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References

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Two Distinct Stages in the Transition from Naive CD4 T Cells to Effectors, Early Antigen-Dependent and Late Cytokine-Driven Expansion and Differentiation

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Efficient peptide presentation by professional APC to naive and effector CD4 T cells in vitro is limited to the first 1–2 days of culture, but is nonetheless optimum for effector expansion and cytokine production. In fact, prolonging Ag presentation leads to high levels of T cell death, decreased effector expansion, and decreased cytokine production by recovered effectors. Despite the absence of Ag presentation beyond day 2, T cell division continues at a constant rate throughout the 4-day culture. The Ag-independent later stage depends on the presence of IL-2, and we conclude optimum effector generation depends on an initial 2 days of TCR stimulation followed by an additional 2 days of Ag-independent, cytokine-driven T cell expansion and differentiation. The Journal of Immunology, 2000, 165: 5017–5026.

A primary CD4 T cell response can be conceptually divided into three distinct phases involving activation, proliferation, and differentiation of responding T cells leading to the generation of large numbers of highly potent effectors with the capacity to rapidly produce high levels of multiple cytokines after reencounter with Ag (1, 2). However, each of these phases may in fact occur sequentially and/or simultaneously. Which stimulatory signals are required to trigger each phase, and the sequence in which they must be delivered to the T cell, are also unclear. For the purpose of these studies, we have defined effectors as activated T cells with the capacity to rapidly produce high levels of multiple cytokines after restimulation (1, 2). We have found that full development of effector function requires 4 days of in vitro culture, (3), and we have also seen a similar requirement for 4 days or more to generate fully activated effector T cells in vivo (4).

We consider the activation phase to be the first step that occurs before division, which commits the cell to enter the cell cycle, requiring a number of signals to promote progression through the cell cycle. There is at least a 24-h lag while a resting (G0) T cell becomes activated and thus competent to progress through the cell cycle (5, 6). During this phase, many genes and their products are expressed in preparation for DNA replication and cell division (7–12). Epigenetic remodeling changes resulting in “on” configurations of polarized cytokine genes account for some of the very earliest changes that occur during T cell activation as the cell undergoes its first round of DNA replication before the cell enters division (10, 13, 14). Therefore, it is notable that cytokines which drive polarization are most effective when present at the initiation of T cell activation before the first round of cell division (15, 16).

Once cell proliferation commences, highly activated T cells divide rapidly and can continue through multiple rounds of division, resulting in an impressive expansion of the initial population (17, 18). Little is known about the critical sequence of external signals required for driving cell division over the later phase of the 4-day period necessary to generate large numbers of highly activated effectors. Differentiation, leading to changes which effect the future functional and survival potential of the progeny cells, also occurs during effector generation. The differentiation of naive CD4 T cells into polarized effectors in vitro is dependent on high doses of high avidity Ag, the interaction of multiple costimulation receptor/coreceptor pairs, growth-promoting cytokines, like IL-2, and polarizing cytokines (1–3, 16, 19, 20). IL-12 or IFN-γ, and the lack of IL-4, generate Th1-polarized effectors (capable of IL-2, IFN-γ, TNF-α, and TGF-β production), whereas the presence of IL-4 and the lack of IFN-γ generate Th2-polarized effectors (capable of IL-4, -5, -10, and -13 production) (16). Although many of the signals required for effector differentiation are known, it is not clear whether these and other differentiation signals are delivered early or late during a primary T cell response to drive the development of functional and phenotypic characteristics critical for optimal effector function. It is likely that various differentiation signals and differentiation-associated changes are available throughout in vivo effector generation at different times. For example, for polarization, the presence of polarizing cytokines are required only during the early phase of effector generation, supporting that the signals required for polarized T cell differentiation begin immediately after stimulation of naive CD4 T cells (21–23). However, the ability of effectors to become competent to produce large amounts of polarized cytokines (i.e., IFN-γ and IL-4) rapidly upon restimulation takes 2–4 days (3, 15, 23, 24). Additionally, the growth-promoting cytokine IL-2 is only produced at maximal levels by the responding CD4 T cells during the later phase of effector generation (1, 2, 21, 24, 25). Another important change that appears to occur during the late phase of effector differentiation includes the induction of susceptibility to activation-induced cell death (AICD), which requires early exposure to IL-2 and at least 2 days of Ag/APC (26, 27).

Although effects of the duration of Ag stimulation on the ability of naive T cells to proliferate has been studied previously, equally important functional characteristics including cytokine production,
activation status, and survival of the generated effectors are commonly overlooked. An additional weakness of these earlier studies is that they involved the use of non-physiological plate-bound anti-TCR/CD3 mAb plus anti-CD28 mAb as an Ag/APC surrogate, which may not lead to an accurate picture of Ag-induced responses in situ (16, 20, 28).

