

## **Migration and Function of Antigen-primed Nonpolarized T Lymphocytes In Vivo**

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### **Abstract**

Upon antigenic stimulation, naive T lymphocytes proliferate and a fraction of the activated cells acquire a T helper cell type 1 (Th1) or Th2 phenotype as well as the capacity to migrate to inflamed tissues. However, the antigen-primed T cells that receive a short T cell receptor (TCR) stimulation do not acquire effector function and remain in a nonpolarized state. Using TCR transgenic CD4<sup>+</sup> T cells in an adoptive transfer system, we compared the in vivo migratory capacities of naive, nonpolarized, Th1 or Th2 cells. Although all cell types migrated to the spleen, only naive and nonpolarized T cells efficiently migrated to lymph nodes. In addition Th1, but not Th2, migrated to inflamed tissues. In the lymph nodes, nonpolarized T cells proliferated and acquired effector function in response to antigenic stimulation, displaying lower activation threshold and faster kinetics compared with naive T cells. These results suggest that nonpolarized T cells are in an intermediate state of differentiation characterized by lymph node homing capacity and increased responsiveness that allows them to mount a prompt and effective secondary response.

Key words: migration • secondary response • T helper cell type 1 • T helper cell type 2 • nonpolarized T cells

### **Introduction**

Naive T lymphocytes traffic through the T cell areas of secondary lymphoid organs in search of antigen presented by dendritic cells (DCs; references 1 and 2). Upon antigen recognition, specific T cells proliferate and, in the presence of polarizing cytokines such as IL-12 or IL-4, differentiate towards Th1 or Th2 cells that produce distinct patterns of cytokines and mediate different types of protective and pathological responses (3, 4). Furthermore, some of the T cells activated during the primary response persist as memory cells that confer immediate protection as well as the capacity to respond more promptly and more vigorously to a secondary challenge (5–8).

The polarization process is overall inefficient, as after antigenic stimulation only a fraction of the proliferating cells acquire effector function, whereas the rest remains in a nonpolarized state (9–12). Several factors have been shown to influence the extent of T cell polarization in vitro. A prolonged antigenic stimulation as well as multiple rounds of stimulation in the presence of high doses of IL-12 or IL-4 generate Th1 or Th2 cells at relatively high frequency

(13, 14). In contrast, a short stimulation in the absence of exogenous cytokines leads to the expansion of T cells that display a nonpolarized phenotype (14). Furthermore, stimulation of T cells in the presence of TGF- $\beta$  results in the selective expansion of T cells that do not display immediate cytokine production capacity, but retain the capacity to differentiate to either Th1 or Th2 when restimulated under appropriate polarizing conditions (15).

Naive T cells express CD62 ligand (CD62L) and CC chemokine receptor 7 (CCR7), which are required to extravasate at the level of the high endothelial venules where the corresponding ligands glycosylation-dependent cell adhesion molecule (GlyCAM) and secondary lymphoid organ chemokine (SLC) are displayed (1, 16–18). CCR7 plays an essential role in this process, as in CCR7-deficient mice naive T cells fail to localize to the lymph nodes (19).

After T cell activation and differentiation to Th1 or Th2, the lymph node homing receptors are downregulated, while tissue homing receptors are acquired (20–24). Indeed, it has been known for a long time that memory T cells home preferentially to nonlymphoid tissues (25). More recently, it has been shown that Th1 and Th2 cells exhibit different migratory capacities in vivo (26, 27) and display distinct pattern of chemokine receptors and adhe-

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sion molecules (28–30). In contrast, the migratory capacities of antigen-primed nonpolarized T cells and their role in the immune response remain to be established.

Using adoptively transferred TCR transgenic T cells we analyzed the *in vivo* homing capacity of *in vitro*-expanded nonpolarized, Th1, and Th2 cells compared with naive T cells. We found that lymph node homing capacity is a characteristic of both naive and nonpolarized T cells, which is markedly decreased in Th1 and lost in Th2. In the lymph nodes, nonpolarized T cells proliferate and acquire effector function in response to antigenic stimulation, displaying lower activation thresholds and faster kinetics compared with naive T cells. These results suggest a role for nonpolarized T cells in immunoregulation and secondary responses.

