Homing to Nonlymphoid Tissues Is Not Necessary for Effector Th1 Cell Differentiation

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The differentiation of naive T cells into effector Th1 cells is a complex process that may proceed in two steps, commitment and development. Initial TCR engagement and IFN-γ signaling instruct the T cells to commit to the Th1 lineage, while subsequent IL-12 and potentially TCR signaling induces final differentiation into irreversible, Th1 effector cells. In agreement with a multistep process of Th1 cell differentiation, effector Th1 cell generation requires repeated TCR and cytokine signaling, thus raising the possibility that commitment and differentiation processes may occur in two distinct anatomical sites, the lymphoid organ and the site of infection, respectively. We tested this possibility using a model of skin sensitization that permits a direct analysis of Ag-specific T cells both within lymphoid organs and at the site of sensitization. We show in this study that Ag presentation in the skin does not induce further differentiation of skin-infiltrating T cells that are highly divided and fully differentiated effector cells. Thus, effector Th1 cell differentiation is completed within lymphoid organs. In addition, we examined the heterogeneity of CD4 T cell responses in vivo through the analysis of the expression, by activated T cells, of different selectins, including P-selectin ligand and CD62L, known to define separable effector populations. We delineated, in lymph nodes, at least five distinct subpopulations of activated CD4 T cells with different phenotypes and recirculation properties. Collectively, these results show that the lymphoid environment orchestrates T cell activation to generate a repertoire of effector T cells with a diversity of effector functions. The Journal of Immunology, 2003, 171: 6355–6362.

Following stimulation with APC, CD4 T cells divide and in parallel differentiate into effector T cells expressing defined sets of cytokines. Both in vitro and in vivo studies have started to elucidate the regulation of these two steps of T cell activation. Transient exposure to Ag for a minimum of 2 h is sufficient for T cell entry into cell cycle (1). Cell division is, however, not observed before 24 h, then progression through cell cycle is very rapid and Ag independent (2–5). The differentiation of naive CD4 T cells into effector Th1 cells is a more complex process that may proceed in two steps, capacitance and development (6). Initial TCR signaling induces transient IFN-γ production by naive CD4 T cells that through STAT-1 activation permits the expression of the transcription factor T-bet, the master switch of naive CD4 T cells that through STAT-1 activation permits the final differentiation into irreversible, Th1 effector cells. In agreement with a multistep process of Th1 cell differentiation, effector Th1 cell generation requires repeated TCR and cytokine signaling, thus raising the possibility that commitment and differentiation processes may occur in two distinct anatomical sites, the lymphoid organ and the site of infection, respectively. We tested this possibility using a model of skin sensitization that permits a direct analysis of Ag-specific T cells both within lymphoid organs and at the site of sensitization. We show in this study that Ag presentation in the skin does not induce further differentiation of skin-infiltrating T cells that are highly divided and fully differentiated effector cells. Thus, effector Th1 cell differentiation is completed within lymphoid organs. In addition, we examined the heterogeneity of CD4 T cell responses in vivo through the analysis of the expression, by activated T cells, of different selectins, including P-selectin ligand and CD62L, known to define separable effector populations. We delineated, in lymph nodes, at least five distinct subpopulations of activated CD4 T cells with different phenotypes and recirculation properties. Collectively, these results show that the lymphoid environment orchestrates T cell activation to generate a repertoire of effector T cells with a diversity of effector functions. The Journal of Immunology, 2003, 171: 6355–6362.