Here, we determine the impact of duration of TCR stimulation on the quantity and quality of effectors generated. To directly assess the requirements of naive CD4 T cells for, and response to, defined durations of Ag presentation, we used a TCR-transgenic mouse model system in which we isolate a homogeneous population of naive Ag-specific CD4 T cells. We evaluate several TCR agonists, including plate-bound anti-TCR mAb and two different physiologically relevant Ag/APC model systems. Because Rogers and Croft (29) have previously reported on the effects of Ag concentration on T cell stimulation, we restricted these studies to optimal concentrations of Ag as previously determined for each Ag to be used in these studies. We know that suboptimal or variable levels of Ag, costimulation, and growth factors (i.e., IL-2) can have major negative impacts on the generation of CD4 effectors; therefore, we chose to limit the scope of these studies to determining the effects of Ag duration under conditions of controlled and optimal Ag dose, costimulation, and IL-2 (1, 19, 29, 30, 31).

With the use of vital dyes and FACS analysis, we have examined the impact of varied durations of TCR stimulation on both T cell division and effector expansion, and confirm that a short duration, 1–2 days, of TCR stimulation promotes optimal proliferation and effector expansion during culture. We also measured the functional capacity of the resulting effectors as the ability to produce high levels of polarized cytokines upon restimulation, and we find that a short duration, 1–2 days, of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote stable polarized cytokine production. In fact, increasing the duration of TCR stimulation beyond 2 days leads to the impact of varied durations of TCR stimulation on both T cell division and effector expansion, and confirm that a short duration, 1–2 days, of TCR stimulation promotes optimal proliferation and effector expansion during culture. We also measured the functional capacity of the resulting effectors as the ability to produce high levels of polarized cytokines upon restimulation, and we find that a short duration, 1–2 days, of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote stable polarized cytokine production. In fact, increasing the duration of TCR stimulation beyond 2 days leads to the impact of varied durations of TCR stimulation on both T cell division and effector expansion, and confirm that a short duration, 1–2 days, of TCR stimulation promotes optimal proliferation and effector expansion during culture. We also measured the functional capacity of the resulting effectors as the ability to produce high levels of polarized cytokines upon restimulation, and we find that a short duration, 1–2 days, of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote stable polarized cytokine production. In fact, increasing the duration of TCR stimulation beyond 2 days leads to the impact of varied durations of TCR stimulation on both T cell division and effector expansion, and confirm that a short duration, 1–2 days, of TCR stimulation promotes optimal proliferation and effector expansion during culture. We also measured the functional capacity of the resulting effectors as the ability to produce high levels of polarized cytokines upon restimulation, and we find that a short duration, 1–2 days, of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote stable polarized cytokine production. In fact, increasing the duration of TCR stimulation beyond 2 days leads to the impact of varied durations of TCR stimulation on both T cell division and effector expansion, and confirm that a short duration, 1–2 days, of TCR stimulation promotes optimal proliferation and effector expansion during culture. We also measured the functional capacity of the resulting effectors as the ability to produce high levels of polarized cytokines upon restimulation, and we find that a short duration, 1–2 days, of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote stable polarized cytokine production. In fact, increasing the duration of TCR stimulation beyond 2 days leads to the impact of varied durations of TCR stimulation on both T cell division and effector expansion, and confirm that a short duration, 1–2 days, of TCR stimulation promotes optimal proliferation and effector expansion during culture. We also measured the functional capacity of the resulting effectors as the ability to produce high levels of polarized cytokines upon restimulation, and we find that a short duration, 1–2 days, of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote stable polarized cytokine production. In fact, increasing the duration of TCR stimulation beyond 2 days leads to the impact of varied durations of TCR stimulation on both T cell division and effector expansion, and confirm that a short duration, 1–2 days, of TCR stimulation promotes optimal proliferation and effector expansion during culture. We also measured the functional capacity of the resulting effectors as the ability to produce high levels of polarized cytokines upon restimulation, and we find that a short duration, 1–2 days, of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote stable polarized cytokine production. In fact, increasing the duration of TCR stimulation beyond 2 days leads to

### Materials and Methods

#### Animals

H-2<sup>ab</sup> (B10.Br) and H-2<sup>ab</sup> (C57BL/6) V<sub>α</sub>1/V<sub>β</sub>3 AND TCR transgene (Tg; I-E<sup>α</sup> restricted), B10.Br.HNT.B10.D2/F1 (V<sub>β</sub>8.3/V<sub>α</sub>2) TCR Tg (I-A<sup>d</sup> restricted), and H-2<sup>ab</sup> B10.Br.B10.D2/F1, mice were used at 2–6 mo of age and were bred in the animal facilities at the Trudeau Institute.

#### Cell isolations

The isolation of pooled spleen and lymph node cells enriched for naive CD4 T cells has been described previously (34). In brief, the pooled spleen and lymph node cells were passed through a nylon wool column, and the nonadherent cells treated with a panel of CD8, heat-stable Ag, and class II MHC-depleting Ab and complement followed by Percoll gradient separation. The purified cell populations were routinely >95% CD4<sup>+</sup> cells, 90–95% of which had a naive phenotype (CD45<sup>hi</sup>, CD62L<sup>hi</sup>, CD44<sup>low</sup>, and CD25<sup>−</sup>) and expressed the TCR Tg.

