Human Monocyte–derived Dendritic Cells Induce Naive T Cell Differentiation into T Helper Cell Type 2 (Th2) or Th1/Th2 Effectors: Role of Stimulator/Responder Ratio

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Abstract

The subset of dendritic cells (DCs) and the nature of the signal inducing DC maturation determine the capacity of DCs to generate polarized immune responses. In this study, we show that the ability of human monocyte-derived DCs (myeloid DC_1) to promote T helper type 1 (Th1) or Th2 differentiation was also found to be critically dependent on stimulator/responder ratio. At a low ratio (1:300), mature DCs that have been differentiated after inflammatory (*Staphylococcus aureus* Cowan 1 or lipopolysaccharide) or T cell–dependent (CD40 ligand) stimulation induced naive T cells to become Th2 (interleukin [IL]-4^+, IL-5^+, interferon γ) effectors. Th2 differentiation was dependent on B7–CD28 costimulation and enhanced by OX40–OX40 ligand interactions. However, high DC/T cell ratio (1:4) favored a mixed Th1/Th2 cell development. Thus, the fact that the same DC lineage stimulates polarized Th1 or Th2 responses may be relevant since it allows the antigen-presenting cells to initiate an appropriate response for the signal received at the peripheral sites. Controlling the number and the rate of DC migration to the T cell areas in lymphoid tissues may be important for the therapeutic use of DCs.

Key words: Th1 • Th2 • dendritic cells • B7-OX40 • interleukin 4

Introduction

The differentiation of naive T cells into Th1 or Th2 effectors is determined by the cytokine environment (IL-12 versus IL-4), the nature and the strength of the T cell receptor–mediated signal, the genetic background, and the type and the activation state of the dendritic cells (DCs)[1] (1–4). Two distinct lineages of human and mouse DCs have been described: myeloid and lymphoid DCs (5, 6). In rodents, myeloid DCs promote Th2 differentiation in vivo and in vitro (7, 8), whereas lymphoid DCs, the major producers of IL-12, are involved in the initiation and maintenance of Th1 responses. In humans, monocyte-derived DCs (DC_1) reportedly induce Th1 cells (9), and the plasmacytoid DCs (DC_2) do not produce IL-12, elicit Th2 responses, and are the major source of IFN-α (10, 11).

The ability of monocytes to differentiate in vitro into DCs under physiological conditions (12) has now been confirmed in vivo (13); monocytes that phagocytosed subcutaneously injected fluorescent microperes, migrate to the lymph nodes and differentiate into DCs (13). It also appears that the nature of the maturation signals received by monocyte-derived immature DCs at the peripheral sites as well as the type of locally released inflammatory mediators are critical to polarize the immune response (14, 15). A relevant question to ask is whether myeloid-derived immature DCs (iDCs), the sentinels of the immune system located at sites of antigen entry (i.e., in nonlymphoid tissues), may keep the flexibility to trigger either a Th1 or a Th2 response after the encounter with an invading pathogen. Here we show that human DC_1 may indeed trigger the development of naive T cells into Th2 effectors, provided that they display a mature phenotype (high level of costimulatory molecules) and are cultured with naive T cells at a low density (1:300) DC/T cell ratio.

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[1]*Abbreviations used in this paper:* CTLA, cytolytic T lymphocyte–associated Ag; DC, dendritic cell; DC_1, human monocyte-derived DC; DC_2, plasmacytoid DC; iDC, immature DC; mDC, mature DC; SAC, *Staphylococcus aureus* Cowan 1.