## Materials and Methods

**Mice and Reagents.** Transgenic BALB/c mice carrying an I-E<sup>d</sup>-restricted TCR specific for the influenza hemagglutinin (HA) 110–119 peptide (SFERFEIFPK; reference 31) were obtained from Dr. A. Rolink (Basel Institute for Immunology, Basel, Switzerland). TCR transgenic mice were crossed with homozygous recombination activating gene (Rag)-2-deficient Balb/c mice (Rag2<sup>-/-</sup>) to generate Rag-2-deficient transgenic mice. Rag-2<sup>-/-</sup>TCR<sup>+</sup> mice were identified by staining with clonotype-specific antibody (31). All breeding was done in the animal colony of the Basel Institute for Immunology. FITC-conjugated anti-CD44, PE-conjugated anti-CD45RB, and allophycocyanin (APC)-conjugated anti-CD62L antibodies were purchased from BD PharMingen. For sorting experiments, the anti-CD62L antibody was used at a very low concentration to minimize a blocking effect.

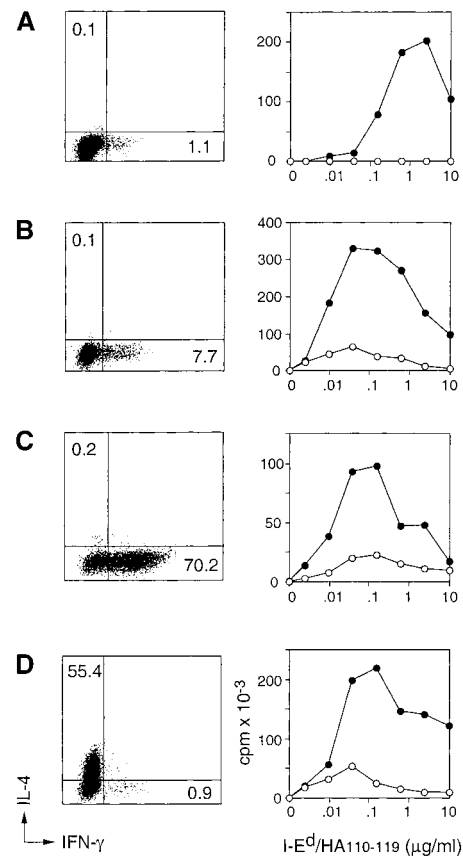
**Generation of Nonpolarized, Th1, and Th2 Cell Lines.** Naive T cells were obtained from lymph nodes of Rag-2<sup>-/-</sup> TCR transgenic Balb/c mice and cultured in tissue culture plates precoated with 10 μg/ml soluble HA<sub>110–119</sub>-I-E<sup>d</sup> complexes and 10 μg/ml of anti-CD28 antibody (BD PharMingen), as described (32). To generate nonpolarized T cells, naive cells were stimulated for 24 h in the absence of exogenous cytokines. To obtain Th1 or Th2 effector cells, naive cells were stimulated for 96 h in the presence of IL-12 (3.5 ng/ml; R&D Systems), or IL-4 (1.5 ng/ml; R&D Systems), respectively. After the indicated period of stimulation, the cells were transferred to uncoated plates and expanded with human recombinant IL-2 (50 IU/ml; Hoffmann-La Roche).

**Proliferation Assay.** T cells (2 × 10<sup>4</sup>) were cultured in flat-bottomed MaxiSorp surface microplate (Nunc) coated with different doses of soluble HA<sub>110–119</sub>-I-E<sup>d</sup> complexes together with 10 μg/ml of anti-CD28 antibody (BD PharMingen) or irrelevant antibody. After 42 h, [<sup>3</sup>H]thymidine (1 μCi/well) was added and the plates were harvested 8 h later.

**Intracellular Cytokine Detection.** Naive T cells and T cell lines or total lymph node or spleen cells from adoptively transferred mice were cultured in medium supplemented with PMA (10<sup>-7</sup> M; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) and incubated at 37°C. After 1 h, Brefeldin A (10 μg/ml; Sigma-Aldrich) was added and the cells were incubated for a further 3 h. The cells were then fixed, permeabilized, and stained as described (33). FITC-conjugated or PE-conjugated anti-IFN-γ and PE-conjugated anti-IL-4 antibodies (all from BD PharMingen) were used.

