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Repeated Antigen Exposure Is Necessary for the Differentiation, But Not the Initial Proliferation, of Naive CD4$^+$ T Cells[1]

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The mechanisms that regulate CD4$^+$ T cells responses in vivo are still poorly understood. We show here that initial Ag stimulation induces in CD4$^+$ T cells a program of proliferation that can develop, for at least seven cycles of division, in the absence of subsequent Ag or cytokine requirement. Thereafter, proliferation stops but can be reinitiated by novel Ag stimulation. This initial Ag stimulation does not however suffice to induce the differentiation of naive CD4$^+$ T cells into effector Th1 cells which requires multiple contacts with Ag-loaded APC. Thus, recurrent exposure to both Ag and polarizing cytokines appears to be essential for the differentiation of IFN-γ-producing cells. Ag and cytokine availability therefore greatly limits the differentiation, but not the initial proliferation, of CD4$^+$ T cells into IFN-γ-producing cells. *The Journal of Immunology*, **2002, 168: 1723–1729.**

Following stimulation by APC, CD4$^+$ T cells divide and in parallel differentiate into effector cells expressing defined sets of cytokines. Both in vitro and in vivo experiments indicate that, in contrast to the massive clonal expansion, the differentiation of activated CD4$^+$ T cells into effector cells is fairly inefficient (1–3). These observations suggest that TCR and cytokine signaling may differently regulate cell division and differentiation.

Activation of naive CD4$^+$ T cells and entry into the cell cycle was shown to require an exposure to Ag for a minimum of 12 h (4). The first division of activated CD4$^+$ T cells is however not observed before 24 h following Ag stimulation. Past this lag time, progression through cell cycle is extremely rapid with a maximum generation time of 4–6 h (5, 6). Whether TCR re-engagement and/or cytokines are required to sustain cell division of activated CD4$^+$ T cells during this intense proliferation phase is still unknown.

The differentiation of naive CD4$^+$ T cells into effector Th1 cells likewise requires a commitment phase during which differentiating cells do not produce the prototype Th1 effector cytokines, IFN-γ and TNF-β. Commitment to the Th1 lineage is regulated by complex signals including ligand density, costimulatory molecules, and cytokines (7–10). Cytokines such as IL-12 play a preeminent role in driving Th1 differentiation (7). Activation of STAT4 upon IL-12 binding to the IL-12R is essential in inducing the commitment of naive CD4$^+$ T cells to the Th1 lineage; although, under some conditions, a STAT4-independent Th1 differentiation may occur (11–13). Expression of the transcription factor T-bet was also shown to be restricted to differentiating Th1 cells and to specifically induce the expression of *IFN-γ* while repressing *IL-4* and *IL-2* gene expression (14). Despite rapid induction of these different transcription factors, commitment to the Th1 lineage requires at least 48 h (15). Whether continuous TCR engagement and/or cytokines signaling are required during this commitment period is still a matter of debate. Indeed, in one study, a 24-h stimulation with plate-bound MHC-peptide complexes in the presence of IL-12 was shown to be efficient at inducing Th1 differentiation (16). In other studies however, a 72-h exposure to TCR and IL-12 stimulation was necessary for full differentiation of effector Th1 cells (5, 13). As both Ag and cytokines may be limiting in the course of an immune response in vivo, it is essential to evaluate to what extent these two parameters are regulating CD4$^+$ T cell responses.

In this report, we determined whether sustained TCR and cytokine signaling are required for clonal expansion and differentiation of effector Th1 cells. We found that transient exposure to Ag induces a program of proliferation that can develop for six to seven divisions, in vivo and in vitro, in the absence of subsequent contact with Ag. In contrast, Ag-driven differentiation of effector Th1 cells requires TCR re-engagement and cytokine signaling, both signals being necessary for the sustained expression of *T-bet* gene. As opposed to CD8$^+$ T cells, for which initial Ag encounter induces a program of proliferation and differentiation that can proceed in the absence of Ag (17, 18), the differentiation, but not the initial burst of proliferation of CD4$^+$ T cells appears therefore tightly regulated by Ag and cytokine availability.

Materials and Methods

Mice

The 3A9 mice are transgenic for a TCR specific for the hen egg lyzosyme (HEL)[3] peptide 46–61 presented by I-A^k (19). The 3A9 mice were maintained on a CBA/J background or, for adoptive transfer, crossed with B10.BR Ly5.1$^+$ congenic mice.

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[3] Abbreviations used in this paper: HEL, hen egg lysozyme; HPRT, hypoxanthine phosphoribosyltransferase; DC, dendritic cell.

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Adoptive transfer

(CBA/J × B10.BR)F_1 recipient mice were injected i.v. with 2.5×10^6 naive or in vitro-activated $CD4^+$ T cells from the 3A9-transgenic mice. Twenty-four hours before adoptive transfer, the mice were immunized by s.c. injection, in the backs of recipient mice, of 200 μg of native HEL protein (Sigma-Aldrich, St. Louis, MO) emulsified in CFA (Sigma-Aldrich) or CFA alone in a 200-μl volume, as previously described (20).

