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T Lymphocytes Potentiate Murine Dendritic Cells to Produce IL-12[1]

Alexandra Rizzitelli, Rolande Berthier, Véronique Collin, Serge M. Candéias, and Patrice N. Marche[2]

IL-12 is mainly produced by CD8α^+ dendritic cells (DCs) and induces Th1 polarization of the immune response. We investigated the influence of lymphocytes on splenic DC (SDC) and thymic DC (TDC) development and on their IL-12 production capacity. First, CD3$\epsilon^{-/-}$ mice, lacking T cells, and RAG-2$^{-/-}$ mice, lacking T and B cells, possess numbers of SDCs, TDCs, and CD8α^+ SDCs similar to wild-type (WT) mice. Second, SDCs and TDCs from CD3$\epsilon^{-/-}$ mice do not secrete IL-12 in vitro after different stimulations, whereas DCs from pT$\alpha^{-/-}$ mice, possessing reduced T cell number, and RAG-2$^{-/-}$ mice, produce an IL-12 level similar to that of WT DCs. We show that T lymphocytes restore the capacity of DCs to produce IL-12 after stimulation in vivo by reconstitution of CD3$\epsilon^{-/-}$ mice with WT T cells and in vitro by coculture of CD3$\epsilon^{-/-}$ DCs with WT T cells. The regulation of IL-12 production occurred at the transcriptional level, with an increase of IL-12p35 transcripts and a decrease of IL-12p40 transcripts. Although IL-4 restores IL-12 production by CD3$\epsilon^{-/-}$ SDCs, anti-IL-4 Abs inhibited only partially the IL-12 production in coculture of CD3$\epsilon^{-/-}$ DCs and WT T cells. Taken together, these data show that T lymphocytes potentiate IL-12 production by DCs and that IL-4 is not solely involved in this regulation. In conclusion, B and T cells exert balanced actions on DCs by respectively inhibiting or promoting IL-12 production. *The Journal of Immunology,* **2002, 169: 4237–4245.**

Dendritic cells (DCs)[3] are professional APCs capable of initiating a primary immune response. Present in low numbers in most of the tissues, DCs capture the Ags in the periphery and then migrate to secondary lymphoid organs where they activate naive T lymphocytes (1). Furthermore, DCs can influence the nature of response initiated, either Th1 or Th2, by varying the cytokines they produce. IL-12, as a polarizing cytokine, has been extensively studied. The bioactive form of IL-12 is a heterodimer of 70 kDa (IL-12p70) comprising two disulfide-linked p40 and p35 subunits, encoded by separate genes (2). Secreted by DCs after activation by bacterial products (3) or T-dependent stimuli (4), IL-12 directs T cells toward the Th1 phenotype (5). In the thymus, DCs have been shown to mediate negative selection of autoreactive T lymphocytes (6). IL-12 is proposed to play a crucial role during negative selection of CD4$^+$CD8$^+$ double-positive thymocytes, but cells which produce IL-12 in the thymus have not yet been identified (7).

DCs play an important role in T lymphocyte development and regulation of their functions, and several lines of evidence support that DCs can respond to signals delivered by T lymphocytes during their interaction. For instance, DCs lose their adhesive and phagocytic capacities after interaction with T cells through a mechanism termed T cell-mediated terminal maturation (8). Furthermore, T cell signals delivered by the ligation of TNF-related activation-induced cytokine and/or CD40 can delay DC apoptosis (9–11). However, the influence of T cells on the development and biological functions of DCs remains controversial. On the one hand, Shreedhar et al. (12) have shown that in RAG-2$^{-/-}$ mice, which are devoid of mature lymphocytes, the number of Langerhans cells is reduced and their APC functions are impaired, as demonstrated by their incapacity to induce contact hypersensitivity. In contrast, De Creus et al. (13) have shown in RAG-1$^{-/-}$ mice that the absence of mature lymphocytes does not modify the development of Langerhans cells or their functions, such as cell migration and Ag presentation. Studies investigating the influence of B lymphocytes on DCs have demonstrated that the absence of B cells, but not of T cells, causes abnormalities in DC distribution in the spleen (14). It has also been shown that in B cell-deficient μMT mice, splenic DCs produce higher levels of IL-12 than do wild-type (WT) DCs. These data suggest that B cells down-regulate IL-12 production by DCs, probably by secreting IL-10 (15).

The aim of this work was to study the influence of lymphocytes on DC development and functions. We have analyzed two populations of DCs: thymic DCs (TDCs), which are usually in constant interaction with developing T cells, and splenic DCs (SDCs), which represent professional APCs in contact with B and T cells in secondary lymphoid organs. We have compared the number, phenotype, and IL-12 production of both types of DC in mice differing in their contents of B and/or T lymphocytes, such as C57BL/6, BALB/c, pT$\alpha^{-/-}$, CD3$\epsilon^{\Delta 5/\Delta 5}$ (CD3$\epsilon^{-/-}$), and RAG-2$^{-/-}$ mice.

Our results show that 1) the total number of TDCs and SDCs is similar in all strains of mice studied, 2) DCs require T cells to acquire the capacity to produce bioactive IL-12p70 in response to activation, 3) this regulation occurs at the level of both IL-12p40 and IL-12p35 transcription, and 4) IL-4 is one of the factors involved in this regulation.

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[3] Abbreviations used in this paper: DC, dendritic cell; WT, wild type; TDC, thymic DC; SDC, splenic DC; HPRT, hypoxantine-guanine phosphoribosyltransferase; TL, T lymphocyte.

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Materials and Methods

Animals

BALB/c mice and C57BL/6 mice were purchased from IFFA-CREDO (L'Arbresle, France). Ly 5.1$^+$ C57BL/6 mice were purchased from the Centre de Développement des Techniques Avancées (Orléans, France). CD3$\epsilon^{\Delta 5/\Delta 5}$ knockout (CD3$\epsilon^{-/-}$) mice (BALB/c or C57BL/6 background) (16, 17), pT$\alpha^{-/-}$ mice (18), and RAG-2$^{-/-}$ mice (19) (both on C57BL/6 background) were maintained in our animal facility and used at 4–10 wk of age.

Reagents

Complete culture medium consisted of IMDM supplemented with 10% heat-inactivated FCS (both from Life Technologies, Grand Island, NY), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), 50 μM 2-ME, 50 U/ml penicillin, and 50 μg/ml streptomycin. Murine recombinant GM-CSF produced by a myeloma cell line transfected with the murine GM-CSF gene (a kind gift of Dr. David Gray, Institute of Cell Animal and Population Biology, University of Edinburgh, Edinburgh, U.K.) was used at 1% in the culture medium. Purified murine recombinant Flt3-ligand was purchased from R&D Systems (Abingdon, U.K.) and used at 20 ng/ml.