**In Vivo Migration Assay.** Freshly isolated naive lymph node cells and T cell lines were labeled with 5-(and 6)-[4-chloro-

methyl]benzoyl]amino)tetramethylrhodamine (CMTMR) or carboxyfluorescein diacetate-succinimidyl ester (CFSE; Molecular Probes), alternatively. For CMTMR labeling the cells were incubated in medium containing 10 μM dye at 37°C for 20 min, washed, and incubated in normal medium at 37°C for a further 20 min. For CFSE labeling the cells were labeled with 5 μM CFSE, as described previously (14). Equal number (5–10 × 10<sup>6</sup>) of naive cells and nonpolarized, Th1, or Th2 effector cells were pooled, resuspended in 500 μl PBS, and injected intravenously in normal nonirradiated Balb/c mice. After 20 h, the mice were killed and peripheral blood, subcutaneous (axillary, inguinal, popliteal, retroauricular, and submandibular) lymph nodes, mesenteric lymph nodes, spleen, peritoneal exudate cells (PECs), bronchoalveolar lavage cells (BAL), lungs, and liver were collected. To obtain PECs, mice were given 1 ml 3% thioglycollate medium intraperitoneally 24 h before cell transfer. From each organ single cell suspensions were prepared and stained with APC-conjugated anti-CD3 antibody (BD PharMingen). The percentage of CD3<sup>+</sup>CFSE<sup>+</sup> or CD3<sup>+</sup>CMTMR<sup>+</sup> cells recovered was assessed by FACS<sup>®</sup> analysis on at least 5 × 10<sup>5</sup> events collected.



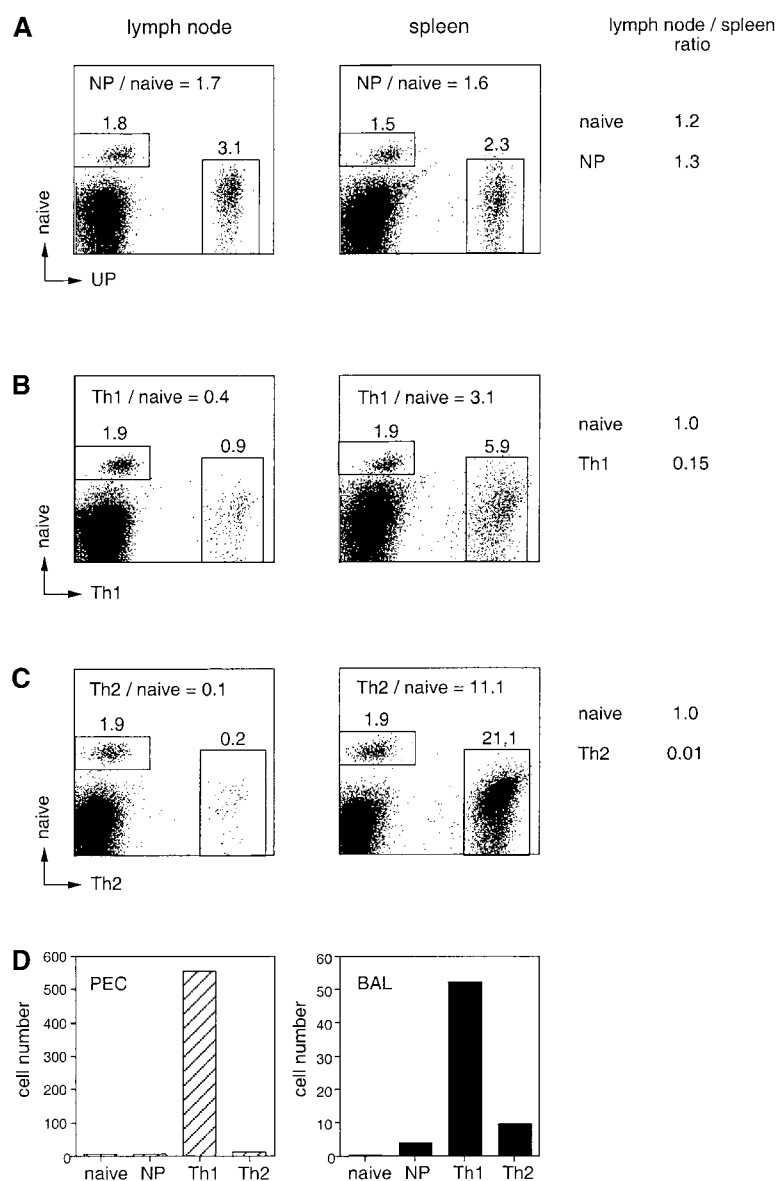
**Figure 1.** Functional characterization of cell populations used for adoptive transfer experiments. Naive T cells (A), nonpolarized T cells generated by a 24-h stimulation in the absence of cytokines (B), Th1 cells generated by a 96-h stimulation in the presence of IL-12 (C), and Th2 cells generated by a 96-h stimulation in the presence of IL-4 (D) were tested for their capacity to produce cytokines after stimulation with PMA and ionomycin (left) and to proliferate in response to different doses of plastic-bound HA<sub>110–119</sub>-I-E<sup>d</sup> complexes in the absence (○) or in the presence (●) of anti-CD28 antibody (right). The percentage of cytokine-producing cells is indicated. One representative experiment out of six.