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Th1 cell differentiation in vivo. To analyze T cell recirculation, we used a model of ear skin sensitization that permits a direct analysis of Ag-specific T cells within lymphoid organs and at the site of sensitization. We show in this study that CD4 T cell responses are heterogeneous, generating at least five distinct subpopulations of activated T cells with different phenotypes, recirculation properties, and functions. Among these different subpopulations detected in LN, skin-homing T cells were confined to a subset of Plig-expressing T cells that had accomplished at least five divisions. We found that Plig-expressing T cells were recruited in inflamed skin whether Ag was present or not. In both cases, skin-infiltrating T cells remain heterogeneous in terms of IFN-γ production, retaining a profile similar to that found in LN. Collectively, these results show that effector T cell functions are determined at the initiation of the immune response in lymphoid organs and that homing to infected tissues is not necessary for further differentiation nor amplification of the pool of effector Th1 cells. In addition, this study shows that CD4 T cell responses are not geared to generate T cells with restricted effector functions, but rather a heterogeneous population of activated T cells with a variety of effector functions.

Materials and Methods

Mice

The 3A9 transgenic mice were maintained on a CBA/J background and, for adoptive transfer, crossed with B10.BR Ly-5.1 congenic mice.

Adoptive transfer and immunizations

LN cells were isolated from 3A9 transgenic mice, and CD4+ T cells were purified by negative selection, as previously described (5). On average, the recovered population was composed of >85% CD4+ T cells, of which less than 3% were CD4+. The cells were labeled with CFSE (Molecular Probes, Eugene, OR), as previously described (5). CBA/J × B10.BR:F1 recipient mice were injected i.v. with 2.5–5 × 106 purified 3A9 CD4+ T cells. Twenty-four hours after adoptive transfer, the mice were immunized by s.c. injection, in the hind footpads, of CFA alone or 60 μg of anti-human IgG (Molecular Probes). P-selectin IgG staining was revealed using an alexa coupled to allophycocyanin, PE, FITC, or biotin, in which case staining before FACS analysis. Triple or quadruple staining was performed, as previously described (5).

Isolation of cells from primed mice

Anesthetized mice were bled, then perfused with PBS, and ears, lungs, and popliteal LN were harvested. Lungs and ears were slightly cut and incubated, for 1 h at 37°C, in Eagle’s Ham’s Amino Acids (EHAA) medium containing 400 U/ml collagenase I (Invitrogen, Grand Island, NY), 0.33 mg/ml DNase I (Sigma-Aldrich), and 2% FCS. Lymphocytes were purified by Ficoll separation (Amsbergh Biosciences AB, Uppsala, Sweden).

Abs and FACS staining

The anti-CD4 (RM4-5), anti-IFN-γ (XMG1.2), anti-Ly-5.1 (A20), anti-CD62L (Mel-14), anti-CD49d (R1-2), anti-CD103 (M290) Ab, and purified P-selectin IgG fusion protein were purchased from BD Pharmingen (BD Pharmingen, San Jose, CA). These Abs were unlabeled or directly coupled to allophycocyanin, PE, FITC, or biotin, in which case staining was revealed using streptavidin-PE or streptavidin-allophycocyanin (BD PharMingen). P-selectin IgG staining was revealed using an alexa fluor 488 anti-human IgG (Molecular Probes). For cytokine staining, cells were either stimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of 5 μM monensin (Sigma-Aldrich) or left unstimulated, in which case 5 μM monensin was added. For TOPRO-3 staining, cells were incubated with 0.5 nM TOPRO-3 (Molecular Probes) before FACS analysis. Triple or quadruple staining was performed, as previously described (5).

