

# Kinetics of dendritic cell activation: impact on priming of T<sub>H</sub>1, T<sub>H</sub>2 and nonpolarized T cells

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To prime immune responses, dendritic cells (DCs) need to be activated to acquire T cell stimulatory capacity. Although some stimuli trigger interleukin 12 (IL-12) production that leads to T helper cell type 1 (T<sub>H</sub>1) polarization, others fail to do so and favor T<sub>H</sub>2 polarization. We show that after activation by lipopolysaccharide, DCs produced IL-12 only transiently and became refractory to further stimulation. The exhaustion of cytokine production impacted the T cell polarizing process. Soon after stimulation DCs primed strong T<sub>H</sub>1 responses, whereas at later time points the same cells preferentially primed T<sub>H</sub>2 and nonpolarized T cells. These findings indicate that during an immune response, T cell priming conditions may change in the lymph nodes, suggesting another mechanism for the regulation of effector and memory T cells.

T helper type 1 (T<sub>H</sub>1) and type 2 (T<sub>H</sub>2) cells represent terminally differentiated effector cells characterized by different cytokine production and homing capacity<sup>1–3</sup>. The generation of either type of response can confer protection against pathogens or lead to immunopathology. T<sub>H</sub>1 and T<sub>H</sub>2 polarization is a stochastic process<sup>4,5</sup>, which is promoted by interleukin 12 (IL-12) and IL-4<sup>6–8</sup>. Other factors that contribute to the T<sub>H</sub>1–T<sub>H</sub>2 balance are the dose of antigen<sup>9</sup>, strength of antigenic stimulation<sup>10</sup>, duration of T cell receptor (TCR) engagement<sup>11</sup> and nature of costimulatory molecules<sup>12</sup>.

After a primary response, T<sub>H</sub>1 and T<sub>H</sub>2 cells persist at low levels as long-term effector memory T cells that can confer protection upon secondary challenge<sup>13–15</sup>. The T cell memory pool is also comprised of a distinct subset of nonpolarized “central memory” T cells that express the lymph node homing receptor CCR7 and lack immediate effector function<sup>16</sup>. The heterogeneity of memory cells indicates that during the primary response, there must be conditions that allow naive T cells to generate at least three functional outputs: T<sub>H</sub>1, T<sub>H</sub>2 and nonpolarized cells.

T cell priming requires the activation of dendritic cells (DCs)<sup>17</sup>. DCs are stimulated by recognition of characteristic patterns of pathogens<sup>18</sup> as well as by inflammatory cytokines<sup>19</sup> and necrotic cells<sup>20</sup>. These stimuli trigger a complex maturation program that results in DC migration from tissues to the draining lymph nodes, enhanced T cell stimulatory capacity and cytokine production<sup>21,22</sup>. The nature of DCs that induce T<sub>H</sub>1 or T<sub>H</sub>2 responses (also called DC1 and DC2, respectively) has been the subject of intense investigation. One study indicated that although myeloid cells give rise to DC1, plasmacytoid monocytes generate DC2 after *in vitro* culture with CD40L<sup>23</sup>. However, it is also clear that myeloid DCs can give rise to either DC1 or DC2, depending on the nature of the maturation stimulus influencing IL-12 production<sup>24</sup>. IL-12, the critical T<sub>H</sub>1 polarizing cytokine<sup>6</sup>, is produced by DCs after stimulation with lipopolysaccharide (LPS), poly(I)•poly(C) or CD40L<sup>25–27</sup>,

but it is not produced in response to tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-1, fungal hyphae and nematode products<sup>28,29</sup>. IL-12 production can be also inhibited in DCs by vitamin D3<sup>30,31</sup> or by agents that increase cyclic AMP such as prostaglandin-E2 or cholera toxin<sup>32–34</sup>.

In this study we investigated whether the kinetics of DC activation might influence the capacity of DCs to prime T cells, not only towards effector T<sub>H</sub>1 or T<sub>H</sub>2, but also towards nonpolarized “central memory” T cells. We found that in response to LPS, DCs produced IL-12 only transiently. Consequently, DCs taken at early points after induction of maturation induced strong T<sub>H</sub>1 polarization, whereas the same cells taken at late time points primed T<sub>H</sub>2 and nonpolarized cells. These findings indicate a dynamic regulation of the generation of effector and memory cells during the immune response.