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Materials and Methods

Generation of Monocyte-derived iDCs and Mature DCs. PBMCs and monocytes were isolated as described (16). To generate iDCs, monocytes were cultured for 1 h in RPMI 1640 with 10 mM Hepes, 2 mM l-glutamine, 100 IU penicillin G, and 100 μg/ml streptomycin. Nonadherent cells were removed, and after washing adherent cells were cultured for 6 d with GM-CSF (25 ng/ml) and IL-4 (25 ng/ml) in complete RPMI 1640 medium supplemented with 10% fetal bovine serum. Some iDCs were prepared using different GM-CSF/IL-4 ratios (10:100; 5:200 ng/ml) with identical results. Mature DCs (mDCs) were generated by stimulating iDCs in complete medium containing GM-CSF and IL-4 for an additional 2 d with: *Staphylococcus aureaus* Cowan 1 strain (SAC, 0.01% vol/vol) (Pansorbin; Calbiochem-Novabiochem); SAC plus IFN-γ (500 U/ml); L-CD40L transfectants (1.25×10^6/well; provided by Dr. J. Banchereau, Schering-Plough, Dardilly, France); IL-1 (1 ng/ml) plus TNF-α (10 ng/ml); LPS (1 μg/ml) (from *Escherichia coli* serotype 0111:B4; Sigma-Aldrich); LPS plus IFN-γ or IFN-γ for 24 h followed by LPS stimulation for 48 h (γ-primed LPS). Phenotype of different mDCs was analyzed using PE-conjugated anti-CD83, -CD40, -CD80, -CD86, and anti–HLA-DR mAbs (ID Labs). The mDC preparations contained <1% $CD14^+$, $CD3^+$, $CD20^+$, and $CD56^+$ cells.

Purification of Human Naive T Cells and DC–T Cell Cocultures. Naive $CD4^+$ T cells were isolated from umbilical cord blood of healthy neonates as described (16). In brief, mononuclear cells were obtained by centrifugation on Lymphoprep (Nycomed Pharma As) and were treated with l-leucine methyl ester to remove monocytes and NK cells. Cell preparation was enriched in T cells by E-rosetting and $CD4^+$ T cells were obtained by treating rosette-forming cells with Lympho-kwik T helper (One Lambda). The resulting populations were >98% viable (trypan blue dye exclusion); >98% $CD3^+$, $CD4^+CD8^-$, and $CD45RA^+$; and contained no detectable $CD45RO^{hi}$, $CD25^+$, $CD19^+$, and $CD56^+$ cells. Primary MLRs were conducted in 96-well U-bottomed tissue culture plates by adding mitomycin C–treated mDCs to 2.5×10^5 allogeneic $CD4^+$ T cells in 250 μl complete culture medium at 1:4 and 1:300 stimulator (DCs)/responder (T cells) ratio. Some cultures were supplemented with neutralizing Abs to block endogenous cytokines: anti–IL-4 mAb clone 8F12 (5 μg/ml) plus anti-IL-4R mAb (5μg/ml) (R&D Systems); anti–IL-12 mAb (clone 8.6, 10 μg/ml; Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ), or isotype-matched IgG; or were supplemented with neutralizing anti-CD40L (clone M92; Immunex), anti-OX40L mAb (clone 5A8, 10 μg/ml [15]), normal human FcIg, or CTLA-4–Fc (5 μg/ml; P.S. Linsley, Bristol-Myers, Princetown, NY). After 4 d of culture, 150 μl of culture supernatant was replaced with fresh medium containing 100 U/ml of IL-2. On days 6 and 8, cultures were split and expanded in the presence of 50 U/ml of IL-2. After 7–10 d of IL-2 expansion, cells were washed, counted, and viable cells were tested for cytokine production. T cells (10^6/ml) were restimulated with anti-CD3 mAb (UCHT1, 200 ng/ml; P. Beverly, University College and Middlesex School of Medicine, London, UK) immobilized on mitomycin C–treated CD32-B7 L double transfectant cells prepared as described (17). Some T cells were restimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml). 24 or 48 h after restimulation, the cell-free supernatants were collected and frozen at −20°C until assayed. Note that in some experiments, DC–T cell cocultures were performed for 6–7 d, washed, and immediately restimulated with anti-CD3 immobilized on mitomycin C–treated CD32-B7 L double transfectant cells.

Flow Cytometry Analysis of Intracellular Cytokines. After IL-2 expansion, T cells (10^6/ml) in six-well tissue culture plates were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) or anti-CD3 immobilized on L–CD32-B7 transfectants for 5 h. Monensin (3 μM; Biosource International) was added for 2 h before staining to prevent cytokine secretion. Cells were collected, fixed, and intracellular cytokines were detected by flow cytometry using a commercial kit (IC Screen™; Biosource International) with FITC-anti–IFN-γ mAb and PE-anti–IL-4 mAb (BD PharMingen). Stained cells were analyzed using a FACScan™ (Becton Dickinson).