Results

Heterogeneity of CD4 T cell responses in vivo

We analyzed the response of CD4 T cells expressing a transgenic TCR specific for the HEL-derived peptide 46–61 presented by I-A^d (3A9) (17). The 3A9 CD4 T cells expressing the Ly-5.1 allele were adoptively transferred into syngeneic Ly-5.2 recipient mice. Twenty-four hours following adoptive transfer, the mice were primed by s.c. injection of HEL protein in CFA. Under those conditions, >90% of the 3A9 CD4 T cells from the draining LN are activated, as evidenced by CD69 and CD44 expression (data not shown) and divide (Fig. 1A). Initial cell division is evident by 32 h postimmunization (data not shown); by 48 h postpriming, 89.6 ± 5% (mean ± SD, n = 3) of the cells have divided one to five times; and by day 6 postpriming, 82 ± 2.6% (mean ± SD, n = 3) have accomplished more than six divisions (Fig. 1A). To further dissect the response of CD4 T cells in vivo, we analyzed the expression, by activated 3A9 T cells, of the LN-homing receptor CD62L, the tissue-homing receptor CD49d (α4 integrin), and the skin-homing receptor Plig, three receptors that are modulated during T cell activation. As negative control, we analyzed the expression level of CD103, an integrin that is preferentially expressed by intraepithelial lymphocytes (18). Naïve 3A9 T cells present in LN of unimmunized or CFA-injected mice are CD62L high, express intermediate levels of CD49d, and are negative for CD103 and Plig expression (Fig. 1B). As expected, skin immunization did not induce any change in CD103 expression by 3A9 T cells present in cutaneous LN (Fig. 1B). Changes in expression levels of CD62L, CD49d, and Plig are only observed in 3A9 T cells that have accomplished more than five divisions (Fig. 1B). Thus, 45% of highly divided 3A9 T cells (35% of all 3A9 T cells) down-modulated CD62L expression, and 16% of highly divided 3A9 T cells (12% of all 3A9 T cells) up-regulated Plig expression (Fig. 1B). In agreement with a previous report, expression of the skin-homing receptor Plig is almost exclusively restricted to CD62L+CD4+ T cells (Fig. 1C) (16). We did not detect significant up-regulation of CD49d expression in activated 3A9 T cells; however, 33% of highly divided 3A9 T cells have down-modulated this integrin, most of which have lost CD62L expression (Fig. 1, B and C). We also analyzed Th1 cell differentiation by intracellular FACS staining for IFN-γ. In this case, 3A9 T cells were stimulated for 4 h with PMA and ionomycin to induce IFN-γ production by differentiated effector T cells. Without such in vitro stimulation, we did not detect significant IFN-γ production by in vivo activated 3A9 T cells (see below). As previously reported, very few IFN-γ-producing effector Th1 cells were detected in vivo. Indeed, by day 4 postpriming, less than 10% of activated 3A9 T cells present in the draining LN produced IFN-γ (Fig. 1D). A similar percentage of IFN-γ-producing Th1 cells was maintained up to 30 days postpriming (data not shown). In agreement with previous reports, all IFN-γ-producing 3A9 T cells were highly divided, and most of them express Plig (Fig. 1D) (4, 16). Indeed, ~27% of the Plig+ and less than 2% of the Plig− 3A9 T cells produced IFN-γ (Fig. 1D).

Based on these different criteria, we could thus identify five different subpopulations of in vivo activated T cells (Fig. 1, B and C). The first population corresponds to CD4 T cells that have accomplished less than five divisions and retain a naïve phenotype (CD62L+, CD49d+, Plig−, IFN-γ−). In addition, among the highly divided 3A9 T cells, we could distinguish four subpopulations that correspond to 3A9 T cells that retain a naïve phenotype (CD62L+, CD49d+, Plig−, IFN-γ−), 3A9 T cells that have down-modulated expression of CD62L and CD49d (CD62L−CD49d−Plig−IFN-γ−), and 3A9 T cells that have up-regulated expression of Plig and may or may not produce IFN-γ (CD62L+CD49d+).
Plig*IFN-γ− or CD62L*CD49d*Plig*IFN-γ+). Interestingly, as observed for cytokine production, and in agreement with previous reports (19, 20), changes in surface phenotype of in vivo activated T cells are only observed for T cells that have accomplished several divisions (Fig. 1B).