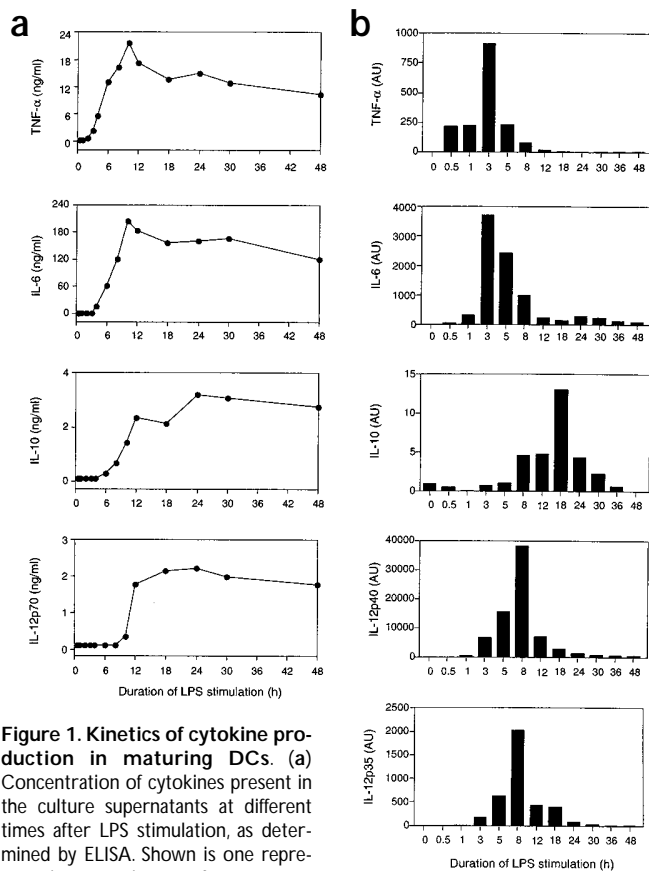
## Results

### Kinetics of cytokine production in maturing DCs

In the first series of experiments, we studied the kinetics of cytokine production by DCs stimulated with LPS (**Fig. 1a**). TNF- $\alpha$  and IL-6 could be detected in the culture supernatant 3 to 4 h later and their amounts increased linearly for 10 h, when a plateau was reached. The kinetics of accumulation is consistent with a rapid induction of synthesis, followed by extinction after 8 to 10 h. In contrast, IL-10 accumulation started at 6 h and its production was sustained until at least 24 h after stimulation. The IL-12p70 subunit was detectable only after 10 h but rapidly reached a plateau 12 to 18 h after LPS stimulation. The kinetics of this accumulation is consistent with a delayed and transient production of IL-12. The absolute amounts of cytokines produced by DCs from different donors in response to LPS stimulation varied considerably, especially IL-10 (see **Fig. 1** legend) but the kinetics were consistently superimposable.

The kinetics of cytokine mRNA levels determined by quantitative real time reverse-transcribed polymerase chain reaction (RT-PCR)

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**Figure 1. Kinetics of cytokine production in maturing DCs.** (a) Concentration of cytokines present in the culture supernatants at different times after LPS stimulation, as determined by ELISA. Shown is one representative experiment of seven conducted with different DC preparations. The kinetics of production were comparable among different experiments, whereas the amounts varied among different donors. Mean and range values of cytokines produced expressed as ng/10<sup>6</sup> cells were: TNF- $\alpha$  = 31 (17–45); IL-6 = 338 (193–500); IL-10 = 14 (3–36); IL-12p70 = 10 (7–13). (b) Kinetics of cytokine mRNA levels in LPS-stimulated DCs as determined by quantitative real time RT-PCR. One representative experiment of three is shown. (AU, arbitrary units.)

were consistent with the kinetics of protein accumulation in the culture supernatant (**Fig. 1b**). In particular, IL-12p40 and p35 mRNAs showed a sharp peak between 5 to 8 h, followed by complete extinction after 18 h. The induction and extinction of mRNA did not change when the cells were stimulated with poly(I)•poly(C) or with IL-1 $\beta$  plus TNF- $\alpha$ , although in the latter case, IL-12p35 mRNA was barely detectable (data not shown).

The above results indicate that different cytokines are produced with distinct kinetics by maturing DCs and that at late time points after induction of maturation, DCs exhaust their capacity to produce cytokines. To test this possibility, we washed DCs 8, 24 and 48 h after LPS stimulation and recultured them for 24 h in medium alone (to mea-

sure the residual cytokine production) or in the presence of CD40L (to measure the capacity to respond to a second stimulation). DCs that were stimulated for 8 h with LPS did not show any residual TNF- $\alpha$  production, although they still produced IL-6, IL-10 and IL-12 (**Fig. 2**). DCs that had been stimulated for 24 or 48 h were no more capable of producing any of the analyzed cytokines, despite low amounts of IL-6 after LPS stimulation for 24 h. In addition, the capacity of maturing DCs to respond to a second stimulus was lost with time after induction of maturation. Thus, CD40L could boost IL-12 production if provided 8 h after LPS stimulation, but was no longer effective after 24 or 48 h (**Fig. 2**). Addition of IFN- $\gamma$  to LPS-stimulated DCs, which is known to increase the production of IL-12<sup>24</sup>, did not significantly affect the kinetics of production (data not shown).