Quantitation of Cytokine Secretion by ELISA or RIA. The amounts of IL-12 p70 and TNF-α in the culture supernatant of activated iDCs were measured after 24 h by a two-site sandwich ELISA as described (16). Anti–IL-12 mAbs were provided by M. Gately (Hoffmann-La Roche). In T cell cultures, IL-2, IL-4, IL-5, IL-13, and IFN-γ measurements were determined using ELISA or RIA as described (15).

Statistical Analysis. The Student's paired *t* test was used to determine statistical significance of the data. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Results

IL-12 p70 and TNF-α Release by Activated Myeloid DCs. Peripheral blood monocytes were differentiated into iDCs after culturing with GM-CSF and IL-4. Stimulation for 24 h with different bacterial products or L-CD40L transfectants induced the release of large amounts of IL-12 p70 and TNF-α (Fig. 1); the lowest levels were secreted after LPS or

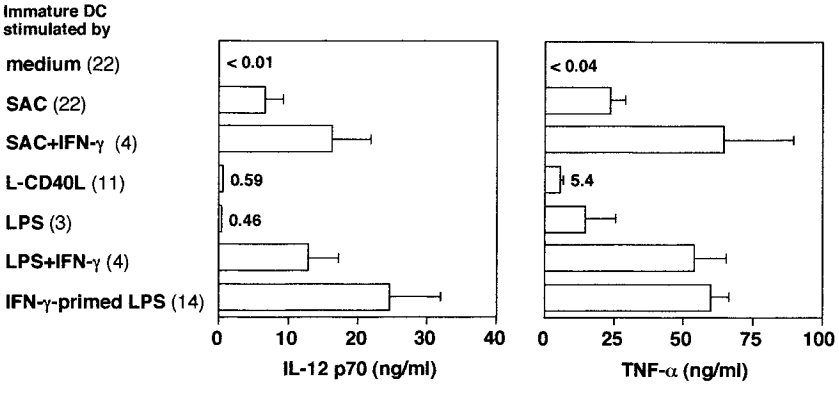

Figure 1. Cytokine production by activated myeloid DCs. IL-12 p70 and TNF-α secretion after 24 h activation of iDC with the indicated stimuli. Results are the mean values ± SEM of at least three independent experiments. The number of separate experiments is indicated in parenthesis.

CD40L stimulation. Note that IFN-γ, added during the culture together with, or 1 d before, the stimulus (γ-primed LPS) significantly enhanced cytokine release ($P < 0.05$). Activation of iDCs with TNF-α and IL-1-α gave similar cytokine levels as LPS stimulation (not shown). The nature of the activating signals did not influence the phenotype of mDC (DC_1) that expressed high levels of CD83, CD40, CD80, CD86, and MHC class II (Tanaka, H., personal observations).

Human DC_1 Promote the Differentiation of Naive T Cells into Th2 at 1:300 Stimulator/Responder Ratio. We next evaluated the nature of secondary allogeneic T cell responses stimulated by iDCs or mDCs differentiated with bacterial (SAC, IFN-γ–primed LPS) or T cell–dependent (CD40L) signals. Umbilical naive $CD4^+CD45RA^+$ T cells were cocultured for 4 d with DCs at stimulator/responder ratios of 1:4 and 1:300. After 7–10 d of expansion in IL-2 (50 U/ml), cells were washed, counted, and restimulated with either anti-CD3 immobilized on L-CD32/B7 transfectants or PMA and ionomycin (Fig. 2). Naive $CD4^+$ T cells cocultured at low ratio (1:300) in primary MLR with mDCs differentiated with SAC or CD40L signals differentiated into typical Th2 cells producing large quantities of IL-4 (up to 1.7 ng/ml) (compared with activated T cells primed with iDCs) and releasing very small amounts of IFN-γ (Fig. 2 A). Similar results were obtained with naive T cells cocultured at 1:300 ratio with LPS, TNF-α plus IL-1, or SAC plus IFN-γ–activated mDCs (not shown). This strong Th2 response was confirmed by flow cytometry since naive T cells matured into single IL-4–producing cells (40–46% positive cells) secreting no IFN-γ (≤3% positive cells) (Fig. 2 B). Conversely, regardless of the nature of the DC stimulation, a high stimulator/responder ratio (1:4) favored a mixed Th1/Th2 response (IFN-γ–positive cells ranging from 13 to 23% and IL-4–positive cells from 23 to 29%) (Fig. 2 B). Note that at 1:4 ratio, γ-primed LPS–activated mDCs appeared to be the most effective APCs, when compared with other mDCs, in triggering large amounts of IFN-γ secretion in the culture supernatant of activated T cells (Fig. 2 A). This correlated with the highest IL-12 production by γ-LPS–activated DCs (Fig. 1). However, at 1:300 ratio, activated T cells mixed with γ-LPS mDCs released up to 1.3 ng/ml of IL-4 and low IFN-γ (Fig. 2 A).