**Trafficking of in vivo activated CD4 T cells**

Due to variable expression of adhesion molecules, these different subpopulations may have different homing capabilities. We therefore analyzed the trafficking of 3A9 T cells from draining LN to the site of immunization. For these experiments, we sensitized mice on the ears because ear-infiltrating cells are more efficiently isolated than footpad-infiltrating cells. Thus, 3A9 recipient mice were first injected with HEL in the hind footpads and rechallenged 3 days later in the ear. Similar activation profiles were observed in mice immunized only once in the ear (Fig. 2C). Thus, at 48 h postimmunization, no infiltrating cells were found into the ear, although the 3A9 T cells within the auricular LN had accomplished up to five divisions. By day 4, when the 3A9 T cells had gone through more than five divisions, skin infiltration was clearly evident and consisted of highly divided 3A9 T cells (Fig. 2A and C). Selective entry into the skin of highly divided 3A9 T cells correlates with Plig expression by these activated T cells. Thus, in agreement with the role of Plig in CD4 T cell recruitment into inflamed skin (21, 22), skin-infiltrating 3A9 T cells were strongly enriched for Plig* T cells (Fig. 2B). Interestingly, although all blood-circulating T cells expressed CD62L, most skin-infiltrating T cells had lost expression of this molecule (Fig. 2B).

We next examined the 3A9 CD4 T cells that were recruited in an inflamed ear. A kinetic study indicates that infiltration into the skin starts at day 3–4 post sensitization, then slowly declines. These kinetics reflect the rate of CD4 T cell division in the LN, as indicated by a time course study of mice immunized only once in the ear (Fig. 2C). Thus, at 48 h post immunization, no infiltrating cells were found into the ear, although the 3A9 T cells within the auricular LN had accomplished up to five divisions. By day 4, when the 3A9 T cells had gone through more than five divisions, skin infiltration was clearly evident and consisted of highly divided 3A9 T cells (Fig. 2A and C). Selective entry into the skin of highly divided 3A9 T cells correlates with Plig expression by these activated T cells. Thus, in agreement with the role of Plig in CD4 T cell recruitment into inflamed skin (21, 22), skin-infiltrating 3A9 T cells were strongly enriched for Plig* T cells (Fig. 2B). Interestingly, although all blood-circulating T cells expressed CD62L, most skin-infiltrating T cells had lost expression of this molecule (Fig. 2B).

The lung was reported to drain significant numbers of activated CD4 T cells as well as memory effector T cells (23). We, however, did not find a significant increase in the percentage of lung-infiltrating T cells following s.c. immunization. In contrast to skin-infiltrating CD4 T cells, the 3A9 T cells found in the lung were not selected for highly divided, Plig* T cells (Fig. 2A, and data not shown). Indeed, the phenotype of the 3A9 T cells found in the lung
and the blood was very similar, suggesting no selective entry or recruitment into that tissue in the absence of local inflammation.

Local Ag load is not necessary for T cell extravasion into skin
Administration of Ag in immunized mice is sufficient to induce a DTH reaction, although this challenge always induces some inflammatory response. Whether Ag is necessary for T cell recruitment in inflamed skin is, however, still a matter of debate. Several in vitro studies indicate that Ag presentation by endothelial cells facilitates transendothelial migration most likely through activation of adhesion molecules (24, 25). Conversely, indirect in vivo studies suggest that T cell may be recruited into inflamed skin even in the absence of Ag (26, 27). We therefore determined whether a local inflammation resulting from skin sensitization with CFA might suffice to recruit 3A9 T cells into the ear. Mice were thus immunized in the hind footpad as before and either analyzed directly or after challenge in the ear with CFA or CFA and HEL. No infiltrating cells could be retrieved from untreated ears (Fig. 3A). Interestingly, 3A9 CD4 T cells were recruited in CFA- or CFA/HEL-sensitized ears (Fig. 3A). The percentage of 3A9 CD4 T cells as compared with total number of CD4 T cells was similar in the two situations (Fig. 3A). The 3A9 CD4 T cells recruited in an inflamed ear, like the 3A9 recruited in a CFA/HEL-challenged ear, were highly divided and expressed Plig (data not shown, and see below). Extravasion into inflamed skin is therefore not determined by Ag, and thus T cell specificity, but selects for activated T cells that express Plig. In agreement, we found a significant number of host-derived CD4 T cells that infiltrate sensitized ears. Like the 3A9 CD4 T cells, they express Plig, but, due to their number, are most likely not all specific for HEL or adjuvant proteins. Nonetheless,