Abs and FACS staining

The Abs anti-CD4 (RM4-5), anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), and anti-Ly5.1 (A20) were purchased from BD PharMingen (San Jose, CA). These Abs were directly coupled to allophycocyanin, PE , FITC , or biotin, in which case staining was revealed using streptavidin-allophycocyanin (BD PharMingen). TO-PRO-3 (Molecular Probes, Eugene, OR) was used at a final concentration of 0.5 nM. For cytokine staining, cells were stimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich), and 10 μg/ml brefeldin A (Sigma-Aldrich) was added for the last 2 h or for the entire 4-h stimulation period when ex vivo T cells were analyzed. Cells were harvested, stained for appropriate surface markers, fixed with 2% paraformaldehyde, and incubated in permeabilization buffer containing the anti-cytokine Ab as previously described (1).

Cell preparation and in vitro activation

$CD4^+$ T cells were purified from the lymph nodes of 3A9-transgenic mice by negative selection using a mixture of Ab composed of an anti-I-E^k (M5/114.15.2), an anti-B220 (RA36B2), an anti-CD8 (H-59-101-2), an anti-FcγRII/III (2.4.G2), and an anti-CD11b (M1/70.15.11.5HL) followed by an incubation with sheep anti-rat IgG magnetic beads (Dynal Biotech, Oslo, Norway). On average, the population recovered was composed of >90% $CD4^+$ T cells, of which <3% were $CD44^+$. The cells were labeled with CFSE (Molecular Probes) as previously described (21). T cell-depleted splenic APCs were prepared by Ab-mediated complement lysis using an anti-Thy1.2 Ab (J1J) and were gamma irradiated at 24 Gy. Naive T cells (5×10^5) were stimulated in 24-well plates with APCs (1×10^6) and 0.6 μg/ml HEL 46–61 peptide. Forty-eight hours later, dividing $CFSE^+CD4^+$ T cells were FACS sorted by excluding cells that were in the most brightest peak (0 division). FACS-sorted populations were >98% pure. Sorted $CD4^+$ T cells (7.5×10^4) were seeded either in the upper or lower compartment of a 12-well Transwell plate with a 0.4-μm membrane pore size (Costar, Cambridge, MA) or in a standard 24-well-plate. When indicated, 5×10^5 APCs were added along with 0.6 μg/ml HEL 46–61 peptide. For anti-CD3 stimulation, plates were coated overnight with 10 μg/ml anti-CD3 (145.2C11) and 1 μg/ml soluble anti-CD28 Ab (37.51) was added during the culture period. When indicated, neutralizing anti-IL-2 (2 μg/ml, JES6-1A12; BD PharMingen), anti-IL-12 (5 μg/ml,C17-6, a generous gift from G. Trinchieri, Schering-Plough Research Institute, Dardilly, France), anti-IL-4 (5 μg/ml, 11B11; BD PharMingen), or anti-IFN-γ (5 μg/ml, XMG1.2; BD PharMingen) Ab were added. Recombinant murine cytokines were IL-2 (10 or 50 U/ml; PeproTech, Princeton, NJ), IL-12 (3.5 ng/ml; PeproTech), or IFN-γ (50U/ml; Genzyme, Cambridge, MA).

RNA preparation and RT-PCR

Total RNA was extracted using the High Pure RNA Isolation kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions, treated with DNase I (Roche Diagnostic) and reverse transcribed using random primers and Superscript II RT (Life Technologies, Grand Island, NY). Real-time PCR was performed on cDNA samples using the Taqman Sybr Green system (PE Biosystems, Warrington, U.K.). Primers used were hypoxanthine phosphoribosyltransferase (HPRT) sense 5′-AGCCCTCTGTGTGCTAAGG-3′, HPRT antisense 5′-CTGATAAAA TCTACAGTCATAGGAATGGA-3′; T-bet sense 5′-CAACAACCCC TTTGCCAAAG-3′, and T-bet antisense 5′-TCCCCCAAGCAGTTGA CAGT-3′. Cycling conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles each corresponding to 15 s at 95°C and 1 min at 60°C. Analysis used the sequence detection software supplied with the instrument. The relative quantitation value is expressed as $2^{-\Delta CT}$ and percentage of maximal $2^{-\Delta CT}$, where ΔC_T is the difference between the mean C_T value of triplicates of the sample and of the endogenous HPRT control.

Results

Role of TCR re-engagement in sustaining T cell division

We analyzed the response of $CD4^+$ T cells isolated from the 3A9 TCR-transgenic mice that are specific for the HEL peptide presented by the class II MHC molecule I-A^k (referred to hereafter as 3A9 $CD4^+$ T cells (19)). To follow T cell division, we used the vital dye CFSE that is equally partitioned to daughter cells at each division (21). To analyze the role of TCR re-engagement or cytokine signaling in further driving the division and differentiation of cycling $CD4^+$ T cells, we stimulated the 3A9 $CD4^+$ T cells for 48 h and FACS sorted the 3A9 $CD4^+$ T cells that had accomplished at least one division. At this time point, on average, 90% of the $CD4^+$ T cells had achieved one to three divisions (data not shown) and did not produce significant levels of cytokines such as IL-2, IL-4, or IFN-γ without restimulation (Fig. 1). Most of these activated 3A9 $CD4^+$ T cells are undifferentiated since they produce IL-2 but little IFN-γ or IL-4 following restimulation with PMA and ionomycin or upon TCR re-engagement (Fig. 1, *b* and *d*). We first determined whether TCR re-engagement and cytokine signaling were necessary to sustain cycling of activated T cells. To mimic as closely as possible the Ag and cytokine environment that may occur during an in vivo response, we used a Transwell system with a membrane pore size of 0.4 μm that allows the passage of soluble factors such as cytokines but not cells. Sorted $CD4^+$ T cells were seeded in the upper and lower chambers of the Transwell while APCs and Ag were only added to the upper chamber. The upper well thus contained $CD4^+$ T cells that had re-engaged their TCR and, as a consequence of this TCR stimulation, may produce cytokines available for themselves and for the $CD4^+$ T cells present in the lower well that did not re-engage their TCR. As shown in Fig. 2*a*, $CD4^+$ T cells present in the upper and lower chambers of the Transwell divided equally within the 48 h of reculture. The continuation of cell division in the lower chamber did not result from carryover of Ag-loaded APC since the $CD4^+$ T cells were FACS sorted to >98% purity. Furthermore, similar results were obtained when the upper and lower wells contained