**CCR7 Desensitization Assay.** CFSE- and CMTMR-labeled cells for each population were incubated (37°C for 40 min) with 15 µg/ml SLC (R&D Systems), or left untreated. The cells were washed and equal numbers of treated and untreated cells were injected together intravenously in normal nonirradiated Balb/c mice. After 20 min, the mice were killed and subcutaneous lymph nodes and spleens were collected. Single cell suspensions were prepared and stained with APC-conjugated anti-CD3 antibody. The percentage of CD3<sup>+</sup>CFSE<sup>+</sup> or CD3<sup>+</sup>CMTMR<sup>+</sup> cells recovered was assessed by FACS<sup>®</sup> analysis on at least 5 × 10<sup>5</sup> events collected.

**In Vitro Migration Assay.** Naive T cells and T cell lines (2 × 10<sup>5</sup> cells/well) were plated in the upper wells of 5-µm diameter 24-well Transwell plates and incubated at 37°C in the presence of medium or titrated doses of SLC (R&D Systems) in the lower wells. After 1 h, the upper wells were removed and the migrated cells were collected and counted. Migration index was calculated as: number of cells migrated/number of cells migrated in the presence of medium alone.

## Results

**Generation of Nonpolarized, Th1, and Th2 Cells.** Naive TCR transgenic Rag-2<sup>-/-</sup> T cells were stimulated with plastic-bound HA<sub>110-119</sub>-I-E<sup>d</sup> complexes and anti-CD28 antibody for different periods of time in the presence or absence of polarizing cytokines and expanded in IL-2 for 10 d. Stimulation for 24 h in the absence of cytokines led to activation and expansion of a cell population that was largely nonpolarized, with only 7% of the cells producing low levels of IFN-γ (Fig. 1 B). In contrast, a 96-h stimulation in the presence of IL-12 or IL-4 resulted in the generation of highly polarized Th1 and Th2 populations with 70% IFN-γ-producing and 55% IL-4-producing cells, respectively (Fig. 1, C and D). All the expanded populations responded to lower doses of HA<sub>110-119</sub>-I-E<sup>d</sup> complexes compared with naive T cells. Furthermore, although naive T cells did not respond in the absence of costimulation, all



**Figure 2.** Nonpolarized, Th1, and Th2 cells show different migratory capacity to lymph nodes, spleen, and inflamed tissues. CMTMR-labeled naive T cells were mixed with an equal number of CFSE-labeled nonpolarized (A), Th1 (B), or Th2 (C) cells and injected intravenously into syngeneic normal mice that were killed after 20 h. (A–C) Distribution of transferred cells in lymph nodes (left) and spleen (right). Percentages of CD3<sup>+</sup>CFSE<sup>+</sup> and CD3<sup>+</sup>CMTMR<sup>+</sup> cells are indicated. One representative experiment out of five. (D) Absolute numbers of transferred cells recovered per 1,000 T cells in PECs (striped bars) and BAL (black bars). One representative experiment out of three. NP, nonpolarized.