It should be noted that 8 and 48 h after LPS stimulation, DCs show similar viability and survival capacity and are comparable in terms of their ability to trigger proliferation of cord blood or adult naïve CD4<sup>+</sup> T cells, as they are at least 30-fold more potent than immature DCs (data not shown). The expression of costimulatory molecules increased progressively during DC maturation and this increase was more pronounced for CD86 than for CD80. As compared to 8-h DCs, 48-h DCs expressed an average of threefold higher amounts of CD80 and fivefold higher amounts of CD86.

Altogether, the above results indicate that with time, maturing DCs, while acquiring high T cell stimulatory capacity, extinguish cytokine production and become unresponsive to further stimulation. Therefore, the kinetics of DC activation define two functional stages in DC lifecycle. We operationally define early DCs that synthesize cytokines as “active” and late DCs that have lost cytokine production as “exhausted.”

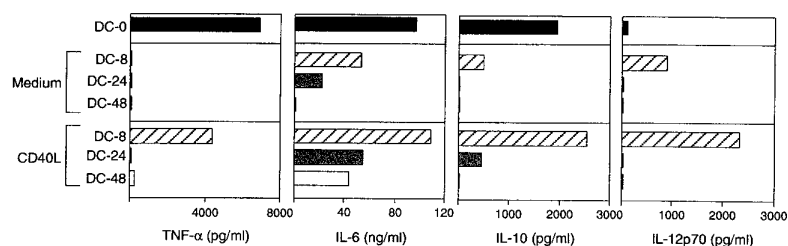
### DC activation kinetics and antigen dose

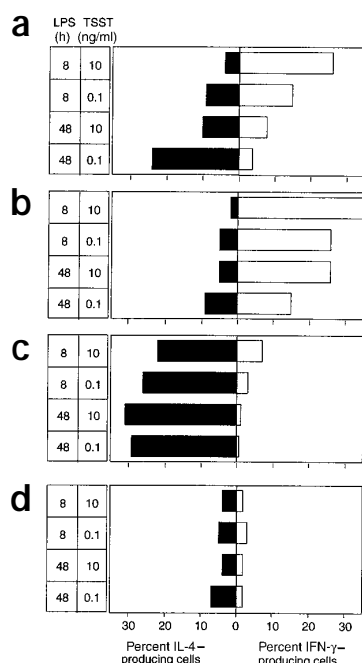
We investigated how the kinetics of DC maturation would impact the class of T cell response generated. Active and exhausted DC, collected 8 or 48 h after LPS stimulation, were pulsed with high or low doses of toxic shock syndrome toxin 1 (TSST-1) and cultured with autologous naïve CD4<sup>+</sup> T cells. The proliferating T cells were expanded with IL-2 and tested for their capacity to produce IL-4 or IFN- $\gamma$  when stimulated. Active DCs preferentially induced T<sub>H</sub>1, whereas exhausted DC induced T<sub>H</sub>2 polarization (**Fig. 3a**). In both conditions, a minority of cells

acquired the capacity to produce both IFN- $\gamma$  and IL-4 (from 2 to 7%), whereas a variable fraction remained nonpolarized. In addition, high TSST-1 doses favored T<sub>H</sub>1 and low doses favored T<sub>H</sub>2.

Exogenous IL-12 or IL-4 only modestly increased the maximum extent of T<sub>H</sub>1 or T<sub>H</sub>2 polarization observed (**Fig. 3b,c**), indicating that DCs are efficient at driving T<sub>H</sub>1 or T<sub>H</sub>2 responses in an autonomous fashion, depending on their kinetics of activation and antigen dose. Exogenous IL-12 or IL-4 did not completely override the intrinsic polarizing effect of DCs. Indeed, exhausted DCs pulsed with low antigen doses still showed relative T<sub>H</sub>2 polarizing capacity even in the presence of IL-12, whereas active DCs showed some T<sub>H</sub>1 polarizing

**Figure 2. Cytokine-producing capacity becomes exhausted following DC maturation.** DCs were stimulated with LPS for 8, 24 or 48 h (DC-8, hatched bars; DC-24, shaded bars; DC-48, open bars), washed and recultured in the absence or in the presence of CD40L. Cytokine production was measured in the 24-h culture supernatant. Cytokine amounts in the 24-h culture supernatant of LPS-stimulated immature DCs are shown for comparison (filled bars). DCs that had been primed for 8 h with LPS, washed and recultured in medium were found to produce higher amounts of IL-12 than did LPS-stimulated immature DCs (1.4- to 8-fold, mean 3.7-fold, in six DC preparations from different donors).