To evaluate the role of the intermediate culture in IL-2 on T cell polarization, DC–T cell cocultures were performed at 1:4 and 1:300 ratios for 6–7 d, washed, and 2.5×10^5 viable T cells were immediately restimulated on anti-CD3 immobilized on L-CD32/B7 transfectants. As shown in Table I, Th effectors differentiated in the absence of expansion in IL-2 secreted low but significant levels of cytokines and displayed polarization profiles similar to those of Th effectors obtained after expansion in IL-2, namely Th2 at 1:300 and mixed Th1/Th2 at 1:4 ratio. Note that the recovery of allospecific T cells was drastically increased (10–25-fold) after expansion in IL-2 and consistently higher (2–4-fold) at 1:4 than 1:300 ratio (Tanaka, H., unpublished observations). Similarly, the number of activated T cells at the end of MLR was higher at 1:4 than 1:300 ratio (% $CD25^+$ cells: 66.1% ± 13 at 1:4 and 38.5% ± 3.1 at 1:300).

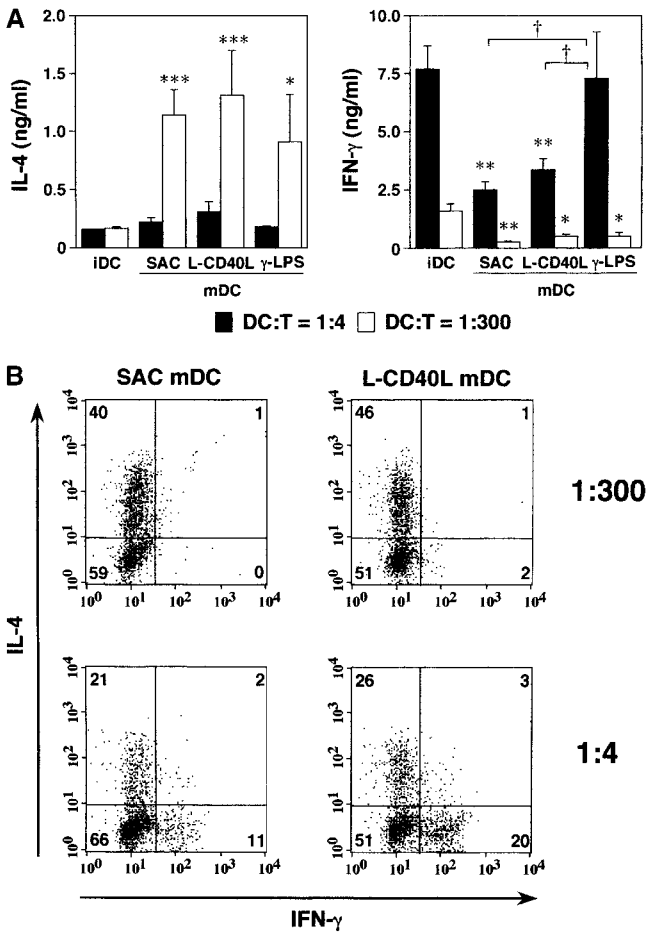

Figure 2. Myeloid DC promotes Th2 development at 1:300 DC/T ratio and favors Th1/Th2 at 1:4 ratio. Human naive $CD4^+$ T cells were cocultured for 4 d with allogeneic mDCs at low (1:300) or high (1:4) stimulator/responder ratio. After IL-2 expansion, T cells were counted and restimulated with anti-CD3 immobilized on L-CD32/B7 transfectants (A) or with PMA and ionomycin for 5 h (B). (A) IL-4 and IFN-γ were measured in 24- or 48-h culture supernatant, respectively, by ELISA or RIA. Data are the mean values of at least four experiments ± SEM (statistical analysis, *mDCs vs. immature DCs, †γ-LPS vs. other mDCs). Similar results were obtained after PMA and ionomycin stimulation. (B) 10^4 cells were analyzed by flow cytometry for IL-4 and IFN-γ expression, and the percentage of positive cells is indicated in the quadrants. Data are one experiment representative of two independent experiments.

To further confirm the polarization of the response toward Th2 at 1:300 ratio, we showed that primed T cells also secreted large amounts of IL-5 (10–15 ng/ml) and IL-10 (~1 ng/ml) (Fig. 3). The production of IL-13, a nontypical Th2 cytokine in humans, was induced in both culture conditions, albeit at a higher level at 1:300 ratio, whereas IL-2 level was not influenced by the cellular ratio.

In agreement with Cella et al. (11), iDCs were much less efficient than mDCs in stimulating T cell proliferation and induced at both cellular ratios a mixed Th1/Th2 cytokine profile as determined at the single cell level (Tanaka, H., personal observations). However, as shown in Fig. 2 A, iDC were poor inducers of Th2 cytokine release, except