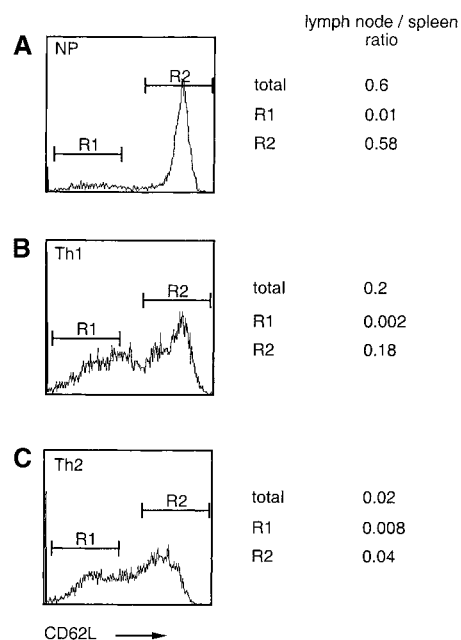
antigen-primed T cells showed comparable dose-response curves in the presence as well as in the absence of costimulation. However, in all cases stimulation through CD28 enhanced the magnitude of the proliferative response (Fig. 1). CD44 and CD25 were upregulated to a comparable extent on all expanded T cells (data not shown). In contrast, CD62L was expressed at high levels on naive and nonpolarized T cells but was downregulated on a fraction of Th1 and Th2 cells (see Fig. 3).

#### Homing Capacity of Nonpolarized, Th1, and Th2 Cells.

To analyze the homing capacity, naive and primed T cells labeled with different dyes were adoptively transferred into syngeneic normal mice. After 20 h, lymph nodes and spleen were collected and the percentage of the transferred cells was determined (Fig. 2, A–C). Compared with naive cells, nonpolarized T cells showed the same capacity to home to lymph nodes (Fig. 1 A) and to localize to T cell areas in both lymph nodes and spleen (data not shown). In contrast, Th1 and especially Th2 cells showed a minimal lymph node homing capacity and an increased localization to the spleen. For each population, the lymph node to spleen ratio can be taken as a measure of the relative capacity to home to these organs. This ratio differs significantly for the four populations analyzed, being  $\sim 1$  for naive, 1.3 for nonpolarized T cells, 0.15 for Th1, and 0.01 for Th2 cells. The analysis of PECs and BAL from adoptively transferred mice showed accumulation of Th1 cells in both and fewer Th2 cells in BAL, whereas virtually no naive or nonpolarized cells were recovered. These results indicate that although effector cells, especially Th1 cells, migrate to inflamed tissues, nonpolarized T cells share lymph node homing capacity with naive cells.

**Differential Expression of CD62L and CCR7 on Nonpolarized and Effector T Cells.** The extravasation of T lymphocytes at the level of high endothelial venules is controlled by the expression of CD62L and CCR7. As shown in Fig. 3, CD62L is expressed at high and homogeneous level on nonpolarized T cells, whereas its expression is heterogeneous on Th1 and Th2 cells. Therefore, we sorted CD62L<sup>lo</sup> and CD62L<sup>hi</sup> cells from nonpolarized, Th1, and Th2 populations and tested their migratory capacity in vivo after coinjection with naive T cells labeled with a different fluorochrome. As expected, in all cases CD62L<sup>lo</sup> T cells failed to migrate to lymph nodes, as indicated by a very low lymph node to spleen ratio. However, even CD62L<sup>hi</sup> T cells isolated from Th1 or Th2 populations did not show increased capacity to enter the lymph node, indicating that their reduced lymph node homing capacity is not simply due to a lower level of CD62L, but may be related to the lack of another essential factor such as CCR7.

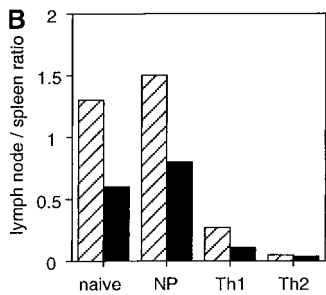
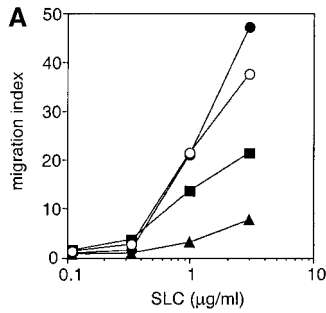
Therefore, we tested naive T cells and primed populations for their capacity to migrate in response to the CCR7 ligand SLC. As shown in Fig. 4 A, naive and nonpolarized T cells efficiently migrated in response to SLC, whereas Th1 showed a reduced and Th2 showed no response. Furthermore, desensitization of CCR7 by preincubation in vitro with high SLC doses reduced the migration of nonpolarized and naive T cells to lymph nodes (Fig. 4 B). Alto-