Purification of TDCs and SDCs

TDCs and SDCs were purified as described previously (20, 21). Briefly, thymi and spleens were digested for 30 min at 37°C with collagenase B (2 mg/ml) plus DNase I (0.4 mg/ml) (Roche Diagnostics, Mannheim, Germany) in PBS-10% FCS and were further dissociated in Ca^{2+}-free HBSS EDTA, 10 mM. Thymic and splenic cells were separated on a Nycodenz (Nycomed, Oslo, Norway) gradient. Low-density cells were enriched for CD11c expression and, for SDCs, were further separated according to CD8α expression, using a multisort anti-FITC kit (Miltenyi Biotec, Paris, France).

Culture conditions for measurement of DC IL-12 production

Purified CD11c$^+$CD8α^+ SDCs (1 $\times$ 10^6 cells/ml), total CD11c$^+$ SDCs (2 $\times$ 10^6 cells/ml), or total CD11c$^+$ TDCs (1 $\times$ 10^6 cells/ml) were cultured for 20 h in complete medium containing GM-CSF and were simultaneously stimulated with different combinations of LPS (1–10 μg/ml), anti-CD40 (1 μg/ml), IFN-γ (20 ng/ml), poly(I:C) (0.5–50 μg/ml), IL-4 (25 ng/ml), anti-IL-4 (20 ng/ml), or isotype control mAbs (20 ng/ml). Then, IL-12 production was measured by the OptEIA kit for mouse IL-12p70 and IL-12p40 (BD PharMingen, San Diego, CA), according to the protocol provided. The Abs used in this test specifically recognize the IL-12p70 heterodimer or the IL-12p40 subunit, either free or linked to p35, in both cases with a detection limit of 30 pg/ml. IL-12 measurements were performed in duplicate on the supernatant of each cell culture.

Immunolabeling procedures

The following mAbs were purchased from BD PharMingen: anti-CD3 (2C11, CyChrome-conjugated), anti-CD8α (53-6.7, CyChrome and biotin-conjugated), anti-CD11b (M1/70, biotin-conjugated), anti-CD11c (HL3, FITC-conjugated and biotin-conjugated), anti-I-A^b that cross-reacts with I-A^d of BALB/c mouse (25.9.17, biotin-conjugated), anti-TCRβ (H57-597, FITC-conjugated), anti-B220 (RA3-6B2, biotin-conjugated), anti-TER119 (TER119, biotin-conjugated), anti-Gr1 (RB6-8C5, biotin-conjugated), anti-CD40 (3/23, purified), anti-IL-4 (11B11, purified), and rat anti-mouse FcγRII/III (2.4G2, purified). Biotin-conjugated mAbs were revealed using streptavidin-PE or streptavidin-CyChrome, also from BD PharMingen. Anti-B7.2 mAbs (RMMP-1, PE-conjugated) were purchased from Caltag Laboratories (Burlingame, CA).

Cell surface phenotype was studied by FACS analysis as described (21). Data from a minimum of 10,000 cells were collected on a FACSCalibur and analyzed using CellQuest software (BD Biosciences, Mountain View, CA).

DC-T lymphocyte coculture

T lymphocytes from the spleen of WT congenic Ly 5.1$^+$ C57BL/6 mice were purified by negative selection using the following biotinylated Abs: anti-B220, anti-CD11c, anti-CD11b, anti-Gr1, anti-Ter119, and streptavidin microbeads on a MACS column (Miltenyi Biotec). The purity of T cells was analyzed by flow cytometry and typically 93% of the cells were TCR$\alpha\beta^+$, $\leq$4% TCR$\gamma\delta^+$, and $\leq$3% NK cells. Among the TCR$\alpha\beta^+$ population, ~50% were CD4$^+$ and 43% were CD8$^+$. Purified T cells were then cocultured with purified CD11c$^+$ SDCs at a ratio of 10:1 in complete medium containing stimuli for 20 h.

Reconstitution of CD3$\epsilon^{-/-}$ mice with normal T lymphocytes

Purified splenic Ly 5.1$^+$ C57BL/6 T lymphocytes were washed twice in PBS, and 15 $\times$ 10^6 cells were injected i.v. in 0.1 ml of PBS into the tail vein of Ly 5.2$^+$ CD3$\epsilon^{-/-}$ mice. Control CD3$\epsilon^{-/-}$ mice were injected with 0.1 ml of PBS alone. After 17 days, mice were treated i.p. with 10 μg/day of human Flt3-ligand (a kind gift of Dr. S. Lyman, Immunex, Seattle, WA) for 9 days. Purification of SDCs and TDCs and measurement of IL-12 production were then performed as describe above.

Quantification of IL-12p35 and IL-12p40 mRNA levels by real-time PCR

After 20 h of stimulation, total cellular RNA was extracted from SDCs by using the RNeasy Protect Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Total RNA (0.5–1 μg) was then reverse transcribed using a Superscript II RNase H$^-$ kit according to the manufacturer's instructions (Life Technologies, Pontoise, France).

Real-time PCR was conducted on a LightCycler System (Roche Diagnostics) as already described (22). Briefly, 2 μl of cDNA, 1$\times$ of LightCycler-DNA master SYBRGreen I, 3 mM $MgCl_2$, 0.5 μM each of sense and anti-sense primers, 0.2 μl (5 U/μl) of *Taq* polymerase, and 0.07 μM TaqStart Ab (Clontech, Heidelberg, Germany) were mixed and H_2O was added up to 20 μl. After a 2-min denaturation step at 95°C, the reactions were cycled 35–40 times for 5 s at 95°C, 10 s at 60°C, and 15 s at 72°C. The oligonucleotides were as follows: IL-12p35 sense, 5′-CACGCTAC CTCCTCTTTTTG-3′; IL-12p35 anti-sense, 5′-CAGCAGTGCAGGAA TAATGTT-3′; IL-12p40 sense, 5′-AAACCAGACCCGCCCAAGAAC-3′; IL-12p40 anti-sense, 5′-AAAAAGCCAACCAAGCAGAAGACAG-3′; hypoxantine-guanine phosphoribosyltransferase (HPRT) sense, 5′-GGTTAAG CAGTACAGCCCCAAAAT-3′; and HPRT anti-sense, 5′-ATAGGCACA TAGTGCAAATCAAAAGTC-3′. Product specificity was determined by melting curve analysis as described in the LightCycler handbook, by visualizing the size of PCR products after electrophoresis in agarose gels, and finally by sequencing the amplified fragments. The amount of IL-12p40, IL-12p35, and HPRT transcripts in each sample have been determined with an intern standard curve of each product in separate experiments. Then, results have been normalized by calculating the number of copies of IL-12p40 and IL-12p35 per thousand HPRT copies found in each sample.

Results

Phenotypic analysis of TDCs and SDCs from WT and lymphocyte-deficient mice

We analyzed the status of TDCs and SDCs in different models of lymphocyte-deficient mice: RAG-2$^{-/-}$ mice, which have no mature B and T lymphocytes; CD3$\epsilon^{-/-}$ mice, which are devoid of mature T cells; and pT$\alpha^{-/-}$ mice, which possess reduced numbers of T lymphocytes (~10% of WT T cell numbers). Results were compared with WT mice.