Table I. *Human DC1 Promotes Th2 versus Th1 Effectors in the Absence of IL-2 Expansion*

	Ratio	IFN-γ	IL-4
Experiment 1	1:4	2.8	0.2
	1:300	0.5	0.7
Experiment 2	1:4	3.8	0.15
	1:300	0.6	0.54
Experiment 3	1:4	2.0	0.1
	1:300	0.2	0.46

Naive T cells (10^6/ml) were stimulated for 6–7 d with mDCs activated by L-CD40L transfectants (Experiment 1 or 2) or SAC (Experiment 3) at 1:4 and 1:300 ratio. After washing, cells were restimulated with anti-CD3 immobilized on L-CD32/B7.1 L transfectants. Cytokines (in ng/ml) were measured after 24 h.

for IL-10 and iDC-primed T cells that released large amounts of IFN-γ at 1:4 ratio, which correlated with high levels of IFN-γ during primary MLR (not shown).

Th2 Differentiation Is Dependent on B7–CD28 Costimulation and OX40–OX40L Interactions. IL-12, a cytokine produced by APCs, skews differentiation towards a Th1 response whereas the Th2-driven response is usually IL-4 dependent (18). Although the precise source of IL-4 in the initiation of a Th2 response is a controversial issue, NK1.1$^+$ cells and naive T cells are good candidates (4, 19). The induction of Th2 effectors by mDCs at 1:300 ratio was not affected by the presence of a combination of neutralizing anti–IL-4 and anti–IL-4R Abs in primary MLR as shown by intracytoplasmic staining (Fig. 4 A) or by ELISA (Tanaka, H., personal observations). The same Abs completely suppressed IL-4–induced CD23 expression by PBMCs (not shown). However, cytolytic T lymphocyte–associated Ag (CTLA)-4–Fc, added during primary culture, reduced by ~50% the number of IL-4–producing cells (Fig. 4 B) and by ~80% IL-4 and IL-5 release in the culture supernatant of activated T cells with no increase in IFN-γ secretion (Fig. 4, B and C). In agreement with our previous studies (15), anti-OX40L mAb, added at priming, significantly suppressed IL-4 and IL-5 production at restimulation (Fig. 4 C). Interestingly, CD40–CD40L interactions did not appear to be critical for Th2 development and IL-2 production was not significantly affected under the above culture conditions (Fig. 4 C). Also shown in Fig. 4 and in agreement with other studies (9, 11), anti–IL-12 mAb strongly inhibited at 1:4 ratio the development of IFN-γ–producing cells (Fig. 4 A) and IFN-γ secretion (not shown) whereas CTLA-4–Fc favored immune deviation towards Th1 effectors (Fig. 4 B).