#### Immunofluorescent staining

All staining was done at 4°C in PBS (Life Technologies, Grand Island, NY) with 1% BSA (Sigma, St. Louis, MO) and 0.1% Na<sub>2</sub>SO<sub>4</sub> (Sigma). FITC-labeled Ab is produced in our laboratory, and PE-biotin- and APC-labeled Ab were purchased where indicated. The following Ab and fluorochrome conjugates were used: PE-labeled anti-CD25 (clone MKAERADLIAYLKQATAK pigeon cytochrome c fragment (PCCF) peptide) (Figs. 1–6). We have compared stimulation of T cells by culture with other costimulatory molecules such as LFA-1, CD48, or heat-stable Ag and molecules, was used as APC at 2:1 T cell:APC. These cells do not express any other costimulatory molecules such as LFA-1, CD48, or heat-stable Ag and were originally generated by R. Germain (National Institutes of Health, Bethesda, MD). T-depleted spleen APC blasts were prepared as described previously (22). Briefly, splenic cell suspensions were depleted of T cells using anti-Thy1.2 (clones HO13.14 and F7DS), anti-CD4 (clone RL172.4), anti-CD8 (clone 3.155), and complement. The resulting cells were small and 90–95% B220<sup>−</sup>. The T-depleted spleen APC were then stimulated at 5 × 10<sup>5</sup> cells/ml for 3 days with LPS (25 μg/ml; Sigma) and dextran (25 μg/ml; Sigma). All APC were treated with 100 μg/ml of mitomycin c (Misc; Sigma) for 60 min at 37°C before use. CD4 effectors were generated by culturing Tg<sup>+</sup> CD4 cells as previously described (22). Briefly, naive Tg<sup>+</sup> CD4 AND T cells (3 × 10<sup>5</sup> cells/ml, Fig. 1) and 3 × 10<sup>3</sup> cells/ml (Figs. 2–6) were cultured with syngeneic T-depleted splenic APC blasts (3 × 10<sup>5</sup> cells/ml, Fig. 2) or DCEK-ICAM APC (1.5 × 10<sup>5</sup> cells/ml, Fig. 1, and 1.5 × 10<sup>3</sup> cells/ml, Figs. 3–6) plus 5 μM KAERADLIAYLQKATAK pigeon cytochrome c fragment (PCCF) peptide (Figs. 1–6). We have compared stimulation of T cells by culture with syngeneic T-depleted splenic APC blasts to that of DCEK-ICAM APC and seen no differences (data not shown). In Fig. 2, 5 μM HNT-NGVTAACSH influenza hemagglutinin (HA peptide; New England Peptide, Fitchburg, MA) was added in addition to the PCCF peptide to stimulate the CD4 HNT T cells. Alternatively, Tg<sup>+</sup> CD4 cells (3 × 10<sup>5</sup> cells/ml, Figs. 1–6) were stimulated with plate-bound anti-V<sub>β</sub>3 (10 μg/ml) and soluble anti-CD28 (10 μg/ml). Recombinant murine cytokines IL-2 and IL-4 were obtained from culture supernatant of X63.Ag8.653 cells transfected with cDNA for the respective cytokines (36). Recombinant murine
IL-12 was a gift from Stanley Wolf (Genetics Institute, Cambridge, MA). IL-2-polarized effectors (Th0) were generated with IL-2 (80 U/ml). Th1 effectors were generated with IL-2, IL-12 (2 ng/ml), and anti-IL-4 (clone 11B11; 10 μg/ml). Th2 effectors were generated with IL-2, IL-4 (200 U/ml), and anti-IFN-γ (clone XMG1.2; 10 μg/ml). Culture durations varied as indicated in each individual experiment. At the end of culture, live T cells were enumerated by direct cell counts of trypan blue-excluding cells.

Cytokine detection
Culture supernatants collected after 24 h, from cultures of effectors (5 × 10⁵ cells/ml) restimulated with Ag/APC (2.5 × 10⁵ cells/ml) or with plate-bound anti-Vβ3 (10 μg/ml), were assayed for the presence of IL-2 in a bioassay with NK-3 cells and for IL-4 by ELISA as previously described (37). IL-2 concentrations are expressed in U/ml (1 U of IL-2 is equal to 1.2 ng). IL-4 concentrations are expressed in nanograms per milliliter.

Alternatively, intracellular cytokine staining (ICCS) was detected in restimulated effectors as previously described (38, 39). In brief, effectors (5 × 10⁵ cells/ml) were restimulated for 24 h with DCEK-ICAM APC (2.5 × 10⁵ cells/ml) with PCCF (5 μM) or restimulated for 4 h with PMA (10 ng/ml; Sigma) and ionomycin (50 ng/ml; Sigma). Negative controls (indicated by dotted lines) indicate ICCS of T cells that are not restimulated and thus do not express the indicated cytokine. Brefeldin A (10 μg/ml final concentration; Sigma) was added 2 h after culture initiation and maintained throughout the ICCS protocol. At the end of the specified restimulation period, cells were collected and surface stained for CD4 and Tg expression as described above. The cells were then divided into separate tubes, washed, fixed in 75 μl of fresh 4% paraformaldehyde (Sigma) plus 25 μl of PBS containing 10 μg/ml brefeldin A, and incubated for 20 min at room temperature. The tubes were then washed once with PBS and resuspended in 50 μl saponin buffer (PBS containing 1% PBS, 0.1% NaN₃, and 0.1% saponin (Sigma), pH 7.4–7.6). PE-labeled antimouse IL-2, PE-labeled antimouse IFN-γ, or PE-labeled antimouse IL-4 was added and incubated with the T cells for 30 min at room temperature. All samples were then washed with PBS/BSA + 0.2% azide and analyzed on a FACSscan or FACScalibur cytometer.