**Figure 3.** The low level of CD62L expression does not account for the decreased lymph node homing capacity of Th1 and Th2 cells. Nonpolarized (A), Th1 (B), and Th2 (C) cells were sorted according to CD62L expression and tested for their capacity to migrate to lymph nodes or spleen as described in the legend to Fig. 2. The lymph node/spleen ratio is indicated. One experiment out of three.

gether, these results indicate that both CD62L and CCR7 expression are maintained on nonpolarized T cells and downregulated on Th1 and Th2 cells.

**Nonpolarized T Cells Respond Rapidly Even in the Absence of Adjuvants to Antigen In Vivo and Acquire Effector Function.** The above results indicate that nonpolarized T cells exhibit a migratory capacity similar to that of naive cells. However, nonpolarized T cells are more responsive to antigenic stimulation in vitro and are relatively independent on costimulation (Fig. 1). Therefore, it was interesting to compare naive and nonpolarized cells for their capacity to respond to antigen in vivo. To address this point, CFSE-labeled naive or nonpolarized cells were transferred into normal mice. After 24 h, the mice were challenged with the specific peptide given subcutaneously in CFA (Fig. 5, A–D). 48 h after challenge, the naive T cells present in the draining lymph nodes had not yet divided (Fig. 5 A). In contrast, nonpolarized T cells had undergone up to three divisions (Fig. 5 B). 5 d after antigen challenge, both naive and nonpolarized T cells had divided more extensively (up to seven or more divisions), but only nonpolarized cells had differentiated to produce IFN- $\gamma$  (Fig. 5, C and D). When mice were boosted with peptide in the absence of adjuvant, naive T cells did not proliferate, whereas nonpolarized cells divided and acquired IFN- $\gamma$  production capacity (Fig. 5, E and F). Similarly, a challenge by intranasal administration of peptide resulted in proliferation of nonpolarized but not of naive T cells in draining lymph nodes (Fig. 5, G and H). Altogether, these results demonstrate that nonpolarized T cells can home to the lymph node and promptly respond to



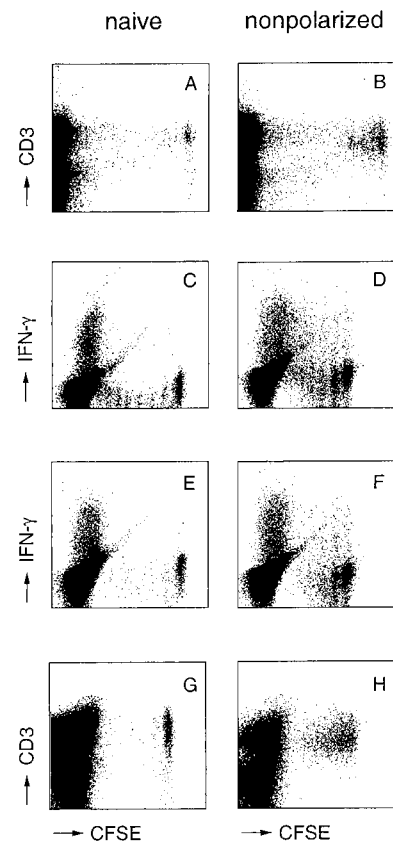
**Figure 4.** Nonpolarized T cells express CCR7 which is required for lymph node homing. (A) In vitro migration to SLC of naive (○), nonpolarized (●), Th1 (■), and Th2 (▲). One representative experiment out of four. (B) Lymph node/spleen ratio of transferred T cells preincubated with medium alone (striped bars) or 15 µg/ml SLC (black bars). NP, nonpolarized.

a secondary challenge even under conditions that are not permissive for the activation of naive T cells.