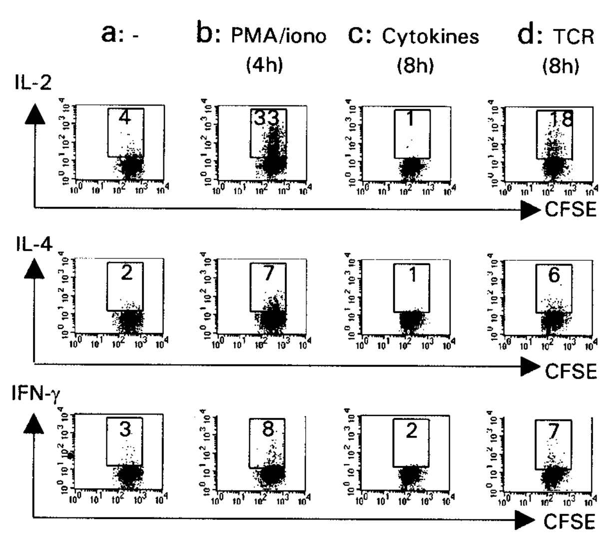

FIGURE 1. Cytokine production by 48-h activated 3A9 $CD4^+$ T cells. CFSE-labeled 3A9 $CD4^+$ T cells were stimulated with APCs plus HEL peptide for 48 h, and dividing $CD4^+$ T cells were FACS sorted based on their CFSE profile. The ability of these activated cells to produce IL-2, IL-4, and IFN-γ either spontaneously (*a*), after 4-h stimulation with PMA/ionomycin (*b*), after 8-h incubation with IL-2, IL-12, and IFN-γ (*c*), or after 8-h stimulation with APC and HEL peptide (*d*) was determined. Similar results were obtained in three independent experiments.

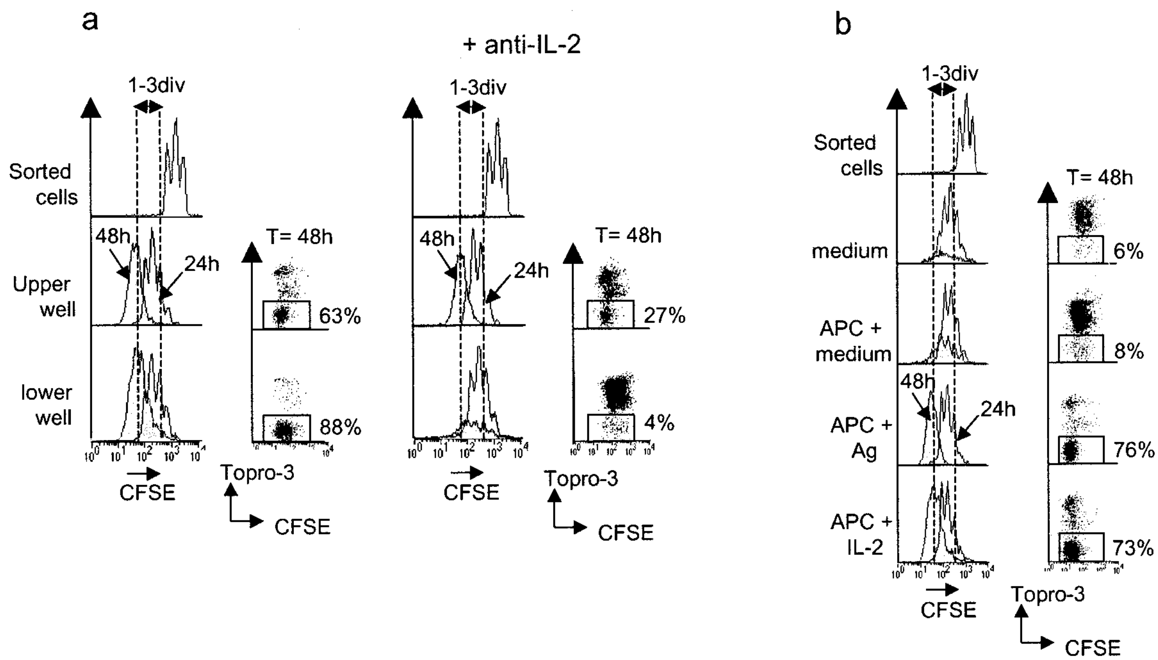

FIGURE 2. Neither TCR re-engagement nor cytokines are necessary to sustain the proliferation of activated CD4$^+$ T cells. CFSE-labeled 3A9 CD4$^+$ T cells were stimulated with APCs plus HEL peptide for 48 h, and dividing CD4$^+$ T cells, having accomplished one to three divisions, were FACS sorted (sorted cells). *a,* Dividing cells were reincubated in the two compartments of a Transwell plate containing APCs plus HEL peptide in the upper well only. When indicated, an anti-IL-2-blocking Ab was added to the culture medium. *b,* Dividing CD4$^+$ T cells were reincubated in medium alone or with APCs either alone or with HEL peptide or with 10 U/ml IL-2 as indicated. The CFSE profile of CD4$^+$ T cells was analyzed at 24 h (open histograms) or 48 h (filled histograms) of secondary culture. The interval between the two dotted lines corresponds to one to three subsequent divisions in addition to the three divisions accomplished by the 48-h activated T cells. Viability was determined by TO-PRO-3 staining and the percentage of viable cells corresponding to TO-PRO-3-negative CD4$^+$ T cells at 48 h after reincubation is indicated. Similar results were obtained in three independent experiments.