Phenotypic analysis showed that 90–99% of the SDCs and TDCs purified from the different mice coexpress CD11c and MHC class II molecules (Fig. 1, *A* and *B*, *top line*). In the thymus (Fig. 1*A*, *top line*), we distinguished two populations of TDCs: one MHC class II^{high} and one MHC class II^{low}, which represent mature and immature TDCs, respectively (23). A total of 71% of WT TDCs coexpress the lymphoid-related marker CD8α and MHC class II molecules (Fig. 1*A*, *bottom line*). These double-positive TDCs were also present in the mutant mice and represent from 62% in CD3$\epsilon^{-/-}$ mice to 83% in pT$\alpha^{-/-}$ mice of the purified DCs. It should be noted that CD8α profiles are different in WT and in lymphocyte-deficient mice. For instance, TDCs from CD3$\epsilon^{-/-}$ and RAG-2$^{-/-}$ mice displayed a maximum of fluorescence intensity up to 3 $\times$ 10^3 logarithmic units (CD8α^{high}), whereas the value obtained in WT mice did not exceed a maximum of 4 $\times$ 10^2 logarithmic units (CD8α^{low}). Therefore, there were differences in TDC phenotype among analyzed mice. In WT mice, two main populations were seen: one was MHC class II^{high}CD8α^{low} and the other was MHC class II^{low}CD8α^- (Fig. 1*A*, *left panel*). CD3$\epsilon^{-/-}$ and RAG-2$^{-/-}$ mice displayed similar TDC population patterns with a continuous gradient of CD8α expression from CD8α^- to CD8α^{high}. In pT$\alpha^{-/-}$ mice, four distinct TDC populations could

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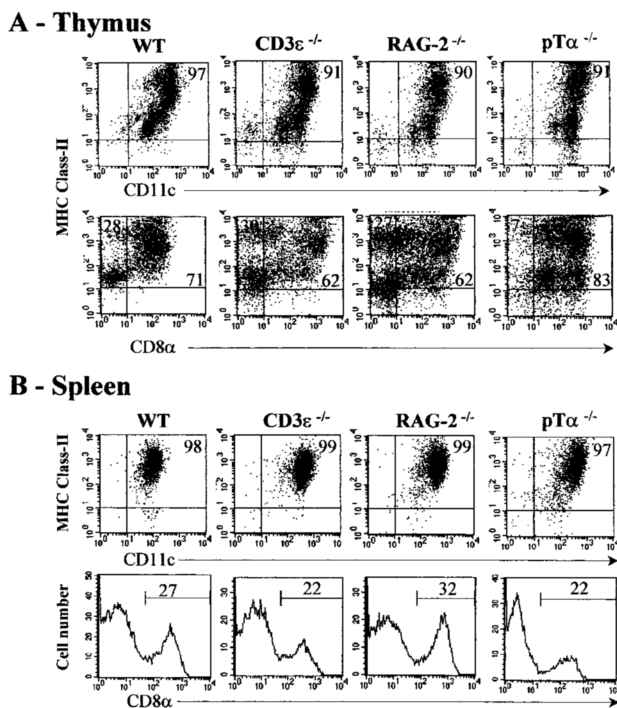

FIGURE 1. Phenotypic analysis of SDCs and TDCs from WT and lymphocyte-deficient mice. Freshly purified TDCs (*A*) and SDCs (*B*) were triple-stained with anti-CD11c FITC, anti-CD8α CyChrome, and anti-I-A^b biotinylated plus streptavidin-PE and were analyzed by FACS. Results are representative of two (pTα$^{-/-}$ mice) to five (CD3ε$^{-/-}$ mice) independent experiments.

be defined according to the level of expression of MHC class II and CD8α molecules, which range from CD8α^{low} to CD8α^{high}. At last, we demonstrated that CD8α expression by TDCs did not result from passive acquisition of T lymphocyte CD8α molecules because TDCs from T lymphocyte-deficient mice could express high levels of CD8α. Taken together, these results showed that TDCs and T lymphocytes develop independently, but lack of T lymphocytes modified the level of expression of CD8α molecules by TDCs as well as the TDC subset distribution.

In the spleen, DCs purified from WT and lymphocyte-deficient mice represented a single homogenous population of MHC class II$^+$CD11c$^+$ cells (Fig. 1*B*, *top line*). Among WT SDCs, 27% expressed CD8α. This proportion did not change significantly in the mutant mice (Mann-Whitney *U* test, $p > 0.05$).

Morphologically, SDCs and TDCs isolated from the different strains of lymphocyte-deficient mice were similar to those of WT mice, with a large cytoplasm and bean-shaped nuclei after May-Grünwald-Giemsa staining and characteristic MHC class II$^+$ cytoplasmic extensions, as shown by immunochemistry (data not shown).

In lymphocyte-deficient mice, thymocyte development was blocked at the CD44$^-$CD25$^+$ stage, leading to a 13- to 19-fold reduction of the thymus cellularity, from $196 \times 10^6 \pm 55 \times 10^6$ thymocytes in WT mice to $10 \times 10^6 \pm 3 \times 10^6$ and $15 \times 10^6 \pm 11 \times 10^6$ thymocytes in CD3ε$^{-/-}$, RAG-2$^{-/-}$, and pTα$^{-/-}$, respectively (Table I). Spleen cellularity was less affected by lymphocyte depletion, except in RAG-2$^{-/-}$ mice, in which a 4-fold reduction ($157 \times 10^6 \pm 39 \times 10^6$ to $38 \times 10^6 \pm 22 \times 10^6$) of splenocytes was seen, which was probably due to the absence of both T and B cells. The number of DCs per organ was then determined by taking into account the percentage of CD11c$^+$ cells obtained by flow cytometry analysis of the low-density fraction and the total cellularity of the organs. In thymus of deficient mice, DC number was slightly diminished, ranging from $626 \times 10^3 \pm 218 \times 10^3$ in WT mice to $130 \times 10^3 \pm 23 \times 10^3$ in pTα$^{-/-}$ mice, but this reduction was in all cases not so drastic as that of total cellularity. From this observation, it resulted that the proportion of TDCs was enriched up to 13-fold, from 0.32% in WT mice to 4.13% in CD3ε$^{-/-}$ mice. In the spleen of deficient mice, DC numbers varied from $3916 \times 10^3 \pm 2230 \times 10^3$ in CD3ε$^{-/-}$ mice to $1379 \times 10^3 \pm 479 \times 10^3$ in pTα$^{-/-}$ mice. Percentage of SDCs was also enriched, but to a lesser extent than in the thymus, ranging from 0.97% (pTα$^{-/-}$ mice) to 2.79% (RAG-2$^{-/-}$ mice). In conclusion, compared with total cellularity, number of DCs was not grossly altered in CD3ε$^{-/-}$, RAG-2$^{-/-}$, and pTα$^{-/-}$ mice compared with WT controls. Our data also showed that a fraction SDCs and TDCs from T lymphocyte-deficient mice were CD8α$^+$, thus demonstrating that this lymphoid-related marker could be expressed by DCs in the absence of T lymphocytes.