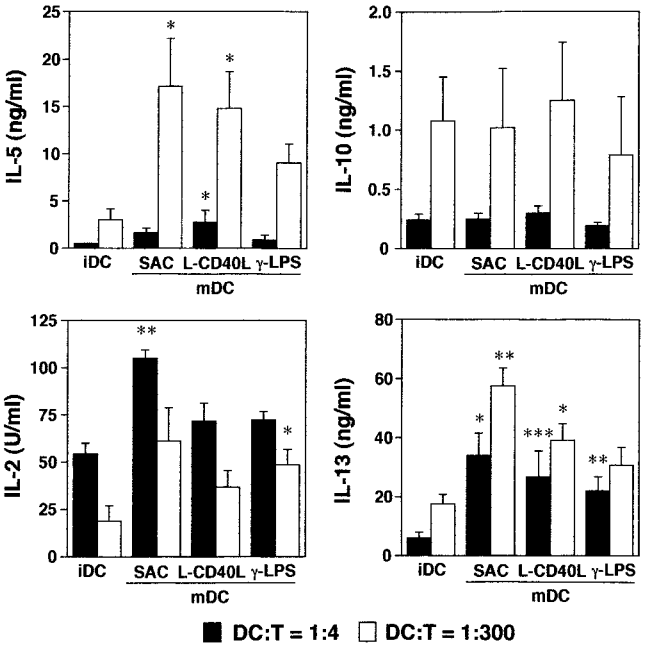

Figure 3. Cytokine profile of effectors primed at 1:4 and 1:300 ratios. Human naive CD4$^+$ T cells were cocultured for 4 d with allogeneic iDCs or mDCs at low and high density ratio, expanded in IL-2, and restimulated with anti-CD3 immobilized on L-CD32/B7 transfectants. IL-2, IL-5, IL-10, and IL-13 were measured by ELISA or RIA in culture supernatants after 24 h (reference 15). Data are the mean values ± SEM of at least four independent experiments.

Discussion

Our results revealed that Th2 differentiation of human naive T cells could be elicited by myeloid-derived DC_1 and was largely dependent on a low (1:300) DC/T cell ratio during primary MLR, demonstrating that Th2-driven responses are not restricted to the human DC_2 lineage (9).

To increase the recovery of allospecific T cells, we performed an intermediate culture in IL-2. Note that Th effectors displayed a similar cytokine profile in the absence (Table I) or presence (Fig. 2 A) of expansion in IL-2. In support of our data that high (1:4) DC_1/T cell ratio favored a Th1 cytokine profile, Rissoan et al. (9) have used 1:2, 1:4, and 1:8 myeloid (DC_1)–naive T cells cocultures, with no expansion in IL-2, to generate Th1 effectors. Also, Cella et al. (11) reported that freshly isolated plasmacytoid DC_2 induced both type 1 and type 2 helper T cells. Taken together, these data do not support an exclusive role of DC_1 for induction of Th1 cells and of DC_2 for Th2.

Th2 development depends on the route of immunization, the nature and the concentration of Ag, and the balance between IL-4 and IL-12 at priming (4, 19). At 1:300 DC/T ratio, alloantigen and IL-12 were present in low quantities and these two conditions reportedly favored a Th2 response. Our results further indicated that a combination of neutralizing anti–IL-4 and anti–IL-4 receptor Abs did not impair the initiation of the Th2 response. Although we cannot completely exclude the presence of residual IL-4, an IL-4–independent generation of Th2 effectors was reported after naive T cells stimulation by low doses of Ag (20) or by plasmacytoid DCs (9), without yet identifying the surface molecules possibly involved during DC–T interactions. Here we show that at 1:300, B7/CD28–CTLA-4