CD4$^+$ T cells recognizing distinct MHC-peptide complexes (data not shown). Finally, naive 3A9 CD4$^+$ T cells when added to the lower chamber were not activated nor did they divide (data not shown). These results indicate that although entry into the cell cycle requires TCR engagement, further progression through cell division, during the 48 h of analysis, does not require TCR re-engagement.

We further determined whether subsequent cycling of activated T cells was dependent on the production, by the restimulated T cells, of cytokine such as IL-2 that is produced following TCR re-engagement (Fig. 1*d*). Anti-IL-2 blocking Ab was therefore added to the Transwell system. Cycling of CD4$^+$ T cells in the upper chamber of the Transwell was similarly sustained whether IL-2 was present or not during the reincubation period, indicating that cycling of cells induced by TCR re-engagement may be IL-2 independent (Fig. 2*a*). Although continuous IL-2 signaling was not necessary to sustain the proliferation of activated 3A9 CD4$^+$ T cells, IL-2 greatly contributed to the survival of these activated T cells in vitro (Fig. 2*a*). Indeed, 73% of the cycling CD4$^+$ T cells died when restimulated in the absence of IL-2 as evidenced by TO-PRO-3 uptake, a dye that specifically stains dead cells. 3A9 CD4$^+$ T cells present in the lower well also progressed through further division whether IL-2 was present or not (Fig. 2*a*). Indeed, in the absence of IL-2, the 48-h activated CD4$^+$ T cells made an additional three divisions within 24 h (Fig. 2*a*). Past this 24-h time period, when deprived of IL-2, the CD4$^+$ T cells present in the lower well did not further divide and most of them died (Fig. 2*a*). We further tested that no other cytokines, such as IL-4, produced by activated T cells or IL-7 or -15 produced by the APC present in the upper well, may contribute to cycling of the CD4$^+$T cells present in the lower chamber. We reincubated sorted cycling CD4$^+$ T cells in medium alone, a condition where none of these cytokines are produced or, as control, in combination with syngeneic APCs with or without IL-2 or HEL peptide. Activated CD4$^+$ T cells further divided in all four culture conditions (Fig. 2*b*). As observed in the Transwell system, activated 3A9 CD4$^+$ T cells made three to four additional divisions within 24 h regardless whether cytokines or Ag were added or not during the restimulation period. Past this 24-h period, 3A9 CD4$^+$ T cells maintained in medium alone stopped cycling and most of them died, in contrast to cells reincubated with IL-2 or Ag-loaded APCs that continued to divide (Fig. 2*b*).

Collectively, these results indicate that once into cycle, CD4$^+$ T cells proceed through several rounds of divisions in the absence of subsequent TCR and cytokine signaling. We further determined whether in vivo cycling of activated CD4$^+$ T cells also proceeded in an Ag-independent fashion. For these experiments, CFSE-labeled 3A9 CD4$^+$ T cells expressing the Ly5.1 allotype were activated for 48 h in vitro, purified to remove any Ag-loaded APCs, and then transferred into syngenic mice expressing the *Ly5.2* allele. Twenty-four hours before adoptive transfer, the recipient mice were injected s.c. with CFA with or without HEL, or left unimmunized. Activated T cells further divided when transferred into unimmunized animals (Fig. 3*a*). Under those conditions, they performed four additional divisions within 48 h and some of them further divided. As observed in vitro, most activated 3A9 CD4$^+$ T cells stopped dividing after 48-h posttransfer into unimmunized hosts, although under those in vivo conditions the CD4$^+$ T cells did not die since the number of 3A9 CD4$^+$ T cells recovered at 48 and 72 h were comparable (Fig. 3*b*). Cell cycle arrest in this case is therefore not resulting from starvation or death of the CD4$^+$ T cells but instead reflects the extent of the programming of proliferation induced by initial Ag encounter. To ensure that the proliferation of activated 3A9 CD4$^+$ T cells when transferred into