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Trafficking of the different subpopulations of activated 3A9 T cells. Recipient mice were adoptively transferred with 2–5 × 10⁶ purified CFSE-labeled 3A9 CD4 T cells expressing the Ly-5.1⁺ allele. A and B. The mice were primed 24 h later in the hind footpads and challenged 4 days after adoptive transfer in the ear with CFA alone (CFA) or together with HEL protein (HEL/CFA). The mice were sacrificed 2 days after challenge. A. The percentage and CFSE profile of 3A9 CD4 T cells present in popliteal LN, blood, lungs, and ears were determined. B. Expression of CD62L, Plig, CD103, and CD49d by 3A9 CD4 T cells present in blood and ears was analyzed by FACS staining. C. Recipient mice were primed once only in the ear and sacrificed 2 or 4 days later. The CFSE profile of gated Ly-5.1⁺ 3A9 T cells in the auricular LN is shown in the left panels. The homing of 3A9 T cells into ears was evaluated by staining total ear infiltrates for CD4 and Ly-5.1 (right panels). One representative experiment of two performed with a total of 8–16 mice is shown.

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** Ag is not required for CD4 T cell entry into inflamed tissues. Recipient mice were adoptively transferred with 2–5 × 10⁶ purified CFSE-labeled 3A9 CD4 T cells expressing the Ly-5.1⁺ allele and primed 24 h later, by hind footpad injection of HEL protein emulsified in CFA. Three days after priming, the mice were either untreated (−) or injected into the ear with CFA alone (CFA) or together with HEL protein (CFA/HEL). The mice were sacrificed 2 days after challenge. A. The percentage of 3A9 CD4 T cells among total CD4 T cells present in popliteal LN, blood, and ears was determined by FACS staining. The mean and SD of three independent experiments performed with at least two mice are shown. B. The corresponding number of 3A9 CD4 T cells in CFA- or CFA/HEL-injected ears was calculated by multiplying the total number of recovered cells with the corresponding percentage of 3A9 T cells. Individual experiments are represented by different symbols. The line represents the mean value of the three different experiments performed.
the presence of HEL increased recruitment of 3A9 T cells into ears (Fig. 3B). Indeed, the total number of 3A9 CD4 T cells in CFA/HEL-sensitized ear was 2.11 ± 0.64 (mean ± SD, n = 3) times that found in CFA-sensitized ear, reflecting a general increase in T cell infiltration. This latter observation suggests that both specific and nonspecific recruitment of T cells into skin are enhanced by local T cell activation.

Final effector Th1 cell differentiation does not occur in the skin

Because activated T cells were recruited in skin even in the absence of Ag, we could examine whether T cell commitment and final effector cell differentiation occur in different anatomical sites, the LN and tissue, respectively. Such a model would predict that the frequency of IFN-γ-producing T cells remains comparable and low in LN or in inflamed tissue, but increases considerably within Ag-draining tissue. We first analyzed mice challenged in the ear with CFA and Ag. The skin-infiltrating 3A9 T cells were enriched for Th1 effector cells because 46% of these cells produced IFN-γ as compared with 7% in LN (Fig. 4A). Because recruitment in the different compartment was regulated by expression of distinct addressing molecules and expression of IFN-γ mainly confined to cells expressing Plig, we determined the frequency of IFN-γ-producing T cells among Plig+ 3A9 T cells. We found that 35.4 and 43.5% of Plig+ 3A9 T cells present in the LN and immunized ear, respectively, produced IFN-γ following 4 h in vitro restimulation (Fig. 4B). Ag stimulation in skin therefore increased only minimally the frequency of IFN-γ-producing cells, suggesting that tissues may not be site of effector Th1 cell generation. To further substantiate this observation, we analyzed IFN-γ production by T cells infiltrating an inflamed tissue containing or not the specific Ag. We found that 35 and 46% of 3A9 T cells present in CFA- and CFA/HEL-challenged ear, respectively, produced IFN-γ following 4 h in vitro restimulation (Fig. 4C). The slight increase in the percentage of IFN-γ-producing 3A9 T cells in Ag-primed ears most likely reflects preactivation of the cells in vivo and not further differentiation. Indeed, 3A9 T cells from Ag-primed ears produced IFN-γ directly ex vivo and produced higher level of IFN-γ following in vitro restimulation (Fig. 4C). Interestingly, 3A9 T cells producing IFN-γ ex vivo are only found in ears injected with Ag and not in Ag-draining LN (Fig. 4, A and B).