Impaired capacity of DCs from CD3ε$^{-/-}$ mice to produce IL-12

To analyze the functionality of DCs in lymphocyte-deficient mice, we next investigated the capacity of freshly purified DCs to produce IL-12, a Th1-polarizing cytokine secreted mostly by CD8α$^+$ DCs in response to a bacterial or a T cell-derived stimulus (24). We wanted to determine whether the absence of T and/or B cells affected the production of IL-12 by DCs upon in vitro stimulation. Thus, DCs were purified from the different types of mice and stimulated with LPS and anti-CD40 for 20 h, and then IL-12p70 (bioactive heterodimeric form of IL-12) and IL-12p40 (regulatory subunit of the protein) activities were measured by ELISA in supernatants.

In our standard culture method, SDCs from WT mice secreted high levels of IL-12p70 (235 pg/ml) and IL-12p40 (1757 pg/ml)

Table I. *Number of DCs in lymphoid organs of WT and lymphocyte-deficient mice*[a]

Lymphoid Organ	Cell Population	WT (n = 3)	CD3ε$^{-/-}$ (n = 6)	RAG-2$^{-/-}$ (n = 3)	pTα$^{-/-}$ (n = 2)
Thymus	All cells (×10^{-6})[b]	196 ± 55	10 ± 3	10 ± 3	15 ± 11
	DCs (×10^{-3})[c]	626 ± 218	413 ± 240	326 ± 116	130 ± 23
	% TDCs[d]	0.32	4.13	3.26	0.86
Spleen	All cells (×10^{-6})	157 ± 39	167 ± 62	38 ± 22	142 ± 32
	DCs (×10^{-3})	3376 ± 2194	3916 ± 2230	1063 ± 554	1379 ± 479
	% SDCs	2.15	2.34	2.79	0.97

[a] Presented results are the means ± SEM of two to six independent experiments.
[b] Thymus and spleen cellularity of each strain of mouse is determined by counting viable cells after collagenase digestion, excluding dead cells with acridin orange/propidium iodide.
[c] Number of DCs in each organ is determined from the percentage of CD11c$^+$ cells in the low-density fraction after FACS analysis and the total organ cellularity of each organ analyzed.
[d] The percentage of TDCs and SDCs is determined by the ratio between mean number of DCs in the organ and mean organ cellularity.

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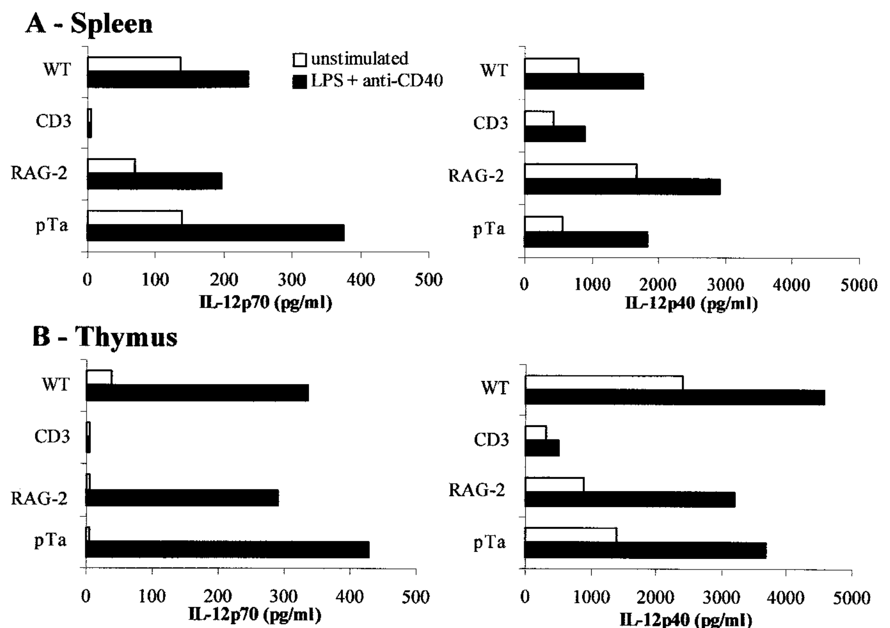

FIGURE 2. Production of IL-12 by purified DCs. $CD11c^+CD8\alpha^+$ SDCs (*A*) and total $CD11c^+$ TDCs (*B*) (both 1×10^6 cells/ml) purified from WT, $CD3\epsilon^{-/-}$, RAG-$2^{-/-}$, and $pT\alpha^{-/-}$ mice were cultured for 20 h without (unstimulated) or with LPS (1 μg/ml) plus anti-CD40 (1 μg/ml). Supernatants were tested for the presence of IL-12p70 and IL-12p40 by ELISA. Detection of cytokine <30 pg/ml was considered zero. Data shown are representative of four ($pT\alpha^{-/-}$ mice) to six ($CD3\epsilon^{-/-}$ mice) independent experiments.

after in vitro stimulation with LPS plus anti-CD40 (Fig. 2*A*). Unstimulated cells secreted detectable amounts of both cytokines, which can be attributed to the presence of GM-CSF in the culture medium, which was reported to provoke IL-12 production (25). In contrast, SDCs from $CD3\epsilon^{-/-}$ mice did not produce significant amounts of IL-12p70 (<30 pg/ml) and little IL-12p40 (894 pg/ml) after LPS plus anti-CD40 stimulation (Fig. 2*A*). The production of IL-12p70 by $CD3\epsilon^{-/-}$ SDCs was not modified when stimulation was conducted with serial doses of LPS in the range of 0.5–2 μg/ml (data not shown). Furthermore, no production of IL-12p70 by SDCs from $CD3\epsilon^{-/-}$ mice was obtained after stimulation with LPS plus IFN-γ, anti-CD40 plus IFN-γ, or poly(I:C) (data not shown). In the absence of stimuli, $CD3\epsilon^{-/-}$ SDCs produced no detectable amounts of IL-12p70 and only 429 pg/ml of IL-12p40. In contrast, IL-12p70 and IL-12p40 production by SDCs from RAG-$2^{-/-}$ and $pT\alpha^{-/-}$ mice were similar to WT. For instance, amounts of IL-12p70 varied from 196 to 376 pg/ml with stimulation and from 69 to 138 pg/ml without stimulation.

TDCs from WT, RAG-$2^{-/-}$, and $pT\alpha^{-/-}$ mice secreted high levels of both of IL-12p70 (291–429 pg/ml) and IL-12p40 (3193–4560 pg/ml) when stimulated with LPS plus anti-CD40 (Fig. 2*B*). As with SDCs, TDCs from $CD3\epsilon^{-/-}$ mice did not produce IL-12p70 under this condition, and secretion of IL-12p40 was reduced approximately 9-fold when compared with WT TDCs. In the absence of stimulation, TDCs from all strains of lymphocyte-deficient mice analyzed secreted an undetectable level of IL-12p70, whereas IL-12p40 was secreted between 306 and 1402 pg/ml. For SDCs and TDCs from each line of mice, similar results were obtained when cells were stimulated with LPS plus IFN-γ or anti-CD40 plus IFN-γ (data not shown).