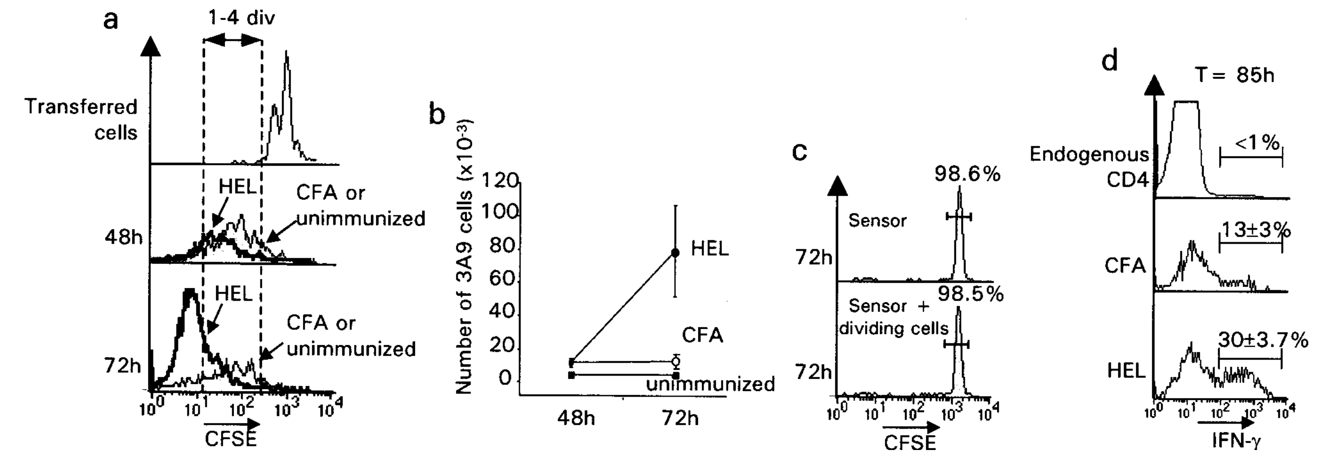

FIGURE 3. Repeated exposure to Ag is not necessary to sustain the division of activated CD4$^+$ T cells but is required for the differentiation of effector Th1 cells. CFSE-labeled 3A9 Ly5.1$^+$CD4$^+$ T cells were activated for 48 h as described in Fig. 1 legend. Activated CD4$^+$ T cells were purified and adoptively transferred into syngenic Ly5.2 recipient mice that were left unimmunized or injected 24 h before transfer with CFA alone or CFA/HEL as indicated. Two and 3 days later, the draining lymph nodes were removed and the CFSE profile (*a*) and the absolute number (*b*) or IFN-γ production at 85 h after a 4-h stimulation with PMA and ionomycin (*d*) of the recovered CD4$^+$Ly5.1$^+$ T cells were analyzed. *d,* IFN-γ production by host CD4$^+$ T cells was also analyzed (endogenous). *c,* Naive CFSE-labeled 3A9 Ly5.1$^+$CD4$^+$ T cells (sensors) were transferred into syngenic Ly5.2$^+$ recipient mice either alone or along with dividing 3A9 Ly5.2$^+$CD4$^+$ T cells. The CFSE profile of the Ly5.1$^+$CD4$^+$ T sensor cells was analyzed 72 h after transfer. One experiment of two performed with two mice per group is presented.

unimmunized hosts occurred in the absence of Ag, we cotransferred naive 3A9 CD4$^+$ T cells along with purified activated 3A9 CD4$^+$T cells. In this case, the 3A9 CD4$^+$ T cells expressing the host Ly5.2 molecule were stimulated in vitro and cotransferred with CFSE-labeled naive 3A9 CD4$^+$ T cells expressing the *Ly5.1* allele. The proliferation of naive 3A9 CD4$^+$ T cells that served as sensors for the presence of residual Ag was then evaluated. As shown in Fig. 3*c*, no proliferation of the sensor cells was detected over the 3-day period of analysis, indicating that indeed, proliferation of the 3A9 CD4$^+$ T cells transferred into unimmunized hosts proceeded in the absence of Ag.

Transient exposure to Ag therefore induces a program of cell division that further develops in an Ag- and cytokine-independent fashion. This programmed proliferation is however limited, lasting for only 24–48 h and allowing only three to four additional divisions. Division can however be reinitiated by restimulation with Ag-loaded APC.

Repeated Ag exposure is necessary for the differentiation of Th1 cells

Having shown that TCR re-engagement was not required to sustain the initial phase of proliferation of activated T cells, we wished to determine whether the same applied for the differentiation of activated T cells into IFN-γ-producing Th1 cells. We adoptively transferred 48-h activated 3A9 CD4$^+$ T cells into mice injected 24 h previously with CFA with or without HEL, as described above. The ability of the 3A9 CD4$^+$ T cells to produce IFN-γ following transfer was determined by intracellular FACS staining upon 4-h in vitro stimulation with PMA and ionomycin. Importantly, the 48-h activated 3A9 CD4$^+$ T cells used for the transfer are mainly undifferentiated, most of them producing no IFN-γ (Fig. 1). The 3A9$^+$CD4$^+$ T cells that were transferred into mice previously immunized with HEL further differentiated into IFN-γ-producing Th1 cells (Fig. 3*d*). In contrast, 3A9 CD4$^+$ T cells transferred in the absence of Ag did not differentiate into IFN-γ-producing cells (Fig. 3*d*).

Altogether, these results indicate that, in contrast to the early burst of proliferation, the full differentiation of effector Th1 cells requires repeated contacts with Ag-loaded APCs.