To further confirm that tissues are not necessary for effector cell differentiation, we prevented T cell homing to the ears and examined the incidence of such treatment on the frequency of effector T cells in lymphoid organs. Under normal circumstances, Plig+ T cells generated in the draining LN could access inflamed skin, where they may be transiently trapped. Then tissue effector cells recirculate through the lymphatic, blood, and lymphoid organs. Thus, due to transient trapping and recirculation of tissue effector, the number of circulating Plig+ T cells may increase when skin homing is hampered. If Th1 cell differentiation is confined to lymphoid organs, such treatment should induce a similar increase in Plig+ IFN+ T cells. However, if tissues are significantly contributing to effector Th1 cell generation, blocking tissue homing should reduce the frequency of recirculating effector Th1 cells. To efficiently block T cell homing into inflamed skin, we treated immunized mice with blocking Ab against E- and P-selectin, the two

Table I. Numbers of effector Th1 cells in lymphoid organs of control mice or mice in which skin homing is hampered

| Group                        | Number of 3A9 (mean ± SD × 10^3) | Number of Plig+ (mean ± SD × 10^3) | Number of Plig+ IFN+ (mean ± SD × 10^3) | % IFN+ among Plig+ (mean ± SD) |
|------------------------------|----------------------------------|-----------------------------------|----------------------------------------|-------------------------------|
| Spleen + LN                  |                                  |                                   |                                        |                               |
| Untreated (n = 8)             | 8.64 ± 4.6                       | 1.09 ± 0.5                        | 3.52 ± 2.21                            | 30.5 ± 6.5                    |
| Treated (n = 8)              | 7.96 ± 1.8                       | 1.16 ± 0.2                        | 3.99 ± 1.26                            | 34.0 ± 5.0                    |
| p Value*                    | 0.612                            | 0.715                             | 0.623                                  | 0.320                         |
| Ears                        |                                  |                                   |                                        |                               |
| Untreated (n = 8)             | 0.29 ± 0.1                       | 0.13 ± 0.05                       | 1.2 ± 0.57                             | 47.6 ± 5.7                    |
| Treated (n = 8)              | 0.1 ± 0.06                       | 0.05 ± 0.03                       | 0.48 ± 0.22                            | 49.1 ± 6.7                    |
| p Value*                    | 0.004                            | 0.0002                            | 0.0006                                 | 0.0008                        |

*Number of Plig+ 3A9 T cells.
†Number of Plig+ IFN+ 3A9 T cells.
‡Mice were i.v. injected with 30 μg of control Ig 48 and 72 h post-ear immunization with HEL + CFA.
§Mice were i.v. injected with 10 μg of UZ4 (anti-E-selectin) and 15 μg of RB40.34 (anti-P-selectin) Ab 48 and 72 h post-ear immunization with HEL + CFA.
\(^{\dagger}\)Two-tailed Student t test.
\(^{\star}\)Percentage of Plig+ IFN+ 3A9 T cells among Plig+ 3A9 T cells.
P-selectin glycoprotein ligand-1 ligands involved in CD4+ T cell extravasation into inflamed skin (22, 28, 29). Such treatment inhibited the homing of 3A9 T cell into skin by 65.75% (Table I). In treated mice, the number of 3A9 T cells in lymphoid organs was also slightly reduced. Because homing of immature dendritic cells into inflamed tissue depends on E- and P-selectin, such reduction of the number of 3A9 T cells in treated mice may reflect reduced clonal expansion due to reduced recruitment of Ag-presenting dendritic cells (30). Nonetheless, in lymphoid organs of mice in which skin homing was hampered, the number of Plig− as well as Plig+/IFNγ 3A9 T cells was slightly increased as compared with control mice (Table I). Although this increase is modest, it may correspond to the low number of effector cells that were missing in inflamed ears of Ab-treated mice (Table I).