**Figure 3. Conditions favoring  $T_H1$  versus  $T_H2$  responses.** Active DCs and high antigen doses favor  $T_H1$ , whereas exhausted DC and low antigen doses favor  $T_H2$  responses. Immature DCs were stimulated with LPS for 8 or 48 h, pulsed with 10 or 0.1 ng/ml TSST-1 and cultured with autologous naive  $CD4^+$  T cells. Cultures were set up in the absence of exogenous cytokines (a) or in the presence of IL-12 (b), IL-4 (c) or TGF- $\beta$  plus neutralizing antibodies to IL-12 and IL-4 (d). T cells were expanded and the percentage of cells producing exclusively IFN- $\gamma$  (open bars) or IL-4 (filled bars) was determined by intracellular staining following stimulation with PMA and ionomycin.

capacity even in the presence of exogenous IL-4. Finally, the polarization to  $T_H1$  or  $T_H2$  was largely dependent on IL-12 and IL-4, respectively, as it was prevented by the addition of neutralizing antibodies (Fig. 3d).

Altogether, these experiments identify active DCs and high antigen doses as the optimal conditions for the generation of  $T_H1$  and exhausted DCs and low antigen doses for the generation of  $T_H2$  responses. However, they also show that not all primed T cells become polarized.

### Induction of nonpolarized T cells

Because nonpolarized T cells represent a distinct subset of memory T cells that, similar to naive T cells, maintain expression of the chemokine receptor CCR7, we asked under which conditions DCs could prime this type of response and what factors might influence the balance between polarized ( $T_H1$  or  $T_H2$ ) and nonpolarized responses. Active DCs and high TSST-1 doses induced high proportions of effector cells (82% IFN- $\gamma$ -producing T cells) and most of the responding  $V_{\beta}2^+$  cells lost CCR7 expression (87%) (Fig. 4a). At a lower antigen dose, active DCs induced a lower frequency of effector cells and a lower proportion of CCR7 $^+$  cells (Fig. 4b). In contrast, in cultures stimulated with exhausted DCs, a sizeable fraction of the proliferating cells retained CCR7 expression (51% at high antigen dose, 76% at low antigen dose) and fewer acquired effector function, mainly  $T_H2$  (Fig. 4c,d). If the most polarizing culture conditions were supplemented with TGF- $\beta$  and neutralizing antibodies to IL-12, the percentage of effector cells decreased markedly, whereas that of CCR7 $^+$  cells increased proportionally (compare Fig. 4a and 4e). These results define the conditions that preferentially generate nonpolarized CCR7 $^+$  T cells: exhausted DCs, low antigen dose and lack of polarizing cytokines. They also show that some nonpolarized T cells are generated even under extreme polarizing conditions.

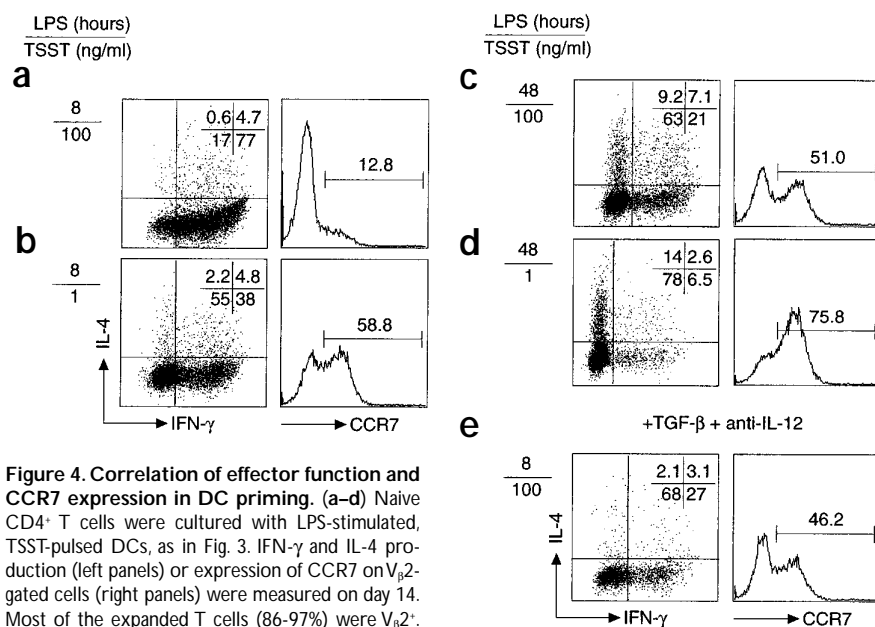
We followed CCR7 expression on proliferating