Sustained TCR and cytokine signaling are necessary for optimal differentiation of effector Th1 cells

The above experiment indicated that the differentiation of CD4$^+$ T cells into Th1 effector cells required repeated contact with Ag-loaded APCs. Since both TCR engagement and IL-12 signaling may result from this interaction, it was essential to determine which of those signals was critical for the further differentiation of activated CD4$^+$ T cells. To address this question, we used the Transwell system described above. Forty-eight-hour activated CD4$^+$ T cells were sorted and reincubated in the upper and lower chambers of the Transwell system. Forty-eight hours later, the fraction of IFN-γ- and IL-2-producing cells was determined after a 4-h stimulation with PMA and ionomycin. Since the transcription of the *IL-2* locus is progressively lost as CD4$^+$ T cells differentiate into IFN-γ-producing Th1 cells (22), we could then more precisely evaluate the differentiation of the distinct CD4$^+$ T cells populations. Importantly, cell division was identical under all culture conditions (Fig. 2*a* and data not shown). As observed in vivo, efficient Th1 differentiation was only observed for cells that re-engaged their TCR. Indeed, 47 ± 3.5% ($n = 3$) of the 3A9 CD4$^+$ T cells in the upper well further differentiated into effector Th1 cells producing IFN-γ but not IL-2 (Fig. 4*Ab*). In contrast, only 16 ± 4.7% ($n = 3$) of the 3A9 CD4$^+$ T cells in the lower well were fully mature Th1 cells producing IFN-γ but not IL-2 and 14 ± 4.2% ($n = 3$) remained undifferentiated producing IL-2 and IFN-γ (Fig. 4*Ac*). Similar results were obtained with higher doses of HEL peptide, indicating that the poor differentiation of the 3A9 CD4$^+$ T cells in the lower well did not result from suboptimal stimulation of the naive CD4$^+$ T cells (data not shown). In addition, the limited differentiation of 3A9 CD4$^+$ T cells present in the lower well into mature Th1 cells was not due to low levels of IL-12 or IFN-γ, as similar profiles where obtained when cycling CD4$^+$ T cells were reincubated with large amounts of these cytokines (Fig. 4*Cb*). IL-12 and IFN-γ were nonetheless essential for the limited maturation of these cells since their differentiation was almost completely abolished when anti-IL-12 and anti-IFN-γ Ab were added during the 48-h reincubation period (Fig. 4*A*, compare *a*, *c*, and *e*).

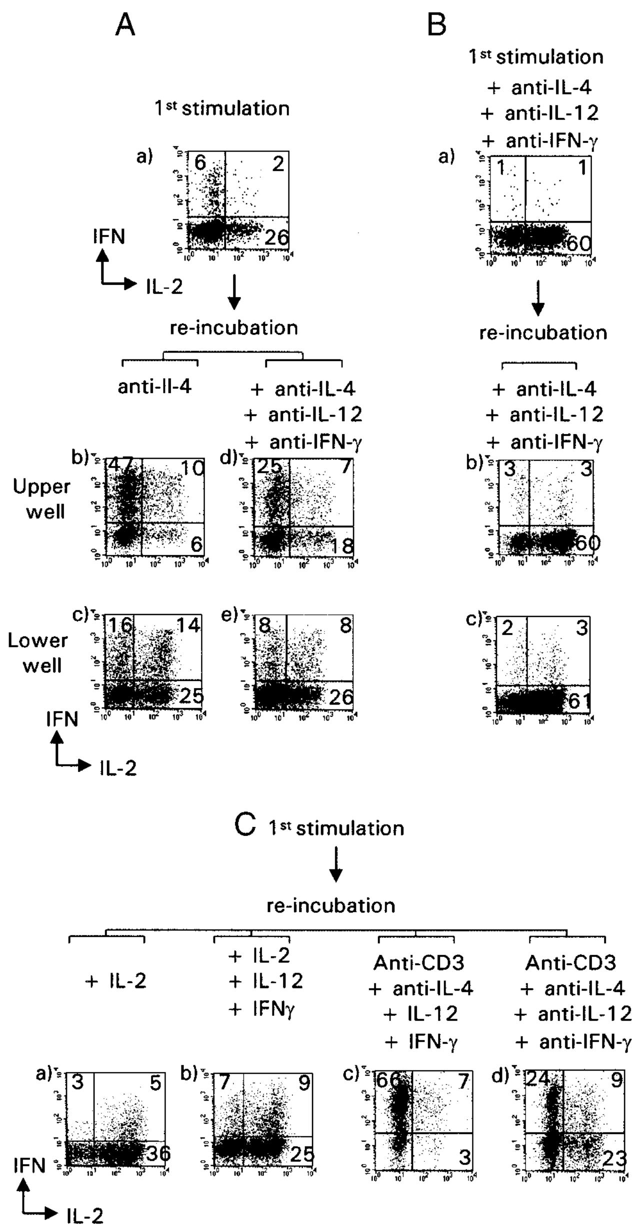

FIGURE 4. Optimal differentiation of naive T cells into effector Th1 cells requires TCR re-engagement and continuous exposure to cytokines. CFSE-labeled 3A9 CD4$^+$ T cells were stimulated as described in Fig. 1 legend. Forty-eight hours later, dividing CD4$^+$ T cells having accomplished at least one division were FACS sorted. *A* and *B*, Sorted cells were reincubated for 48 h in the two compartments of a Transwell plate containing APCs and HEL peptide in the upper well only. Cultures were performed in the presence of anti-IL-4 Ab alone or along with anti-IL-12 and anti-IFN-γ Ab as indicated. *C*, Sorted cells were reincubated in the presence or absence of plate-bound anti-CD3 Ab as indicated. The medium was supplemented with a combination of IL-2 (50 U/ml), IL-12 (3.5 ng/ml), IFN-γ (50 U/ml), anti-IL-4, anti-IL-12, and anti-IFN-γ Ab as indicated. The production of IL-2 and IFN-γ was revealed by intracellular FACS staining after a 4-h stimulation with PMA and ionomycin. The numbers in each quadrant correspond to the percentage of cells producing IL-2 and IFN-γ or both, expressed as the mean percentage of three independent experiments.