Collectively, these results show that differentiation of effector Th1 cells mainly occurs within the LN, and that these effectors are preferentially sequestered from the blood in the inflamed tissue. The local presence of Ag within the skin does not induce further expansion or differentiation of infiltrating CD4 T cells, but stimulates cytokine production by infiltrating T cells and, as a consequence, a DTH reaction (data not shown).

Tissue effector cells are not end stage, but can further expand

Effector Th1 cells are generally viewed as short-lived, end-stage products of T cell activation due to their increased susceptibility to apoptosis and low survival in vivo (23, 31, 32). Rapid death of effector Th1 cells in the skin might mask ongoing proliferation and differentiation in that tissue. We therefore evaluated the survival and proliferation of in vivo generated tissue-infiltrating 3A9 T cells. Cells were collected from CFA- or CFA/HEL-challenged ears, and the in vitro survival/proliferation of the corresponding 3A9 T cells was compared with that of LN resident 3A9 T cells. Total cell populations were used in these experiments and may therefore contain APC and Ag when isolated from immunized mice. To analyze T cell division, the recovered cell populations were CFSE labeled before initiation of the culture. Cell death was monitored by TOPRO-3 staining, a dye that integrates into the DNA of dead cells. The survival of 3A9 T cells was comparable for the three populations analyzed (Fig. 5). In addition, 3A9 T cells proliferated when Ag was present. Thus, most 3A9 T cells isolated from Ag-challenged ears or draining LN had divided more than three times within 4 days (Fig. 5). In contrast, most 3A9 T cells isolated from CFA-challenged ears did not proliferate, unless in vivo pulsed APC were added to the culture (Fig. 5). This latter finding indicates that the observed proliferation did not result from initial stimulation in vivo within the LN, but rather from TCR re-engagement during the in vitro incubation period.

Collectively, these results indicate that effector T cells recruited into the skin are not short-lived and can be reinduced to proliferate when confronted with Ag.

Discussion

Generation of a productive immune response in vivo relies on the expansion, differentiation, and adequate localization in lymphoid organs and infected tissues of Ag-specific T cells. The coordinate regulation of these different parameters is critical, as it will permit appropriate homing of specialized effector T cells. Through the analysis of CD4 T cell differentiation and homing in a model of skin sensitization, we analyzed the site and regulation of these different parameters in vivo.

Phenotypic and functional analysis of Ag-specific T cells during an immune response in the LN indicates that at least five different subpopulations of activated T cells are generated. Previous studies of the proliferation and cytokine production by activated T cells had suggested a heterogeneity of CD4 T cell responses in vivo (3, 14, 23, 33). Further analysis of integrin and selectin receptor expression by in vivo activated CD4 T cells reinforced this notion (this study) (16, 19). How heterogeneous CD4 T cell responses are in vivo is still an open question and cannot be inferred from the limited studies performed to date. Such heterogeneity most likely has a biological significance and may reflect the different functions CD4 T cells have in the regulation of an immune response. In agreement, B cell helper and inflammatory CD4 effector T cells are segregated on the basis of Plig and CD62L expression (this study) (16). The function of the other subpopulations identified by this study that retain a naïve phenotype when considering the different adhesion molecules analyzed, but show different numbers of cell division, is still unknown. They might have other helper functions such as CD8 helper or other regulatory functions, or may constitute part of the long-lived memory population. Further analysis of the cytokines they may produce and their homeostasis may help dissect their in vivo function. The Plig−/CD62L− population that represents a large fraction of activated T cells most likely has broader functions than merely B cell helper. Recent studies indicate that CD62L−CD8+ T cells may correspond to effector memory T cells with tissue-homing abilities, suggesting that at least some of CD62L−CD4+ T cells may likewise constitute part of the effector memory CD4 T cell pool (34–37).