Results and Discussion

Kinetics of CD4 effector expansion during the proliferative phase
In order for naive CD4 T cells to progress from a resting (G₀) state to an activated effector state and initiate division, they must transit from G₀ into the cell cycle. Because the transition from a resting G₀ state into the cell cycle involves expression of multiple genes and their products in a coordinated fashion, this often results in a delay between entry into the cell cycle and the first round of cell proliferation (5, 6). To analyze the rate of division of naive CD4 T cells following Ag stimulation, we stained naive CD4 T cells with the vital dye CFSE, which binds to intracellular proteins and is partitioned to daughter cells with each division (35, 40). Analysis of the CFSE profiles on each of the 4 days following culture initiation indicates that naive CD4 T cells have a delay of ~24 h between initial TCR stimulation and the first round of cell division (Fig. 1, a and b), but that after this initial lag period the cells progress through multiple cell cycles at a constant rate of 7–10 h/cell division throughout the remaining 3 days of culture (Fig. 1b).

After day 2, only cells that have divided are detected, indicating either that all of the cells have been stimulated to undergo division or that cells which have not divided fail to survive (21). Moreover, when we compare the predicted to the actual effector recovery for each day during effector generation, with the calculations based on the starting cell number of 3 × 10⁵ cells and the average number of cell divisions (Fig. 1, a and b) for each day, we find that the actual effector recovery is much lower than the predicted effector recovery (Fig. 1c). This suggests that there are significant levels of cell death which occur throughout effector generation, leading to lower effector recoveries than would be predicted if all of the cells generated survived the 4-day culture period. These results suggest that although the CD4 T cell proliferative phase has the potential to generate ~250-fold cell expansion, conditions in vitro are such that the viable effector expansion is limited to ~10- to 20-fold (Fig. 1c). The nature of this cell death is unclear, but we and others also observe that naive CD4 T cell expansions in vivo in response to peptide Ag in adjuvant are also less than would be predicted by the CFSE profiles (E. Roman, G. E. Huston, and S. L. Swain, unpublished observations; Refs. 41–43).

One possible explanation for limited CD4 expansion, despite the high rate of cell division, is that Ag presentation is of limited duration in vitro and cells need repeated or ongoing TCR triggering to persist after division. Several groups have reported that DNA synthesis, measured via [³H]thymidine incorporation, or cell
Efficient Ag presentation is restricted to the first 1–2 days of culture

We know from previous studies that MitC-treated APC disappear from culture by 48 h, as measured by surface staining for ICAM-1 and class II (17, 46). This phenomenon only occurs during culture conditions where Ag presentation occurs. MitC-treated APC cultured with T cells and without Ag or MitC-treated APC cultured with Ag and without T cells survive for 4 days of culture, whereas MitC-treated APC cultured with T cells and Ag are rapidly deleted from culture (data not shown). However, since it is possible that membrane fragments of no longer intact APC might still present Ag, we sought to determine the time at which cultures of MitC-treated APC are no longer capable of effectively stimulating naive CD4 T cell proliferation or cytokine production by effectors in standard in vitro cultures. We developed a cell mixing system where we initiate cultures of BODIPY Red-labeled naive HNT T cells, specific for influenza HA peptide, with an activated APC population containing not only B cell blasts, but also a small number of other APC populations including macrophages and dendritic cells. We then loaded the splenic APC blasts with HA and PCCF peptides such that the APC are capable of efficient presentation of both the influenza HA peptide to HNT T cells and the PCCF peptide to AND T cells within the same culture. At various time points after initiation of the HNT T cell:Ag/APC culture, we isolated fresh naive AND T cells from spleens and lymph nodes from AND TCR Tg mice, labeled these cells with CFSE, and introduced them to the ongoing HNT T cell:Ag/APC cultures. Three days after the introduction of the AND T cells to the ongoing HNT T cell:Ag/APC cultures, the T cells were harvested and analyzed for cell division and effector expansion. With these experiments, we were able to easily identify the two populations of T cells with anti-TCR β-chain-specific Ab, and based on their BODIPY Red vs CFSE staining, measure the capacity of the newly added AND T cells to respond to whatever APC were still actively presenting Ag at various time points after initiation of the HNT T cell:Ag/APC cultures, and verify that the proliferative capacity of the HNT T cells added at the initiation of the T cell:Ag/APC cultures was not perturbed by the addition of the fresh naive AND T cells.