cells as a function of cell division using naive T cells labeled with 5- (and 6-) carboxy fluorescein diacetate succinimidyl ester (CFSE). Most of the T cells stimulated by allogeneic active DCs lost CCR7 expression after five or six divisions (Fig. 5a), indicating that, similar to the acquisition of effector function<sup>4,5</sup>, loss of CCR7 is dependent on division. However, despite the same number of divisions, a large proportion of T cells stimulated by exhausted DCs maintained CCR7 expression (Fig. 5b).

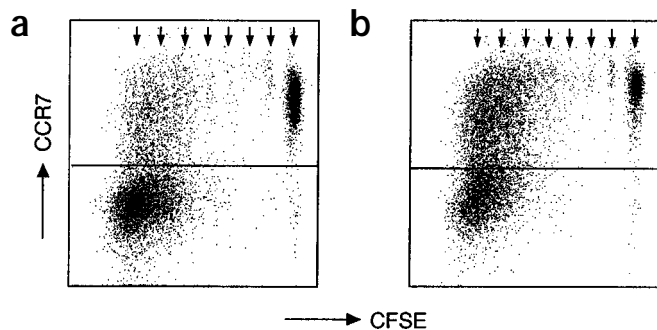
### Effect of duration of T cell-DC interaction

Data collected with plastic-bound peptide-major histocompatibility complexes (MHCs) and TCR transgenic mouse T cells has shown that sustained TCR stimulation is required to drive polarization to  $T_H1$  or  $T_H2$ , whereas a short stimulation selectively expands nonpolarized T cells<sup>11</sup>. Therefore, we asked whether limiting the duration of the T cell-DC interaction might favor the generation of nonpolarized human T cells. Naive T cells were labeled with CFSE and cultured with allogeneic DCs. After 40 h, T cells were isolated by cell sorting and recultured in the presence or absence of the same DCs. As shown in Fig. 6, a 40-h contact with DCs was sufficient to induce T cell proliferation. However, by day 9, the responding isolated T cells made fewer divisions (Fig. 6b) than did T cells that were recultured with the same DCs after sorting (Fig. 6a). Although expression of CCR7 was lost on most cells continuously stimulated by DCs, it was maintained on cells exposed to DCs for only 40 h. This difference was also observed in cells that underwent more than six divisions, indicating that the maintenance of CCR7 is not simply the consequence of fewer divisions. In addition, the expression of CCR7 was also maintained on T cells that were left in culture for up to 30 days (data not shown). To correlate CCR7 expression with cytokine production, CCR7 $^+$  and CCR7 $^-$  cells that had undergone the same number of divisions were sorted. Whereas IL-2 was produced in comparable amounts by both cell types, IL-4 and IFN- $\gamma$  were produced preferentially by CCR7 $^-$  cells (Fig. 6c).

We conclude that a transient T cell-DC interaction limits the clonal expansion and favors the generation of nonpolarized T cells that



**Figure 4. Correlation of effector function and CCR7 expression in DC priming.** (a-d) Naive  $CD4^+$  T cells were cultured with LPS-stimulated, TSST-pulsed DCs, as in Fig. 3. IFN- $\gamma$  and IL-4 production (left panels) or expression of CCR7 on  $V_{\beta}2$ -gated cells (right panels) were measured on day 14. Most of the expanded T cells (86-97%) were  $V_{\beta}2^+$ . (e) Cultures were set up in the presence of TGF- $\beta$  and neutralizing antibodies to IL-12.



**Figure 5. Loss of CCR7 as a function of cell division and DC state.** CFSE-labeled naïve T cells were stimulated with allogeneic active (a) or exhausted DCs (b). From day 5 on, cells were expanded in IL-2 and analyzed on day 11 for cell division and expression of CCR7.

maintain the expression of CCR7.