Thus, when developed in the absence of T cells but in the presence of B cells ($CD3\epsilon^{-/-}$ mice), DCs from thymus and spleen were unable to secrete bioactive IL-12. When only 10% of T lymphocytes were present as in $pT\alpha^{-/-}$ mice or in the absence of both T and B lymphocytes as in RAG-$2^{-/-}$ mice, IL-12 production was similar to that in WT mice.

DC phenotype after in vitro stimulation

To determined whether the impaired IL-12p70 production by SDCs from $CD3\epsilon^{-/-}$ mice after in vitro stimulation (Fig. 2*A*) was linked to a general defect of activation, we next examined the expression of the activation marker B7.2. We compared the expression of B7.2 proteins on SDCs from $CD3\epsilon^{-/-}$ and WT mice, before and after in vitro activation by LPS plus anti-CD40. Freshly purified SDCs from WT and $CD3\epsilon^{-/-}$ mice did not express detectable levels of B7.2 (Fig. 3, *thin line*). Strikingly, after activation, 37.1% of SDCs from WT and 30.2% from $CD3\epsilon^{-/-}$ mice were $B7.2^+$ (Fig. 3, *thick line*). Thus, it seems that impaired IL-12 secretion by $CD3\epsilon^{-/-}$ SDCs was not related to a defect in the signal transduction induced by LPS plus anti-CD40.

IL-12 production by DCs from $CD3\epsilon^{-/-}$ mice is restored by T cells: in vitro and in vivo analysis

To establish the possible activating role of T lymphocytes on DC IL-12 secretion, we complemented the lack of T lymphocytes in $CD3\epsilon^{-/-}$ mice with purified T cells from WT mice (≥93% $CD3^+$,$TCR\alpha\beta^+$), either by addition into $CD3\epsilon^{-/-}$ SDC cultures or by injection into $CD3\epsilon^{-/-}$ recipient mice.

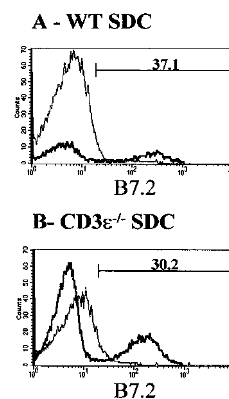

FIGURE 3. Phenotype of SDCs after in vitro stimulation. Purified SDCs from WT (*A*) and $CD3\epsilon^{-/-}$ mice (*B*) were cultured in vitro for 20 h with LPS plus anti-CD40 and then were analyzed by FACS. Expression B7.2 on SDCs before (*thin line*) and after stimulation (*thick line*) is shown. The percentage of positive cells after stimulation is indicated. Data are representative of four independent experiments.

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First, we looked at the production of IL-12 after coculturing T cells with SDCs in vitro. For these experiments, SDCs were purified from Flt3-ligand-treated CD3$\epsilon^{-/-}$ mice to increase the number of DCs necessary to do IL-12 measurements. Purified CD11c^{+} SDCs were then cocultured in vitro with splenic T cells purified from WT congenic mice. As shown previously (Fig. 2*A*), CD3$\epsilon^{-/-}$ SDCs cultured without T cells produced no bioactive IL-12, even after Flt3-ligand treatment. However, they secreted a higher level of IL-12p40 than did CD3$\epsilon^{-/-}$ SDCs purified from non-Flt3-ligand-treated mice: 894 vs 4387 pg/ml (Fig. 4*A*). Thus, Flt3-ligand treatment had no effect on IL-12p70 secretion by DCs from CD3$\epsilon^{-/-}$ mice, whereas it enhanced the production of IL-12p40 with and without stimuli. In the presence of T cells in the culture, stimulated SDCs from CD3$\epsilon^{-/-}$ mice then produced bioactive IL-12p70 (478 pg/ml) in the same range as SDCs from WT mice (Fig. 4*A*). An increase of IL-12p40 production was also observed up to 25,600 pg/ml in these conditions (Fig. 4*A*). Lower but significant increases of both IL-12p70 (58 pg/ml) and IL-12p40 (2126 pg/ml) were obtained without stimulation.

Second, for in vivo analysis, CD3$\epsilon^{-/-}$ mice were reconstituted by i.v. injection of splenic T cells purified from WT congenic mice. Seventeen days later, mice were treated with Flt3-ligand, and then SDCs were purified and IL-12 production was measured after in vitro stimulation without further addition of T cells. Fifteen million splenic T cells were injected. They were found in the spleen ($18.6 \times 10^6 \pm 4.4 \times 10^6$/spleen), in the blood ($1.58 \times 10^6 \pm 0.96 \times 10^6$/ml), and a lower number can be retrieved from the thymus ($0.26 \times 10^6 \pm 0.13 \times 10^6$/thymus) (data not shown). Homeostatic proliferation of injected T lymphocytes probably explained that $>15 \times 10^6$ T cells are found in grafted animals (26). As we can see in Fig. 4*B*, T cell reconstitution restored the capacity of IL-12p70 production by CD3$\epsilon^{-/-}$ SDCs after stimulation with LPS plus anti-CD40 up to 1010 pg/ml. This level of IL-12p70 production was similar to that obtained with WT SDCs under the same conditions. T cell reconstitution of CD3$\epsilon^{-/-}$ mice increased IL-12p40 secretion from 8,327 to 13,901 pg/ml after LPS plus anti-CD40 stimulation and lowered the level of IL-12p40 secretion from 4,666 to 4,146 pg/ml without in vitro stimulation (Fig. 4*B*). Similar results were obtained with SDCs stimulated by IFN-γ plus anti-CD40 and with TDCs under the same conditions (data not shown).

Thus, both experiments using either addition of T cells into DC cultures or T cell reconstitution of CD3$\epsilon^{-/-}$-deficient mice demonstrated that the presence of T cells potentiates IL-12 production by DCs.

IL-12 mRNA expression by SDCs from CD3$\epsilon^{-/-}$ mice

To determined whether the deficiency in IL-12 production by DCs from CD3$\epsilon^{-/-}$ mice and the recovery of production after complementation with T cells were correlated with changes in IL-12 transcripts, levels of IL-12p40 and IL-12p35 mRNA were quantified