When naive AND T cells were added to HNT T cell:Ag/APC cultures at day 0, <10% of the AND T cells could be detected as nondivided cells under all three polarizing conditions (Fig. 2a), indicating that these culture conditions are fully capable of stimulating optimal effector generation. However, when the naive AND T cells were added after 1 day of Ag/APC culture, ~40% of the AND T cells recovered after 3 days had not divided, and adding naive AND T cells to Ag/APC cultures at day 2 resulted in little AND T cell division (Fig. 2a). Our results indicate that although the original HNT T cells proliferate normally, regardless of the time of naive AND T cell introduction to the cultures (data not shown), the ability of the splenic APC blasts to stimulate cell division (Fig. 2a) and expansion (Fig. 2b) of naive AND T cells declined during the first day of culture and was negligible by 2 days after the original Ag/APC cultures were initiated.

To determine whether cytokines present during effector polarization might prolong the ability of the splenic APC blasts to present Ag to naive CD4 T cells, we designed this experiment under Th0 (IL-2 only)-, Th1-, or Th2-polarizing conditions. The ability of the Ag/APC cultures to stimulate naive AND T cell proliferation was similar under all three polarization conditions (Fig. 2a). Therefore, the cytokines present during in vitro effector polarization did not enhance the ability of the splenic APC blasts to present Ag to naive CD4 T cells. We repeated this experiment using the DCEK-ICAM fibroblast APC line and have seen no difference in the ability of the two APC populations to stimulate naive CD4 AND T cell expansion and differentiation (data not shown; Refs. 1, 2, 30).

The recovery of the AND T cells after 3 days in culture mirrored their division. Naive AND T cells added at the initiation of HNT T cell:Ag/APC culture (day 0) expanded markedly by 3 days, whereas AND T cells added after 1 or 2 days of Ag/APC culture underwent little expansion or even declined over the 3 days (Fig. 2b). Th2-polarizing conditions supported slightly greater effector recoveries as compared with IL-2-polarizing (Th0) and Th1-polarizing conditions, suggesting that the presence of IL-4 in the cultures may enhance T cell survival. However, all AND T cell effector recoveries had decreased to numbers below starting cell conditions where Ag presentation occurs. MitC-treated APC cultured with T cells and without Ag or MitC-treated APC cultured with Ag and without T cells survive for 4 days of culture, whereas MitC-treated APC cultured with T cells and Ag are rapidly deleted from culture (data not shown). However, since it is possible that membrane fragments of no longer intact APC might still present Ag, we sought to determine the time at which cultures of MitC-treated APC are no longer capable of effectively stimulating naive CD4 T cell proliferation or cytokine production by effectors in standard in vitro cultures. We developed a cell mixing system where we initiate cultures of BODIPY Red-labeled naive HNT T cells, specific for influenza HA peptide, with an activated APC population containing not only B cell blasts, but also a small number of other APC populations including macrophages and dendritic cells. We then loaded the splenic APC blasts with HA and PCCF peptides such that the APC are capable of efficient presentation of both the influenza HA peptide to HNT T cells and the PCCF peptide to AND T cells within the same culture. At various time points after initiation of the HNT T cell:Ag/APC culture, we isolated fresh naive AND T cells from spleens and lymph nodes from AND TCR Tg mice, labeled these cells with CFSE, and introduced them to the ongoing HNT T cell:Ag/APC cultures. Three days after the introduction of the AND T cells to the ongoing HNT T cell:Ag/APC cultures, the T cells were harvested and analyzed for cell division and effector expansion. With these experiments, we were able to easily identify the two populations of T cells with anti-TCR β-chain-specific Ab, and based on their BODIPY Red vs CFSE staining, measure the capacity of the newly added AND T cells to respond to whatever APC were still actively presenting Ag at various time points after initiation of the HNT T cell:Ag/APC cultures, and verify that the proliferative capacity of the HNT T cells added at the initiation of the T cell:Ag/APC cultures was not perturbed by the addition of the fresh naive AND T cells.

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numbers when AND T cells were added on either day 2 or 3 of Ag/APC culture. Overall, these results suggest that MitC-treated splenic APC blasts are no longer capable of supporting naive T cell proliferation and effector generation when cultured for more than 2 days with Ag-specific T cells, regardless of the T cell-polarizing cytokine milieu.