The differentiation of the 3A9 CD4$^+$ T cells in the upper compartment was also affected by blocking IL-12 and IFN-γ (Fig. 4*Ad*). Indeed, under those conditions, 25 ± 9.1% (n = 3) of the 3A9 CD4$^+$ T cells differentiated into effector Th1 cells as compared with 47 ± 3.5% when IL-12 and IFN-γ were present (Fig. 4*A*, compare *b* and *d*). Importantly, however, under those conditions, very few cells had an immature IL-2$^+$IFN-γ$^+$ phenotype. To ensure that under those conditions IL-12 signaling did not occur, we restimulated the 48-h activated 3A9 CD4$^+$ T cells with plate-bound anti-CD3, in the absence of APCs that may produce IL-12 and further added anti-IL-12 and anti-IFN-γ blocking Ab. Under those conditions, differentiation of the 3A9 CD4$^+$ T cells into mature Th1 cells also occurred, although less efficiently than when cytokines were also present (Fig. 4*C*, *c* and *d*). As expected, in the total absence of IL-12 and IFN-γ, that is when anti-IL12 and anti-IFN-γ blocking Ab were added in both the primary and secondary stimulation periods, no IFN-γ-producing cells developed (Fig. 4*B*).

These results confirm our in vivo study showing that differentiation of effector Th1 cells is a two-step process requiring multiple contact with Ag-loaded APCs. They further suggest that IL-12/IFN-γ and TCR signaling have complementary and synergistic roles in driving the full differentiation of effector Th1 cells. Signaling through the TCR seems however more efficient in driving full differentiation of effector Th1 cells that produce IFN-γ but not IL-2. Interestingly, it was recently shown that the transcription factor T-bet was essential for Th1 cell differentiation and could induce *IFN-γ* gene expression and repress *IL-2* gene expression (14). We therefore analyzed T-bet expression in the different populations showing distinct cytokine profile.

Sustained high level of T-bet *gene expression correlates with Th1 differentiation*

T-bet gene expression was measured by quantitative RT-PCR in the different culture conditions described above. The level of expression of T-bet mRNA was very low in naive 3A9 CD4$^+$ T cells and was up-regulated by a 48-h stimulation in the presence but not in the absence of IL-12 and IFN-γ, suggesting that induction of *T-bet* gene expression in naive CD4$^+$ T cells requires both TCR and cytokine signaling (Fig. 5). Sustained high levels of T-bet mRNA expression correlated with full differentiation of Th1 effector cells (Fig. 5). Indeed, cycling CD4$^+$ T cells restimulated with anti-CD3 in the presence of IL-12 and IFN-γ that did efficiently differentiate into effector Th1 cells express a high level of T-bet mRNA. Likewise, cells restimulated with anti-CD3 in the absence of cytokines, of which 33% produce IFN-γ also express high levels of T-bet mRNA. In contrast, 3A9$^+$ T cells that remained undifferentiated, i.e., when maintained for an additional 48 h in medium containing or not polarizing cytokines, had reduced levels of T-bet mRNA expression. In addition, this study shows that both TCR and cytokine signaling are necessary for the induction of T-bet expression in naive T cells. In committed CD4$^+$ T cells however, expression of T-bet can be maintained by TCR signaling only.

Discussion

In this article, we present evidence that proliferation and differentiation of CD4$^+$ T cells are differently regulated by TCR signaling. We found that transient exposure to APC will induce a program of proliferation that can proceed in the absence of Ag both in vitro and in vivo. Iezzi et al. (4) showed that a 20-h stimulation with plate-bound MHC complexes is sufficient to commit naive T cells to proliferation. Our findings extend this observation and further suggest that the programmed proliferation induced by transient exposure to Ag can proceed in an Ag- and cytokine- independent fashion. Indeed, we found that activated T cells continue to divide even when reincubated in medium alone. This suggests that the continuous presence of IL-2, -4, -15, or IL-7, four γ-chain-dependent cytokines that have been involved in the proliferation and

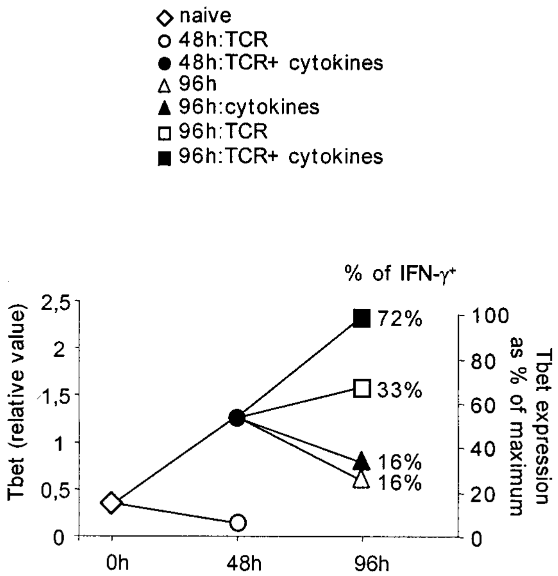

FIGURE 5. The efficiency of Th1 differentiation correlates with the level of T-bet mRNA expression. CFSE-labeled 3A9 CD4$^+$ T cells were stimulated as described in Fig. 1 legend in the presence (48 h: TCR) or absence (48 h: TCR + cytokines) of anti-IL-12 and anti-IFN-γ Ab. Forty-eight hours later, dividing CD4$^+$ T cells were FACS sorted based on their CFSE profile. Sorted cells were reincubated for 48 h with IL-2 only (96 h), with IL-2/IL-12/IFN-γ (96 h: cytokines), with anti-CD3, anti-IL-12, and anti-IFN-γ Ab (96 h: TCR) or anti-CD3 + IL-12 + IFN-γ (96 h: TCR + cytokines). RNA was extracted from the different populations and the level of T-bet mRNA expression was determined by quantitative RT-PCR. The relative mean value (*left axis*) of three independent measurements was calculated as indicated in *Materials and Methods*. In addition, the relative value expressed as percentage of the maximal value (*right axis*) is also presented. The percentage of IFN-γ-producing cells in the different populations of cells analyzed at 96 h is indicated. One experiment of two with similar results is presented.