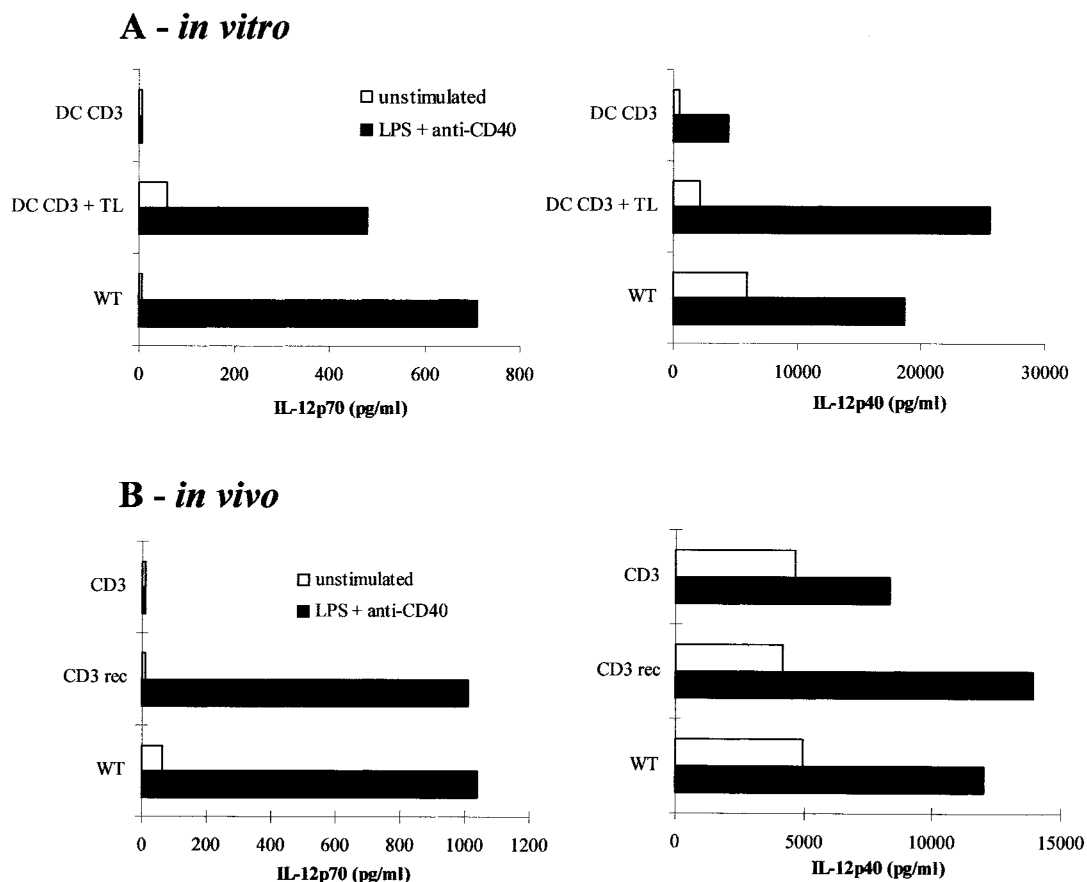

FIGURE 4. Activating role of T lymphocytes on IL-12 production by SDCs from CD3$\epsilon^{-/-}$ mice. Activating role of T lymphocytes (TLs) was assessed in vitro by coculturing TLs with SDCs from CD3$\epsilon^{-/-}$ mice (*A*): CD11c^{+} SDCs (2×10^6 cells/ml) and splenic TLs purified from WT congenic mice were mixed at a ratio of 1:10 and stimulated or not with LPS (1 μg/ml) plus anti-CD40 (1 μg/ml) for 20 h. Then the supernatants were tested for IL-12p70 and IL-12p40 presence by ELISA. These results are representative of three separate experiments. In vivo investigation (*B*) consists of the reconstitution of CD3$\epsilon^{-/-}$ mice with congenic WT splenic TLs. CD11c^{+} SDCs from WT, CD3$\epsilon^{-/-}$, and reconstituted CD3$\epsilon^{-/-}$ mice, all treated with Flt3-ligand, were purified as described and cultured for 20 h with with LPS (1 μg/ml) plus anti-CD40 (1 μg/ml). These results are representative of five independent experiments.

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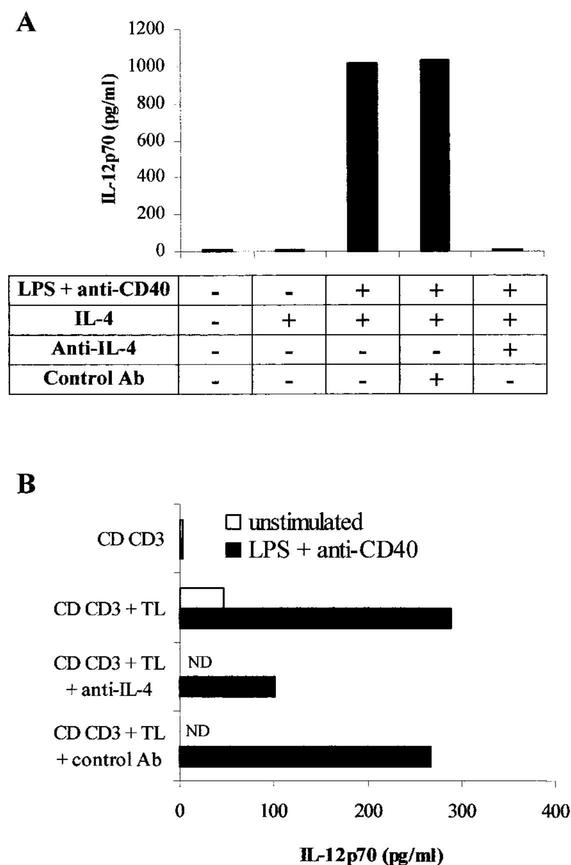
by real-time PCR. SDCs were purified from CD3$\epsilon^{-/-}$ mice, which were reconstituted or not with T lymphocytes and stimulated in vitro for 20 h with LPS plus anti-CD40. Total RNA samples were converted into cDNA and the number of copies of IL-12p40, IL-12p35, and HPRT transcripts present in each sample were determined using an intern standard curve of each product. Then, results were normalized by calculating the number of copies of IL-12p40 and IL-12p35 per thousand HPRT copies found in the same sample. Without stimulation, numbers of IL-12p40 and IL-12p35 mRNA copies present in CD3$\epsilon^{-/-}$ and WT SDCs were low and did not change significantly when CD3$\epsilon^{-/-}$ mice were reconstituted (Fig. 5). Nevertheless, upon stimulation with LPS plus anti-CD40, 6580 ± 3 copies of IL-12p40 mRNA were detected in CD3$\epsilon^{-/-}$ SDCs, whereas only 363 ± 36 copies of IL-12p40 mRNA per thousand copies of HPRT were present in WT SDCs. At the same time, 12 ± 8 copies of IL-12p35 mRNA per thousand copies of HPRT were found in SDCs from CD3$\epsilon^{-/-}$ mice. This last value was similar to that obtained in stimulated SDCs from WT mice (8 ± 1 copies of IL-12p35 mRNA per thousand copies of HPRT). These results suggested that absence of bioactive IL-12 production by SDCs from CD3$\epsilon^{-/-}$ mice was not solely due to a defect in IL-12p35 mRNA transcription, because a similar level of this mRNA was present in CD3$\epsilon^{-/-}$ and WT SDCs, the latter producing a normal level of bioactive IL-12.

Reconstitution of CD3$\epsilon^{-/-}$ mice with normal T cells induced a 12-fold decrease of the IL-12p40 transcript number down to 544 ± 12 copies. In parallel, IL-12p35 gene expression was induced >11-fold, up to 141 ± 18 copies. Thus, we could suppose that the restoration of IL-12 production by SDCs from CD3$\epsilon^{-/-}$ mice that have been reconstituted with T cells was linked to a modification of the balance between IL-12p40 and IL-12p35 subunit expression.