Since naive CD4 T cells require a significantly greater amount of TCR stimulation and costimulation to enter into the cell cycle and rapidly produce high levels of cytokines, as compared for 4-day effectors, we examined the ability of freshly prepared (day 0) and 1-day Ag/APC cultures to stimulate 4-day effectors to produce polarized cytokines (1, 30). We generated 4-day Th0-or Th2-polarized effectors and restimulated them with freshly prepared DCEK-ICAM APC loaded with PCCF peptide (Fig. 3, positive control, filled histograms), freshly prepared DCEK-ICAM APC without Ag (negative control, dotted lines), or 1-day cultures of DCEK-ICAM APC loaded with PCCF peptide (solid lines). Twenty-four hours after the 4-day Th0 and Th2 effectors were added to the various APC cultures, we harvested the T cells and measured intracellular cytokine production by effectors. Because we needed to culture APC with Ag and T cells in order for the APC to be deleted from the cultures, and these T cells could potentially contribute secreted cytokines to culture supernatants used in ELISA cytokine detection methods, we used ICCS to ascertain the cytokine production of the 4-day Th0 or Th2 effectors while gating out any cytokine contribution of the naive T cells used to generate the 1-day Ag/APC cultures. When 4-day effectors were stimulated for 24 h with freshly prepared Ag/APC, they were induced to secrete readily detectable levels of appropriate cytokines (positive control), whereas 4-day effectors stimulated for 24 h with freshly prepared Ag without Ag do not produce detectable levels of cytokines (negative control). Th0 effectors typically produce high levels of IL-2 and slightly lower production of IFN-γ, whereas Th2 effectors produce measurable levels of IL-4 as determined by ICCS (Fig. 3, shaded histograms, positive controls). These results can be mimicked by the recovery of cytokines in culture supernatants from purified 4-day effector populations (data not shown). In contrast, when 4-day Th0 or Th2 effectors are added to Ag/APC cultures which have interacted for as little as 1 day with naive T cells, they exhibit significantly reduced intracellular cytokine production (Fig. 3, solid lines). These results indicate that the ability of Ag/APC to stimulate cytokine production by even highly activated effectors is lost as early as 1 day after in vitro culture of Ag/APC with T cells. Together, these results reveal that efficient Ag presentation to both naive and effector T cells by professional APC is limited to the first 1–2 days in standard in vitro culture systems (Figs. 2 and 3), and any potential residual APC are incapable of supporting naive T cell proliferation and cytokine production or even the response of highly activated effectors.

**Prolonged Ag stimulation leads to detrimental effects on effector expansion and function**

In all cases discussed thus far in these studies, the APC used were MitC treated; therefore, their short life span was expected, but it is important to note that under these standard in vitro culture conditions used by many groups of investigators, Ag presentation is of short duration but nonetheless results in highly efficient T cell expansion and effector generation. However, in vivo there is the potential for APC to interact with T cells throughout the process of effector generation and to potentially provide T cells with prolonged or repeated TCR stimulation. Since our in vitro Ag/APC culture system results in the disappearance of APC between 1 and 2 days, to determine whether increased durations of exposure to Ag/APC results in more efficient effector generation, we examined the effects of increasing the duration of Ag presentation on effector generation by supplementing cultures with additional Ag/APC throughout the 4-day culture period. We incubated naive CD4 T cells under IL-2-polarizing (Th0) and Th2-polarizing conditions and supplemented the cultures with fresh peptide-pulsed APC once at day 0 (normal culture conditions), twice at days 0 and 1, three times at days 0–2, and four times at days 0–3. The effectors present after 4 days were then analyzed for effector recovery and their ability to secrete cytokines upon restimulation.

The results indicate that effector recovery in both the IL-2-polarized (Th0) (Fig. 4a) and Th2-polarized (Fig. 4b) subsets is significantly decreased when standard in vitro T cell cultures are supplemented with extended durations of Ag/APC exposure. Although the Th2-polarized subset benefited slightly from one additional Ag/APC supplement, extended durations of Ag/APC exposure resulted in markedly decreased effector recoveries (Fig. 4b, shaded bars). Interestingly, the viable cells recovered after 4 days all maintained similar proliferation profiles, as measured by CFSE dye loss (data not shown), suggesting that the decrease in effector recovery which results from extended durations of Ag/APC exposure is not due to an impairment in proliferation potential of surviving cells. Therefore, we hypothesized that the decreased effector recovery (Fig. 4) indicates that effectors stimulated with >2 days of Ag presentation undergo high-level death as they respond. Using forward scatter and side scatter (FSC × SSC) FACS profiles of the CD4 Tg cells, we were able to show that, in fact, the percentages of cell death increased from 11 to 55% for the IL-2 (Th0)-polarized effectors and from 18 to 34% for the Th2 effectors as Ag/APC exposure was extended. These numbers may seem too low to account for the low cell recoveries, but the cell death measurements taken at day 4 do not reflect the integrated effect of cell death during the culture. Dead cells typically disappear from culture within 24–36 h of their death, and therefore a cell that dies at days 2–3 of culture would not be detectable on day 4. Additionally, if a cell dies on day 2 its potential capacity to expand had it lived must be taken into account when adjusting for cell recovery at day 4. Thus, the cell recovery at day 4 has the potential to be much lower than what would be expected based on calculations of percentages of cell death.
In these studies, we provide exogenous IL-2 at the beginning of T cell culture (day 0) to promote optimal T cell expansion. However, previous studies have shown that production and accumulation of autocrine IL-2 by naive T cells takes 20–30 h following stimulation with optimum levels of TCR stimulation and costimulation (Ref. 11; L. Haynes and S. L. Swain; unpublished data). IL-2 is known to regulate AICD such that T cells previously exposed to IL-2 undergo apoptosis rapidly following Ag receptor stimulation (26, 47). Moreover, the kinetics of IL-2 addition to culture has been shown to have profound effects on the susceptibility and rescue of T cells from Fas-mediated AICD (17). Therefore, we examined the effects of increasing the duration of Ag presentation on effector function by supplementing cultures with additional Ag/APC throughout the 4-day culture period, as described in Fig. 4, and adding exogenous IL-2 on day 0 (normal culture conditions), 1, or 2. We found there to be no difference in the kinetics of decreased effector recovery and increased cell death caused by prolonged Ag presentation when the time of exogenous IL-2 addition was delayed from day 0 to day 1 or 2 (data not shown).