## Discussion

We have shown that in addition to the nature of the maturation stimulus, the kinetics of activation can influence the capacity of DCs to induce different types of T cell responses. There are two sets of experiments that point to this conclusion. First, DCs produce IL-12 during a narrow time window, 10 to 18 h after stimulation with LPS, and afterwards become refractory to further stimulation. Second, DCs taken early after activation induce strong  $T_H1$  responses, whereas the same DCs at later time points preferentially prime  $T_H2$  and nonpolarized T cells that retain CCR7 expression. These results indicate that the T cell priming conditions might change in lymph nodes during the primary response, generating a variety of effector and memory T cells.

The fact that cytokines and chemokines are produced by DCs with distinct kinetics indicates that they might act sequentially in different microenvironments<sup>35</sup>. For instance, TNF- $\alpha$ , IL-1 $\beta$  and MIP-1 $\beta$ <sup>35</sup>, which are produced 2 to 8 h after stimulation, may be released when DCs are still in peripheral tissues. Together, they may boost recruitment of DC precursors to sustain antigen sampling and presentation in the lymph nodes<sup>3</sup>. In contrast, IL-12 is produced with slower kinetics and may therefore be released in lymph nodes, consistent with its action on developing T cells.

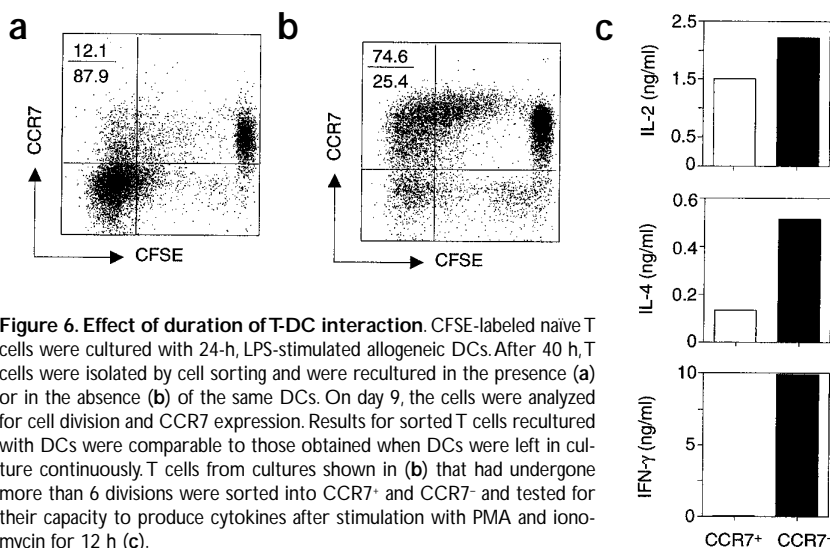
Another aspect of these kinetics is that cytokines produced by maturing DCs may regulate the production of other cytokines in an autocrine or paracrine fashion. We observed that DCs washed 8 h after LPS stimulation produced higher amounts of IL-12 than did DCs that were not washed. This effect might be due to the removal of IL-10 or TNF- $\alpha$ , which have been reported to inhibit IL-12 production<sup>36–38</sup>. This interpretation is supported by the fact that neutralizing IL-10-specific antibodies enhance 5- to 30-fold LPS-induced IL-12 production (data not shown). It would be interesting to explore to what extent the cascade of cytokine and chemokine production may provide positive and negative feedback signals for DC regulation. For instance, it has been reported recently that MIP-1 $\beta$  can directly stimulate IL-12 production by mouse lymphoid DCs<sup>39</sup>.

The exhaustion of cytokine production in DCs is reminiscent of a phenomenon that has been described in macrophages as LPS tolerance, or the down-regu-

lation of endotoxin-driven responses following a first exposure to LPS<sup>37</sup>. We found that DC exhaustion can be induced not only by LPS but also by other maturation stimuli, such as poly(I)•poly(C) and TNF- $\alpha$  plus IL-1 $\beta$ , and is not prevented by IFN- $\gamma$ . Additionally, DCs become unresponsive not only to the homologous stimulation, but also to CD40L. Thus, exhaustion is a general phenomenon that applies to various maturation stimuli and may also apply to other cell lineages. For instance, plasmacytoid monocytes cultured *in vitro* with CD40L were found to induce  $T_H2$  responses and have been consequently named DC2s<sup>23</sup>. These cells were exhausted by CD40L stimulation for several days and failed to produce considerable amounts of cytokines, except IL-8. Other studies in which exhaustion was avoided showed that plasmacytoid monocytes can induce  $T_H1$  or mixed responses<sup>40–42</sup>. Thus, we suggest that kinetics of DC activation, rather than DC lineage, might be the basis of at least some of the DC1-DC2 phenomenology described so far.