## Discussion

We have shown that activated nonpolarized T cells generated in vitro by a short antigenic stimulation of naive T cells in the absence of exogenous cytokines display intermediate properties between naive and effector cells. Like effector T cells, nonpolarized T cells show a low activation threshold and respond in vitro in the absence of costimulation; like naive T cells they express the lymph node homing receptors CD62L and CCR7. When transferred to syngeneic mice, nonpolarized T cells home to lymph nodes and spleen as efficiently as naive cells. In contrast, Th1 populations efficiently localized to inflamed organs and to spleen, where they represent the prominent T cell subset, but showed reduced homing to lymph nodes. Finally, Th2 cells, although homing almost exclusively to the spleen, displayed a negligible capacity to enter lymph nodes. Thus, nonpolarized T cells represent the only subset of activated cells that can encounter antigen-carrying DCs in the lymph nodes. Furthermore, we have shown that upon antigenic challenge, nonpolarized T cells proliferate in the lymph nodes, draining the site of antigen injection with a faster kinetics than naive T cells and rapidly acquire the capacity to produce IFN- $\gamma$ , even when the antigen is provided in the absence of adjuvant. Altogether, our results indicate that nonpolarized T cells are in an intermediate state of differentiation characterized by lymph node homing capacity and increased responsiveness that allows them to mount a prompt and effective secondary response.

The conditions that favor the generation and expansion of polarized T cells are not completely understood. We have shown that, by controlling the duration of TCR stimulation and the cytokine milieu, nonpolarized, Th1, or



**Figure 5.** Rapid and efficient response of nonpolarized T cells to antigen in vivo. CFSE-labeled naive T cells (left) or nonpolarized T cells (right) were transferred into syngeneic normal mice. After 24 h, the mice were immunized. (A–D) Mice were challenged by subcutaneous administration of HA<sub>110–119</sub> in CFA and cells from draining lymph nodes were analyzed after 48 h (A and B) or 5 d (C and D). (E and F) Mice were challenged by subcutaneous administration of HA<sub>110–119</sub> in PBS and cells were analyzed after 5 d. (G and H) Mice were challenged by intranasal administration of HA<sub>110–119</sub> in PBS and the cells from draining lymph nodes were analyzed after 5 d. The dot plots show division of CFSE-labeled cells vs. CD3 expression or vs. IFN- $\gamma$  production after stimulation with PMA and ionomycin. One representative experiment out of three.

Th2 cells can be preferentially generated (14). Thus, a short antigenic stimulation in the absence of exogenous cytokines leads to the expansion of activated nonpolarized T cells, whereas a prolonged stimulation in the presence of IL-12 or IL-4 allows generation of fully polarized Th1 and Th2 cells, respectively. The expansion of homogeneous populations of primed T cells is a necessary prerequisite to analyze and appreciate differences in their migratory capacities. Indeed, when DCs were used for priming, the T cell populations generated showed a more heterogeneous profile with variable proportions of polarized and nonpolarized cells (34; and data not shown). This is consistent with the probabilistic nature of the T–APC interaction and the heterogeneity of DC types and cytokines available that impact on T cell differentiation (15, 35–37). Indeed, it has been shown that depending on the DC activation state and the duration of DC–T cell interaction, different proportions of nonpolarized as well as polarized Th1 or Th2 cells are gen-

erated (34). Furthermore, it has been recently demonstrated that antigen-stimulated CD8<sup>+</sup> T cells can be maintained in a nonpolarized memory state by culture in IL-15, whereas they acquire effector function when cultured in high doses of IL-2 (data not shown).

The precise role of APCs and cytokines in promoting generation of different T cell subsets *in vivo* remains to be established. However, it is likely that during a primary response the dynamic changes in DC type and stimulatory capacity together with the changes in cytokine milieu will lead to the generation of both polarized and nonpolarized T cells, although in different proportions or in different phases of the response. Indeed, in human peripheral blood a subset of “central memory” T cells can be identified, characterized by lack of immediate effector function and by the expression of lymph node homing receptors CD62L and CCR7 (38). Because of their differential homing capacity, nonpolarized and polarized T cells will realize an effective division of labor upon a secondary encounter with the antigen. Whereas polarized T cells will migrate to inflamed tissues mediating immediate protection, nonpolarized T cells will interact with antigen-loaded DCs in the lymph nodes, giving rise to new waves of effector cells. Therefore, nonpolarized T cells represent a strategic reservoir of antigen-specific precursors for mounting rapid and effective secondary responses.

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