The ability of effectors to rapidly produce high levels of multiple cytokines after restimulation is an important functional attribute which is critical to regulate the immune response to an invading pathogen. Therefore, we also tested the ability of the surviving viable 4-day effectors to produce polarized cytokines. Because T cell recovery was much lower in the cultures with prolonged Ag/APC exposure, we readjusted the 4-day effectors recovered to equivalent cell concentrations before restimulation with Ag/APC for 24 h. We measured IL-2 levels produced by IL-2-polarized (Th0) effectors (Fig. 4a) and IL-4 levels produced by Th2-polarized effectors (Fig. 4b). Equivalent numbers of effectors obtained from cultures with prolonged Ag/APC stimulation produced much lower levels of cytokines compared with effectors resulting from the standard (shorter) Ag/APC exposure (Fig. 4). Together, these studies show that exposure to Ag/APC beyond 2 days during effector generation not only leads to decreased effector recovery, which is most likely due to increased cell death, but also decreases the ability of surviving T cells to function properly and produce polarized cytokines upon restimulation. Thus, the susceptibility of restimulated developing effectors to cell death may limit the cytokine accumulation upon restimulation or select for less differentiated T cells.

Supplementing T cell cultures with Ag/APC beyond 2 days was detrimental to the recovery and function of resulting effectors; however, it is possible that this result might be due to the effects of culture crowding by the added APC or some other negative impact of adding fresh APC to the culture. Therefore, we used plate-bound anti-TCR mAb stimulation plus soluble anti-CD28 mAb costimulation conditions to independently define the optimal duration of Ag presentation. These studies were designed to provide optimal stimulatory conditions throughout the culture period with the only variable being the duration of TCR stimulation. Naive T cells were incubated in anti-Vβ3 mAb-coated wells with soluble anti-CD28 mAb and IL-2 alone (Th0) or under Th2-polarizing conditions. The T cell cultures were then transferred to noncoated tissue culture wells, without washing, over a broad range of time points. Effector recovery, effector death, and polarized cytokine-producing capacity of the resulting effectors were measured at the end of a total 4-day culture period.

Our results indicate that the highest level of effector recovery results from 2 days of TCR stimulation in both the IL-2-polarized (Th0) (Fig. 5a) and Th2-polarized (Fig. 5c) populations, with shorter and longer durations of TCR stimulation resulting in lower effector recovery. Th0 effectors (Fig. 5a and c) responded to Ag/APC stimulation resulting in low numbers of effectors (Fig. 5, a and c), and stimulation with anti-TCR mAb for <2 days resulted in effectors that appeared poorly activated as indicated by changes in activation marker expression and small cell size, whereas exposure to anti-TCR mAb beyond 2 days resulted in effectors that did display a highly activated phenotype (data not shown). We hypothesized that the decrease in effector recovery resulting from increased durations of Ag/APC exposure (Figs. 1c and 4) is due to AICD as effectors are reexposed to Ag/APC or anti-TCR Abs, rather than due to a decrease in induction of cell proliferation. Therefore, we examined effector death, as measured by propidium iodide (PI) uptake or FSC × SSC FACS profiles of the cultures. Indeed, longer durations of TCR stimulation resulted in high levels of effector death (Fig. 5, a and c). Taken together, the decreased effector recovery and increased cell death (Fig. 5, a and c) by cultures stimulated with >2 days of TCR stimulation support the hypothesis that prolonging Ag presentation results in high levels of T cell death, which directly results in decreased effector recovery rather than increased effector responses.

FIGURE 4. Negative effect of prolonged Ag stimulation with Ag/APC. Naive CD4 AND T cells were CFSE labeled and cultured at 3 × 10^6 T cells/culture with 1.5 × 10^6 DCEK-ICAM pulsed with PCCF peptide under IL-2-polarizing (Th0) or Th2-polarizing cytokine environments. A total of 1.5 × 10^6 freshly prepared PCCF peptide-pulsed DCEK-ICAM was added once at day 0 only, twice at days 0 and 1, three times at days 0–2, or four times at days 0–3. a, Th0 effector recoveries of live Vβ3^+ (AND)/CD4^+ T cells were determined at day 4 of culture by direct cell counts. Cell death was measured by FSC × SSC FACS profiling and determined to be 11, 36, 52, and 55%, respectively. IL-2 production was measured by bioassay of supernatants recovered after 24 h of restimulation of 4-day Th0 effectors with PCCF peptide-pulsed DCEK-ICAM. b, Th2 effector recoveries of live Vβ3^+ (AND)/CD4^+ T cells were determined at day 4 of culture by direct cell counts. Cell death was measured by FSC × SSC FACS profiling and determined to be 18, 13, 23, and 34%, respectively. IL-4 production was measured by ELISA of supernatants recovered after 24 h of restimulation of 4-day Th2 effectors with PCCF peptide-pulsed DCEK-ICAM. Data are representative of four independent experiments.
We also analyzed the ability of effectors generated in the anti-TCR mAb cultures to be restimulated to produce polarized cytokines. We found that a slightly longer Ag presentation of 2½ days resulted in IL-2-polarized (Th0) effectors best capable of producing high levels of IL-2 upon restimulation, with lesser and longer durations of Ag presentation resulting in lower levels of cytokine production (Figs. 4a and 5b). Whereas 2 days of Ag presentation resulted in Th2 effectors best capable of producing high levels of IL-4 upon restimulation (Figs. 4b and 5d). The need for a shorter TCR stimulation in the Th2-polarizing cultures could be due to the presence of IL-4, which promotes a more rapid activation as compared with IL-2 alone that is present in Th0 cultures (D. M. Jelley-Gibbs and S. L. Swain, unpublished observations).