We have shown that in parallel with the loss of IL-12 production, DCs switch from a  $T_H1$ - to a  $T_H2$ -inducing mode. Although IL-12 production certainly accounts for this change, one has to consider other factors, such as the strength of TCR stimulation and costimulatory molecules that can affect  $T_H1$ - $T_H2$  polarization. In agreement with previous reports, we found that high doses of agonist favor  $T_H1$ , whereas lower doses favor  $T_H2$  responses<sup>9,10</sup>. Also, in line with the selective role of CD86 in  $T_H2$  polarization<sup>12</sup>, we observed that exhausted DCs express a higher CD86-to-CD80 ratio than do active DCs. Thus, the DC1 or DC2 phenotype, or the  $T_H1$ - $T_H2$  polarizing capacity of DCs, is dynamically regulated by the nature of maturation stimuli and the kinetics of activation.

The  $T_H1$ - $T_H2$  polarizing process is intrinsically inefficient, as not all activated T cells acquire effector function. Nonpolarized T cells represent a distinct subset of central memory T cells with characteristic expression of L-selectin and CCR7<sup>16</sup>. Therefore, it is important to define physiological conditions that might lead to their clonal expansion. Stimulation of mouse naïve T cells in the presence of TGF- $\beta$  has been shown to result in the selective expansion of nonpolarized T cells<sup>43</sup>. In addition, it was found that a shorter TCR stimulation in the absence of IL-12 and IL-4 can also prime nonpolarized T cells<sup>11</sup>. In this work, we have defined conditions in which DCs can prime nonpolarized T cells that retain CCR7 expression. These conditions are similar to those that promote  $T_H2$  responses (exhausted DCs and low doses of



**Figure 6. Effect of duration of T-DC interaction.** CFSE-labeled naïve T cells were cultured with 24-h, LPS-stimulated allogeneic DCs. After 40 h, T cells were isolated by cell sorting and were recultured in the presence (a) or in the absence (b) of the same DCs. On day 9, the cells were analyzed for cell division and CCR7 expression. Results for sorted T cells recultured with DCs were comparable to those obtained when DCs were left in culture continuously. T cells from cultures shown in (b) that had undergone more than 6 divisions were sorted into CCR7<sup>+</sup> and CCR7<sup>-</sup> and tested for their capacity to produce cytokines after stimulation with PMA and ionomycin for 12 h (c).



antigen). In addition, in agreement with previous reports, we demonstrate a role for exogenous TGF- $\beta$ , as well as for transient T-DC interactions. These results indicate that longevity of DCs<sup>44,45</sup> might represent a critical factor in the priming of T<sub>H</sub>2 *versus* nonpolarized cells.

Altogether, our results indicate that the kinetics of DC activation and migration can influence the type of effector and memory T cells generated. IL-12 can be induced by some stimuli, such as bacteria, viruses or T cells, but not by parasites. However, because IL-12 is produced by DCs only transiently, its availability in the lymph nodes will be dependent on a continuous flux of recently activated DCs from the inflamed tissues. In the early phases of the immune response, when lymph nodes receive a high input of active DCs, the priming will be biased towards T<sub>H</sub>1. However, when the influx of active DCs ceases, the exhausted DCs will rapidly outnumber the active ones, thus creating favorable conditions for the development of T<sub>H</sub>2 and, eventually, of nonpolarized T cells that build up the central memory pool. The kinetics of DC activation add a further degree of complexity and flexibility to the regulation of effector T cell responses and the generation of memory cells.

## Methods

**Media and reagents.** The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 50  $\mu$ g/ml kanamycin (Gibco, Grand Island, NY),  $5 \times 10^{-5}$  M 2-mercaptoethanol (Merck, West Point, PA) and 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) or 5% human serum (Swiss Red Cross). Human recombinant IL-2 and IL-4 were produced as described<sup>46</sup>. Recombinant IL-12 and TGF- $\beta$ , and neutralizing antibodies to IL-12 were purchased from R&D Systems (Minneapolis, MN). Recombinant TNF- $\alpha$  and IL-1 $\beta$ , and neutralizing antibodies to IL-4 were purchased from PharMingen (San Diego, CA).

**DC preparation.** Monocytes were purified by positive sorting using anti-CD14 conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). DCs were generated as described<sup>19</sup> by culturing monocytes in RPMI-10% FCS supplemented with granulocyte-macrophage colony-stimulating factor (50 ng/ml; Leukomax, Novartis, Basel, Switzerland) and IL-4 (1,000 U/ml) for 3–5 d. Cells were stimulated by addition of 20 to 100 ng/ml of LPS (from *Salmonella abortus equi*, Sigma) or CD40L-transfected J558 cells (at a ratio of 1:5, provided by P. Lane).