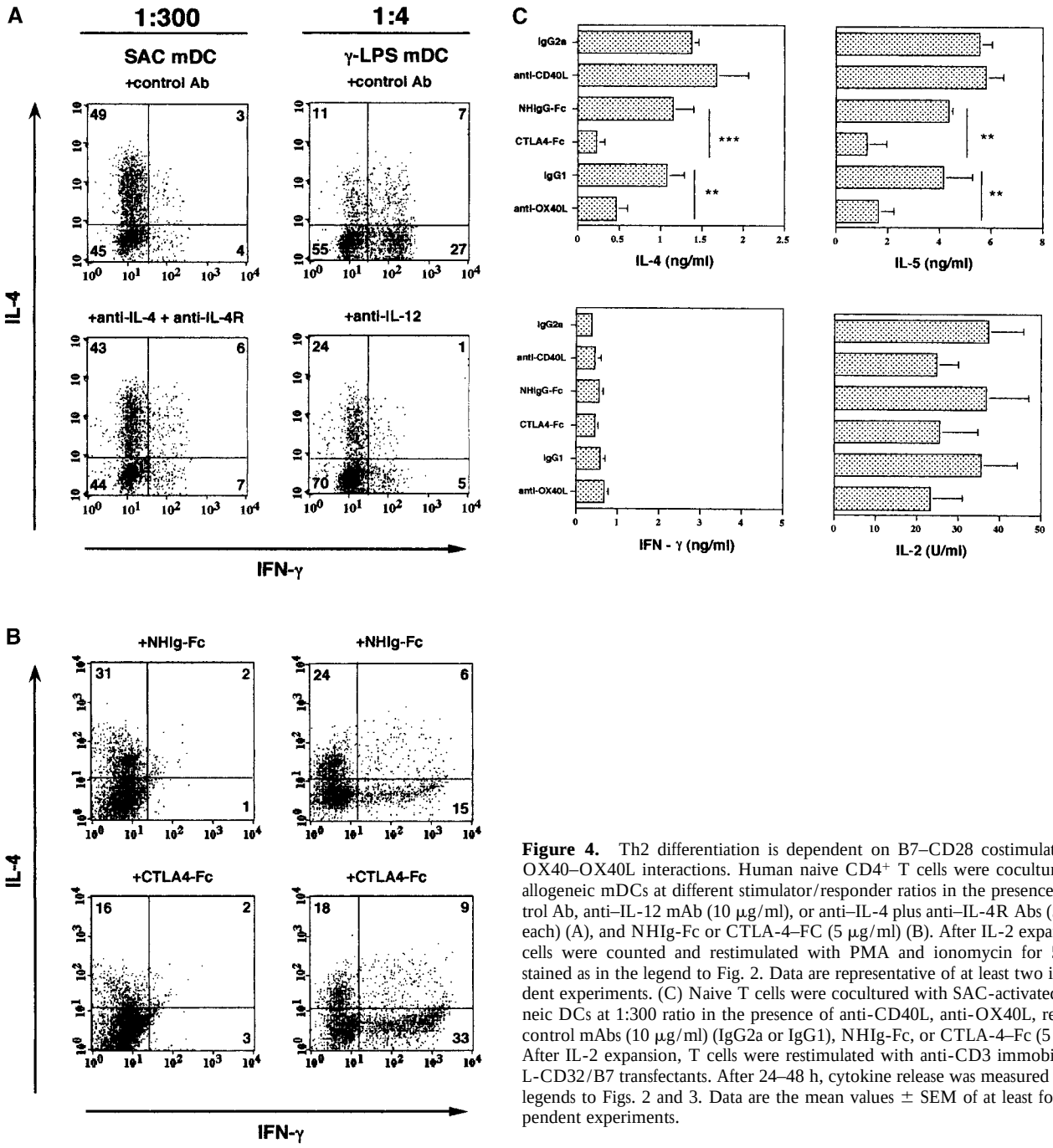

Figure 4. Th2 differentiation is dependent on B7–CD28 costimulation and OX40–OX40L interactions. Human naive $CD4^+$ T cells were cocultured with allogeneic mDCs at different stimulator/responder ratios in the presence of control Ab, anti–IL-12 mAb (10 μg/ml), or anti–IL-4 plus anti–IL-4R Abs (5 μg/ml each) (A), and NHIg-Fc or CTLA-4–FC (5 μg/ml) (B). After IL-2 expansion, T cells were counted and restimulated with PMA and ionomycin for 5 h and stained as in the legend to Fig. 2. Data are representative of at least two independent experiments. (C) Naive T cells were cocultured with SAC-activated allogeneic DCs at 1:300 ratio in the presence of anti-CD40L, anti-OX40L, respective control mAbs (10 μg/ml) (IgG2a or IgG1), NHIg-Fc, or CTLA-4–Fc (5 μg/ml). After IL-2 expansion, T cells were restimulated with anti-CD3 immobilized on L-CD32/B7 transfectants. After 24–48 h, cytokine release was measured as in the legends to Figs. 2 and 3. Data are the mean values ± SEM of at least four independent experiments.

blockade by CTLA-4–Fc prevented differentiation of naive T cells into Th2 with no immune deviation towards Th1. Several reports indicated that CD28 influences Th2 differentiation (21–25). Engagement of CD28 by B7 molecules promotes Th2 development, whereas CTLA-4 is a potent inhibitor of the Th2 differentiation pathway (21). We previously reported that CD3 and CD28 costimulation of purified human naive $CD4^+$ T cells induced IL-4 production at priming and at restimulation, skewing the cytokine profile towards Th2 (22). These results are in keeping with Kalinski et al. (23) and King et al. (24) but are in apparent contradiction with Rissoan et al. (9). $CD4^+$ T cells in CTLA-4–deficient mice secreted high levels of IL-4 and IL-5 upon TCR activation and anti-CD28 costimulation compared with wild-type T cells (25). CD28 costimulation increased IL-4R sensitivity by enhancing phosphorylation of Janus kinase 3 (Jak3), the IL-4Rα chain, and signal transducer and activator of transcription 6 (STAT-6) after IL-4 stimulation (26). Finally and in support of our data, the B7/CD28–CTLA-4 costimulatory pathway plays a role in the pathogenesis of allergen-induced airway hyperresponsiveness in a murine model of allergic asthma (27). Ad-