Role of IL-4

It has been established that IL-4 increased bioactive IL-12 production by enhancing IL-12p35 subunit and down-regulating IL-12p40 subunit secretion in human and mouse DCs (25). We have demonstrated previously that the reconstitution of CD3$\epsilon^{-/-}$ mice with WT T cells modifies IL-12 gene expression in a way similar to that of IL-4. Thus, we wondered whether IL-4 played a role in the restoration of IL-12 production by SDCs from reconstituted CD3$\epsilon^{-/-}$ mice and whether IL-4 by itself can restore IL-12p70 production by SDCs from CD3$\epsilon^{-/-}$ mice.

In CD3$\epsilon^{-/-}$ SDCs, IL-12 secretion was restored when IL-4 was added together with LPS plus anti-CD40, whereas IL-4 alone had no effect (Fig. 6*A*). This result was specific to IL-4 because its effect was completely inhibited by anti-IL-4 Ab but not by control mAb. Thus, SDCs from CD3$\epsilon^{-/-}$ mice were intrinsically able to produce bioactive IL-12, but the active secretion of IL-12 requires strong stimulation. LPS plus anti-CD40 stimulus alone thus was not powerful enough and required a help which could be provided by IL-4.



LPS + anti-CD40	-	-	+	+	+
IL-4	-	+	+	+	+
Anti-IL-4	-	-	-	-	+
Control Ab	-	-	-	+	-

FIGURE 6. Role of IL-4 on IL-12 production by SDCs from CD3$\epsilon^{-/-}$ mice. *A*, CD11c$^+$ SDCs (2×10^6 cells/ml) from CD3$\epsilon^{-/-}$ mice treated with Flt3-ligand were purified and cultured for 20 h without or with different combinations of LPS (1 μg/ml), anti-CD40 (1 μg/ml), IL-4 (25 ng/ml), anti-IL-4 (20 ng/ml), and control Ab (20 ng/ml). Data shown are representative of three independent experiments. *B*, CD11c$^+$ SDCs (2×10^6 cells/ml) and splenic TLs purified from WT congenic mice were mixed at a ratio of 1:10 and stimulated or not with LPS (1 μg/ml) plus anti-CD40 (1 μg/ml) for 20 h in presence or absence of anti-IL-4 or control Ab. Results are representative of three independent experiments. In both cases, supernatants were tested in duplicate for the presence of IL-12p70 by ELISA. Detection of cytokine <30 pg/ml was considered zero. ND, Not determined.

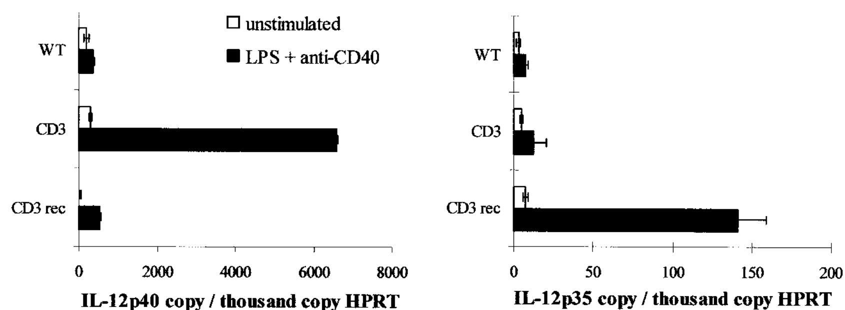


FIGURE 5. Determination of IL-12p40 and IL-12p35 mRNA levels by real-time PCR. SDCs from WT, CD3$\epsilon^{-/-}$, and reconstituted CD3$\epsilon^{-/-}$ mice treated with Flt3-ligand were incubated either in medium alone (unstimulated) or in the presence of LPS (1 μg/ml) plus anti-CD40 (1 μg/ml). After 20 h, mRNA was extracted, reverse transcribed, and amplified by real-time PCR using primers specific for IL-12p40, IL-12p35, or HPRT. IL-12p40 and IL-12p35 mRNA levels were quantified and normalized against HPRT mRNA level using intern standard curves. Data shown are the mean of triplicate quantification and are representative of four independent experiments.

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Furthermore, we analyzed the effect of the anti-IL-4 mAb during the coculture of CD3$\epsilon^{-/-}$ SDCs and WT T lymphocytes. As shown previously, CD3$\epsilon^{-/-}$ SDCs alone, stimulated or not, did not produce bioactive IL-12 (Fig. 6*B*). IL-12p70 secretion was restored up to 288 pg/ml when CD3$\epsilon^{-/-}$ SDCs were stimulated in the presence of WT T cells. We measured the amount of IL-4 present in the coculture supernatant and found that it did not exceed 32 pg/ml (data not shown). Addition of 20 ng/ml anti-IL-4 mAb, but not a control mAb, partially inhibited IL-12p70 production to 101 pg/ml. As shown in Fig. 6*A*, this quantity of anti-IL-4 mAb was able to completely inhibit 25 ng/ml IL-4, which was far more than the IL-4 amount found in the coculture supernatant. In conclusion, even if IL-4 alone could restore IL-12 production by CD3$\epsilon^{-/-}$ SDCs in vitro, it was not the only molecule involved in DC/T cell coculture experiments.

Discussion

Cellular cooperation between cells of the immune system is fundamental for the development of an appropriate immune response. We have studied the influence of T and B lymphocytes on the development and functions of DCs, key actors in the initiation of the specific immune response.

Several studies in different models of T lymphocyte-deficient mice indicate that the absence of T cells does not alter the number of DCs. For instance, TCR$\alpha^{-/-}$ mice, which are devoid of mature T lymphocytes, possess a normal number of DCs in spleen as compared with WT mice (14). Furthermore, bone marrow grafts from Notch$^{-/-}$ mice, which lack early T cell progenitors, generate a normal number of DCs in the thymus of the recipients (27). In this study, we show that pT$\alpha^{-/-}$ mice, which have a reduced number of T cells, and CD3$\epsilon^{-/-}$ mice, which have no TCR$\alpha\beta$, no TCR$\gamma\delta$ T cells, and no T NK cells, have normal numbers of DCs in thymus and in spleen as compared with WT mice, despite a strong reduction in their thymus sizes. Our data in RAG-2$^{-/-}$ mice, lacking both T and B lymphocytes, indicate minor, if any, differences in numbers of TDCs and SDCs. It should be noted that other studies reported modifications of DC counts in T and B lymphocyte-deficient mice. This discrepancy can be explained by different experimental procedures, such as the use of different strains of mice, RAG-1$^{-/-}$ vs RAG-2$^{-/-}$ (28), or the enumeration of DCs after overnight culture (14). The absence of B and/or T lymphocytes does not modify the phenotype or the morphology of the SDCs and TDCs. Our data show that CD8α is a true marker of DCs and that its expression in spleen and thymus is independent of the presence of T lymphocytes. Interestingly, TDCs were previously described to be all CD8α^{-} in 5-day-old RAG-2$^{-/-}$ mice (29). Here, we show expression of CD8α by DCs from 2-mo-old animals. Taken together, these results suggest that the synthesis of CD8α by TDCs appears late after birth in the absence of T lymphocytes. This observation is in agreement with the model that CD8α expression is developmentally regulated on TDCs rather than a lineage marker (27).