**Sorting and priming of naive T cells.** Naive CD4<sup>+</sup> T cells were sorted by negative selection using the following antibodies: anti-CD45R0 (UCHL-1, IgG2a), anti-CD8 (OKT8, IgG2a), anti-CD16 (B73.1, IgG1), anti-CD14 (144, IgG2b), anti-CD20 (HB9645, IgG; all from ATCC, Rockville, MD). Purity of the sorted population was 90–99% in all the experiments. Contaminating cells were CD4<sup>+</sup> CD45RA<sup>+</sup> CCR7<sup>+</sup>, mostly likely natural killer cells. Titration experiments showed that an excess of DCs could bias the response towards T<sub>H</sub>1, whereas a small number of DCs favored the development of T<sub>H</sub>2 and nonpolarized T cells. Sorted naive T cells were cultured at a ratio of 10:1 with allogeneic DCs or autologous DCs pulsed with TSS-1 (Toxin Technology, Sarasota, FL). After 5 d, proliferating cells were expanded with IL-2 and analyzed between day 8 and 14. In some experiments, naive T cells were labeled with CFSE (Molecular Probes, Eugene OR) as described<sup>47</sup>. For the detachment experiments, the CFSE-labeled T-DC cultures were collected after 40 h, treated with PBS with 0.5 mM EDTA to release cell aggregates and sorted. Cells were then expanded in IL-2-containing medium.

**Cytokine detection.** Cytokine production was measured in the supernatants of DCs or T cells by ELISA using matched paired antibodies specific for IL-2, IL-4, IFN- $\gamma$ , TNF- $\alpha$ , IL-6, IL-10, and IL12 p70 (all from PharMingen). DCs were stimulated in 10% FCS medium with LPS (20–100 ng/ml), followed by restimulation with CD40L (5:1) in 24-well plates at  $3 \times 10^5$  cells/ml. T cells were stimulated in the same medium with  $10^{-7}$  M phorbol 12-myristate 13-acetate (PMA) plus 1  $\mu$ g/ml of ionomycin (Sigma) at  $5 \times 10^5$  cells/ml for 8–12 h. For cytokine detection at the single cell level, T cells were stimulated with PMA plus ionomycin for 5 h. Brefeldin A (10  $\mu$ g/ml, Sigma) was added during the last 3 h. Cells were fixed with 2% paraformaldehyde, permeabilized with PBS containing 2% FCS and 0.5% saponin and stained with fluorescein isothiocyanate (FITC)-labeled IFN- $\gamma$ -specific and phycoerythrin (PE)-labeled IL-4-specific monoclonal antibodies (PharMingen).

**FACS analysis.** Cell staining for CCR7 was conducted using the rat mAb 3D12<sup>48</sup>, followed by PE- or biotin-conjugated, affinity-purified, isotype-specific goat anti-rat (Southern Biotechnology Associates, Birmingham, AL) and streptavidin-allophycocyanin (Molecular Probes, Eugene, OR). Staining for V $\beta$ 2 was conducted using a PE-conjugated specific mouse monoclonal antibodies (Immunotech, Westbrook, ME). Samples were

analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) using propidium iodide to exclude dead cells.

**Real-time RT-PCR.** Total RNA was isolated from DCs using the RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA using random hexamers and the TaqMan Reverse Transcription kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Real-time quantitative RT-PCR was done as described<sup>48</sup>. A relative quantification of TNF- $\alpha$ , IL-6, IL-10, IL-12p35 and IL-12p40 mRNAs was done on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer) using predeveloped TaqMan reagents according to manufacturer's instructions. Amplification of 18S rRNA was done for each experimental sample as endogenous control to account for differences in the amount and quality of total RNA added to each reaction. Thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 2-step PCR consisting of 15 s at 95 °C and 1 min at 60 °C. All samples were amplified in duplicate. Threshold cycle,  $C_t$ , which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increased above a threshold level. For each sample, the amount of target mRNA (TNF- $\alpha$ , IL-6, IL-10, IL-12p35, IL-12p40) was expressed as an  $n$ -fold difference relative to the amount of target mRNA isolated from nonstimulated DCs (calibrator). The formula used to determine these values is  $2^{-\Delta\Delta C_t}$ , where  $\Delta C_t$  is determined by subtracting the average 18S rRNA  $C_t$  value from the average target  $C_t$  value. The calculation of  $\Delta\Delta C_t$  involves subtraction by the  $\Delta C_t$  calibrator value from the target the  $\Delta C_t$  value.

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