survival of naive or memory T cells (23–26), is not necessary to sustain the proliferation of these activated CD4$^+$ T cells (Figs. 1 and 2). This observation is in agreement with the γ-chain independence of the clonal expansion of naive T cells in vivo (27). The continuous presence of IL-2 in vitro is however essential for survival of the activated 3A9 CD4$^+$ T cells. This may suggest that in vivo, other not yet defined receptor(s) may deliver the necessary survival signals to activated CD4$^+$ T cells. The programmed proliferation induced by initial Ag stimulation is however not endless and will cease after a total of six to seven divisions when proliferation can be reinduced by re-exposure to Ag. This early programmed proliferation may however ensure a significant clonal expansion (64- to 128-fold increase) of rare Ag-specific T cells even when Ag is limiting, as during the early phase of an infection.

In sharp contrast, the differentiation of naive CD4$^+$ T cells into effector Th1 cells requires repeated contact with Ag-loaded APCs. Indeed, we found both in vitro and in vivo that when Ag and cytokines are removed 48 h after primary stimulation, activated T cells did not further differentiate and produced mainly IL-2 but limited IFN-γ. Clearly, both cytokine- and TCR-derived signals were essential at this stage to induce optimal differentiation. Nonetheless, differentiation, although less efficient, was also induced by TCR re-engagement or cytokine signaling alone. In lymphoid organs, the most likely source of IL-12 is dendritic cells (DC). Mature DC do not constitutively produce IL-12 but are induced to do so by innate and T cell-derived signals including MHC class II and CD40 ligation (28–31). Under these conditions, CD4$^+$ T cells would be exposed to IL-12 mainly upon direct interaction with DC. Differentiation of CD4$^+$ T cells in vivo may be therefore mainly driven by Ag availability. In agreement, 48-h activated T cells did not differentiate into IFN-γ-producing Th1 cells when transferred into hosts preinjected with CFA, that due to microbial constituents ought to induce IL-12 production (32).

The observed difference in the duration of TCR stimulation required to induce proliferation or differentiation of naive CD4$^+$ T cells does not simply reflect difference in the kinetics of the two processes. Indeed, naive 3A9 CD4$^+$ T cells did not differentiate efficiently into effector Th1 cells when Ag is retrieved at 48 h, but IL-12 and IFN-γ signaling is maintained for an additional 48–72 h. Recurrent TCR engagement is thus more likely required to sustain expression of specific transcription factors and to induce the epigenetic modifications that are critical for Th1 effector cells differentiation. In agreement, our study shows that sustained expression of T-bet correlates with full differentiation of effector Th1 cells. Furthermore, our results suggest that the critical role for TCR re-engagement in inducing effector Th1 differentiation is in maintaining a high level of *T-bet* gene expression.

The regulation of Th1 differentiation greatly diverges from that of CD8$^+$ T cells. Indeed, in CD8$^+$ T cells, initial Ag encounter will induce a program of proliferation and differentiation into IFN-γ-producing Tc1 cells that can proceed in the absence of Ag (17, 18). This programmed differentiation of CD8$^+$ T cells may reflect the rapid induction of *IFN-γ* gene expression, reaching maximal levels by 48 h of activation, as well as its IL-12 and STAT-4 independence (33). In addition, transient exposure to Ag-loaded APCs suffices to induce optimal differentiation of effector Th2 cells when IL-4 signaling is maintained (Ref. 6 and M. Bajénoff and S. Guerder, unpublished observation). It is intriguing that CD4$^+$ T cells have developed specific regulatory circuits to control selectively Th1 effector cell development. Although the biological significance of such tight regulation, by Ag and cytokine, of Th1 effector cell development is unknown, it is tempting to speculate that it may simply reflect the function of effector Th1 cells as compared with undifferentiated or committed CD4$^+$ T cells in memory responses. We found that 48-h activated T cells are committed to the Th1 lineage since they rapidly differentiate into IFN-γ-producing effector cells upon restimulation. As opposed to effector cells, they are reversible (M. Bajénoff and S. Guerder, unpublished observation). They may therefore provide a pool of memory cells able to rapidly respond to novel Ag challenge with flexible effector function. This population may correspond to the recently described lymphoid memory cells that were shown to produce IL-2 only but not IFN-γ upon 2-h stimulation (34, 35). In contrast, cells that were stimulated several times with Ag-loaded APCs may preferentially home to nonlymphoid tissue where, as effector memory cells, they may produce rapidly IFN-γ upon restimulation, serving as an immediate barrier to novel infection (34, 35). Clearly, unraveling the contribution of these different memory populations to the control of recurrent infection may help understanding why Th1 cell differentiation is tightly regulated by Ag and cytokine availability.

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