 5′-nuclease PCR assay [P. M. Holland *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7276 (1991)], using a Perkin-Elmer ABI Prism 7700 Sequence Detection System (SDS; ABI–Perkin-Elmer, Foster City, CA). Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 s. The following amplicons were used and analyzed with 6-carboxy-fluorescein–labeled predeveloped Taqman assay reagents (Perkin-Elmer, Foster City, CA): IL-1α, IL-1β, IL-4, and IL-12p40. Cytokine amplicons spanned at least one intron/exon boundary. An 18S ribosomal RNA amplicon was analyzed with a labeled probe (Perkin-Elmer, Foster City, CA) and used as an internal control for quantitation of the total amount of cDNA in a multiplex reaction. Seven 10-fold dilutions of plasmids (10 ng/ml) containing cytokine cDNAs were used to create a standard curve for quantitation of cytokine cDNA using the SDS software, then these values were adjusted for the amount of total cDNA. Values are expressed as femtogram of cDNA per 50 ng input total RNA.
22. Purification of naïve T cells and DC–T cell cocultures. $CD4^+CD45RA$ T cells were incubated with a cocktail of mAbs, including IOM2 (CD14); ION16 (CD16); ION2 (HLA-DR) (Immunotech); NKH1 (CD56); OKT8 (CD8) (Ortho); 4G7 (CD19); UCHL1 (CD45RO); and mAb 89 (CD40). This was followed by incubation with anti-mouse immunoglobin-coated magnetic beads and magnetic depletion. This was repeated two times to create >96% pure $CD4^+$ naïve T cells. T cells were cocultured with allogeneic DC1 or DC2 at a 2:1, 4:1, and 8:1 ratios in Yssel's medium (Irvine Scientific, Santa Ana, CA) containing 10% FBS in 24-well culture plates for 6 days with or without: (i) polyclonal antibody to IL-4 (BDA11, 15 μg/ml; R&D Systems); (ii) mAb to IL-4 (MP4-25D2; Pharmingen); (iii) antibody to IL-12 (AB-219-NA, 25 μg/ml; goat polyclonal immunoglobin G, R&D Systems); and (iv) IL-12 (5 ng/ml; R&D Systems). T cells were also cultured with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml; Pharmingen) coated on culture plates for 6 days. After 6 days of priming, T cells were restimulated with anti-CD3 and anti-CD28 for either 4 or 24 hours.
23. Immunostaining of cytokines on cytospins. Cytospin slides were fixed in cold paraformaldehyde for 5 min and washed in Hank's balanced salt solution. For IL-12p40 staining, the slides were incubated with mAb 609 (5 μg/ml; R&D Systems) or an isotype control mAb for 45 min. The binding of antibody was revealed by a Vectastain ABC kit (Vector Laboratory, Burlingame, CA). DAB chromogen was used to reveal the peroxidase activity. For IFN-γ staining, slides were incubated with mAb B27 (J. Abrams, DNAX Research Institute of Molecular and Cellular Biology) followed by staining with the Vectastain ABC kit.
24. Flow cytometry analysis of intracellular cytokines. After 6 days of DC–T cell coculture, T cells were reactivated with anti-CD3 and anti-CD28 for 5 hours. Brefeldin A (1 μg/ml; Sigma) was added into the cultures for 2 hours before the staining to prevent cytokine secretion. Cells were washed and fixed with paraformaldehyde and incubated with the following antibodies (Pharmingen): anti-IL-4–phycoerythrin (PE) plus anti-IFN-γ–FITC; anti-IL-10–PE plus anti-IFN-γ–FITC; and anti-IL-4–FITC plus anti-IL-10–PE. Samples were analyzed on a FACScan (Becton-Dickinson).
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27. pDC2 were cultured in complete medium alone (RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 40 mg/ml gentamycin) or with different cytokines. Cytokines used were IL-3 (10 ng/ml), IL-4 (50 U/ml), IL-10 (100 ng/ml), and IL-13 (50 ng/ml) from Schering-Plough Research Institute, Kenilworth, NJ, and IFN-γ (25 ng/ml) and IL-2 (20 U/ml) from Genzyme, Boston, MA. Irradiated CD40L-transfected L cells were used at a concentration of one L cell per four DC2 cells. Nontransfected or CD32-transfected L cells were used as controls. We cultured 3×10^4 cells per well in triplicate in 96-well plates. After 3 days, cells were pulsed with 1 μCi of ^{3}H-thymidine for 12 hours before harvesting and of counting. Viable and dead cell numbers at 1, 3, and 6 days after culture were determined by trypan blue exclusion assay.
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