DCs polarize T lymphocytes by producing IL-12, which promotes Th1 responses. IL-12 production can be affected by exogenous factors such as microbial infections or by stress, suggesting that IL-12 production strongly depends upon the general status of the environment of the DCs (30). Therefore, we investigated the putative role of T lymphocytes on IL-12 production by analyzing DCs from different lymphocyte-deficient mice. Analysis of mice lacking both T and B cells revealed that DCs from RAG-2$^{-/-}$ mice have normal IL-12 production, as previously shown for DCs from SCID mice (31). However, we provide evidence that SDCs and TDCs purified from CD3$\epsilon^{-/-}$ mice, lacking only T lymphocytes, are unable to produce IL-12 after in vitro stimulation by LPS plus anti-CD40. IL-12 production is recovered after either addition of T lymphocytes in DC cultures or after T lymphocyte transfer in CD3$\epsilon^{-/-}$ mice. These complementation experiments, first, ascertain that the invalidation of the CD3ϵ gene does not alter the potentiality of DCs to produce IL-12 and, second, demonstrate the role of T lymphocytes in promoting IL-12 production by DCs. It should be noted that CD3$\epsilon^{-/-}$ mice contain CD8α^{+} DCs, which are known to be the main IL-12-producing cells. Thus, the lack of IL-12 production by DCs in T lymphocyte-deficient mice cannot be explained by the absence of CD8α^{+} DCs, and this determined that the DC phenotype is dissociated from IL-12 capacity production. Furthermore, the inability of these CD8α^{+} DCs to produce IL-12 cannot be attributed to a lack of response to activation signals because these cells can express B7.2 molecules after LPS plus anti-CD40 stimulation.

Absence of IL-12 production by CD3$\epsilon^{-/-}$ SDCs is not due to a defect in IL-12p35 gene transcription because a similar level of mRNA is obtained in SDCs from CD3$\epsilon^{-/-}$ and WT mice after stimulation. However, SDCs from CD3$\epsilon^{-/-}$ mice possess much more IL-12p40 mRNA transcript than do SDCs from WT mice. Despite this, SDCs from CD3$\epsilon^{-/-}$ mice still produce less IL-12p40 protein than do SDCs from WT mice. Thus, protein production is not proportional to transcription of the gene, suggesting that posttranscriptional modifications may occur that prevent, first, the production of a large amount of IL-12p40 subunit and/or, second, the association of the IL-12p35 and IL-12p40 subunits to form bioactive IL-12.

IL-12p40 can also be associated with IL-23p19 to form bioactive IL-23 (32). It is theoretically possible, because IL-12p40 is produced in large excess compared with IL-12p35, that it is used to form IL-23 in CD3$\epsilon^{-/-}$ mice. However, by quantitative RT-PCR, we never detect IL-12p19 transcripts (with a detection limit of seven copies per thousand copies of HPRT) in SDCs from CD3$\epsilon^{-/-}$ mice with or without reconstitution with T lymphocytes and with or without stimulation by LPS and anti-CD40 (data not shown). Therefore, lack of IL-12p70 secretion in T lymphocyte-deficient mice is not compensated by IL-23 and is probably due to posttrancriptional regulation, as proposed by Carra et al. (33) for human DCs.

Reconstitution of CD3$\epsilon^{-/-}$ mice with T lymphocytes increases IL-12p35 mRNA levels, whereas IL-12p40 is diminished. IL-4 similarly influences the transcription of IL-12 genes in DCs. For instance, addition of IL-4 during stimulation strongly enhances the secretion of bioactive IL-12p70 and inhibits the production of the IL-12p40 homodimer (34, 35). These IL-12 modulations are regulated at the transcriptional level of IL-12p35 and IL-12p40 genes (35).

If SDCs from CD3$\epsilon^{-/-}$ mice are stimulated with CD40-ligand plus IFN-γ in the presence of IL-4, these SDCs produced IL-12p70 (36). We have obtained similar results with CD3$\epsilon^{-/-}$ SDCs stimulated with LPS plus anti-CD40 in the presence of IL-4 (Fig. 6*B*). Then, we assessed the role of IL-4 during the coculture of SDCs from CD3$\epsilon^{-/-}$ mice and T lymphocytes. Because anti-IL-4 mAb only partially inhibits IL-12 production, this suggests that IL-4 is not the only molecule involved in this regulation.

Finally, the comparison of IL-12 production levels by DCs purified from mice differing in their content of B and/or T lymphocytes suggests that T and B cells have opposite effects on DCs. It has been demonstrated that B cells could inhibit the production of IL-12 by SDCs via the secretion of IL-10 (15). Therefore, the impaired IL-12 production of SDCs in mice that lack T cells, such as CD3$\epsilon^{-/-}$ mice, could be attributed in a first instance to the inhibition by B cells. The analysis of pT$\alpha^{-/-}$ mice, which possess 10% of normal levels, and CD3$\epsilon^{-/-}$ mice reconstituted with WT

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T cells shows that few T cells allow DCs to recover their potentiality to secrete IL-12 in response to activation. T cell action may occur at two different levels, first on B cells by altering their inhibitory action on DCs and second directly on DCs by preparing them to respond to activation. The direct action of T cells is supported by the fact that DCs isolated from CD3$\epsilon^{-/-}$ mice, which are unable to produce IL-12, retrieve their potentiality to secrete IL-12 after stimulation when purified T cells are added. Moreover, Tzehoval et al. (37) have shown that injection of mature T cells in athymic mice restored the functionality of macrophages and that Ag presentation by Langerhans cells from adult SCID and RAG-2$^{-/-}$ mice was restored upon T cell reconstitution (12). Taken together, these observations suggest that T lymphocytes educate the immunogenic functions of APCs.

Recently, two groups have shown that DCs and T lymphocytes can form a functional immunological synapse in the absence of exogenous Ag (38, 39). This synapse includes the molecules required in cellular interactions necessary to achieve prolonged survival and activation of naive T cells. In these synapses, signals delivered by DCs are transmitted to the nucleus of T cells because they result in transcription of IL-12Rβ2 and IFN-γ and slow proliferation (38). However, little is yet known concerning the events that take place in the DCs in response to the contact with T cells in the absence of exogenous Ag. It is possible that T cell signals, allowing DCs to be fully competent to produce IL-12, take place in these kinds of synapses because they occur in the absence of Ag-specific T cells.

In conclusion, the absence of T and/or B cells does not dramatically affect the development of SDCs and TDCs. However, production of IL-12 by these cells is under the control of both B and T lymphocytes, which display antagonist actions. In a normal situation, when both T and B cells are present, DCs are fully able to produce IL-12 in response to stimulation and induce the development of Th1 lymphocytes. In the absence of both T and B lymphocytes, DCs can secrete IL-12, supporting the concept that DCs are constitutively potent to be activated. B lymphocytes hamper the potentiality of DCs to produce IL-12, thus they act as negative regulators. Finally, T lymphocytes restore the capacity of DCs to secrete IL-12, so T lymphocytes potentiate IL-12 production by DCs.

Acknowledgments

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