Analysis of the circulation and homing of activated 3A9 T cells reveals some unexpected findings. It was surprising to find that
CD62L–CD49d+ 3A9 T cells, which represent ~40% of the 3A9 T cells in LN, were excluded from the circulating pool. It is yet unclear whether this later subpopulation of activated T cells is selectively retained in the LN or whether CD62L+ and CD49d+ down-modulation is transient for in vivo activated T cells. Indirect evidence suggests that a fraction of these cells up-regulates CD62L and is part of the circulating pool of highly divided CD62L+ 3A9 T cells. Some of them, however, may express chemokine receptors that trap them transiently in the B cell area or other area of the LN, thus excluding them from the circulating pool. The absence of circulating CD62L–CD49d+ 3A9 T cells is quite surprising in view of the recent finding that CD62L+CD8+ T cells are found in the blood of mice or humans and may constitute the pool of effector memory cells (34–37). One possible explanation for this discrepancy may come from our observation that the CD4+ T cells isolated from skin had lost CD62L expression. The observation that all Plig+ T cells in LN and all circulating T cells in blood express CD62L suggests that expression of this receptor is down-modulated in tissue due to either local inflammation or TCR stimulation within the skin. We would thus propose that circulating CD62L– T cells are not derived from the CD62L+ population in LN, but in fact tissue emigrant effector CD4 T cells, leaving the infection site when Ag and inflammation are contained. Lack of expression of CD62L would exclude them from LN. They would thus patrol between tissue, spleen, and blood until regaining expression of CD62L and most likely CCR7. In agreement with previous reports, we found that recruitment into the skin critically relies on expression by T cells of Plig (16, 21, 28). We also show that Ag is not required for T cell extravasation into skin. As a consequence, T cells recruited in an inflamed tissue need not be specific for the infectious agent. This may explain the massive infiltration of irrelevant T cells found in tissue target of an immune or autoimmune response. Ag-independent homing of T cell into inflamed skin may at first glance appear unavailing. This process may, however, ensure recruitment of memory effector cells into an infectious site rapidly after infection and before Ag spreading.

We also show that tissues are not necessary for Th1 cell differentiation. The absence of Th1 cell differentiation in skin cannot be explained by a rapid death of effector T cells because skin-infiltrating T cells survived well in vitro and further divided when confronted with Ag. Instead, lack of further differentiation into the skin may reflect the inability of committed T cells to home to that tissue. We have shown previously that Th1 cell differentiation requires recurrent T cell stimulation by polarized APC (5). Thus, initial stimulation of CD4 T cells will induce a first round of three to four divisions and Th1 cell commitment. Subsequent exposure to APC will induce further differentiation of the committed Th1 cells into IFN-γ-producing cells and also a new round of T cell division. Because Plig expression is induced when the T cells have accomplished at least six divisions, committed T cells should not express Plig and thus home into the skin. Interestingly, changes in most homing and chemokine receptor expression are likewise observed when T cells have accomplished at least five divisions (this study) (19, 20). Similarly, high and irreversible cytokine profile is observed only in T cells that have divided several times (4, 7, 19, 20). These observations suggest that homing properties and effector functions are coordinately regulated, further suggesting that, in general, only differentiated effector cells are recruited into tissues. Nevertheless, our study shows that effector T cell differentiation is restricted to lymphoid organs, and that tissues are not amplifying the pool of effector cells. Thus, the lymphoid environment will define the diversity of effector CD4 T cell and the representation of each effector subpopulation.

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