ministration of CTLA-4–Ig before Ag sensitization decreased airway hyperresponsiveness and IL-4 production in the bronchoalveolar lavage with no change in IFN-γ. Besides B7/CD28 molecules, other costimulatory interactions, including CD40–CD154, RANK–RANKL, and OX40–OX40L, have an important role in DC–T cell interactions (28). In the present study, we confirmed and extended with different mDCs our previous observations that blocking OX40–OX40L interactions inhibited Th2 development (15). In vivo studies confirmed the involvement of the OX40–OX40L pathway in DC–T cell interactions but its differential role in Th2 versus Th1 varies according to the in vivo models (28, 29).

iDCs, widely distributed in nonlymphoid tissues, are specialized in Ag capture, but a fundamental function of these cells is their capacity to migrate (5, 30). The rate of DC migration from blood to tissues and tissues to lymph nodes is considerably increased by inflammatory stimuli (30–33). Systemic administration of LPS or TNF-α resulted in a drastic decrease of DCs from the peripheral tissues or marginal zone of the spleen followed by transient accumulation of mDCs in the T cell zones (31–34). Different inflammatory chemokines and chemokine receptors secreted or expressed by DCs and endothelial cells participated in each step of DC migration (30, 35). The migratory and stimulatory DCs recruited under inflammatory conditions are myeloid derived whereas resident lymphoid DCs may be responsible for induction of tolerance in the absence of inflammation (30). The current concept is that, besides the nature of the DCs, the quantity and the quality of the costimulatory molecules and cytokines they express determine the balance between Th1 and Th2 effector cells that will be generated (36). Our results further suggest that the ratio between mature myeloid DCs and naive T cells appears as important to polarize the immune response. Therefore, finding factors and understanding the mechanisms that regulate the rate and the number of DCs that migrate from skin, mucosa, and interstitial tissues to their final destination in the T cell areas will be relevant for opening new avenues for therapeutic intervention (37).

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