Cytokine dependence during Ag-independent late phase of effector generation

Our next set of experiments was aimed at examining the mechanism responsible for driving the constant rate of cell division seen in the late phase (days 2–4) of our standard in vitro cultures (Fig. 1), despite the clear lack of Ag presentation beyond the early phase of effector generation (days 0–2). We hypothesized that the Ag-independent constant rate of cell division observed in the late phase of cell expansion is either driven by growth-promoting cytokines, like IL-2, or is a consequence of early and irreversible programming events within the T cell, or a combination of both. To test whether IL-2 is required to drive the late phase of effector generation, naive CD4 cells were cultured with Ag/APC under standard Th2-polarizing conditions for 2 days. The T cells were then washed to remove any exogenous and secreted IL-2, CFSE labeled, and recultured for an additional 2 days with either 1) culture supernatants added back, 2) fresh T cell media plus exogenous IL-2, or 3) fresh T cell media plus anti-IL-2Rα-blocking Ab. To obtain high levels of synchronized CFSE labeling, we CFSE labeled the T cells at day 2 of culture before dividing the cells into the three respective groups. Because all of the T cells are already cycling, we observed a relatively synchronized cell proliferation profile unlike that seen when naive or resting T cells are CFSE labeled and then cultured to proliferate. Naive or resting T cells enter the cell cycle in an asynchronous manner which has a delay of 24–32 h, and then they divide at a relatively constant rate of every 6–8 h (Fig. 1). Alternatively, actively cycling cells (those seen on days 2–4 of culture) appear to be relatively synchronized in their cell cycles. This synchronized cell cycling results in CFSE profiles which are less defined for specific division peaks. However, the numbers of divisions each cell has undergone can be easily calculated by measuring the mean fluorescent intensity (MFI) of the CFSE as dye loss in each daughter cell being equal to exactly 50% of that of the parent cell. For example, if the MFI of CFSE labeling on day 2 is 1000, the MFI of a cell that had divided exactly 50% of that of the parent cell. For example, if the MFI of CFSE labeling on day 2 is 1000, the MFI of a cell that had divided once time would be 500, twice would be 250, and three times would be 125. Using these mathematical calculations, we were able to determine the average number of divisions occurring between days 2 and 4 of effector generation. At the end of the 4-day culture period, the effector recovery and CFSE profiles were examined, and the ability of the resulting viable effectors to produce polarized cytokines upon restimulation was measured by ICCS.

Under conditions where effectors received no ongoing IL-2 stimulation (blocking with anti-IL-2Rα Ab for the last 2 days of culture), there was a greatly reduced effector recovery (Fig. 6a), which was paralleled by a decreased rate of cell division between days 2 and 4 as determined by CFSE dye loss (Fig. 6b). The decreased effector recovery of ~50% of that found when IL-2 was present was accounted for by a reduction of an average number of cell divisions of 4.25 divisions for the cultures containing IL-2, to 2 divisions for the cultures without IL-2. We quantitated cell death of these cultures by FSC × SSC FACS profiling and found no
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significant increases in cell death as a result of blocking IL-2. Therefore, instead of proliferating every 8 h, effectors cultured during the late phase of effector generation with anti-IL-2Rα-blocking Ab only underwent one round of proliferation approximately every 16 h (Fig. 6b). Moreover, effectors cultured under conditions where IL-2 stimulation was blocked were capable of producing little IL-2 and no IL-4, whereas cultures reconstituted with their own supernatants or with exogenous IL-2 produced normal levels of IL-2 and IL-4 (Fig. 6c). Therefore, not only was the rate of cell proliferation during the final 2 days of culture reduced by the lack of IL-2 stimulation, but the resulting effectors were incapable of optimum effector function, as measured by cytokine production. This supports the hypothesis that there are two distinct phases of cell activation and expansion such that effectors first undergo a 1–2-day Ag-dependent phase of cell activation and expansion, which is then followed by an Ag-independent and IL-2-driven late phase of effector differentiation and expansion into fully functional effectors (Figs. 1 and 6) (25). This pattern suggests that mechanisms may well exist in situ to ensure that once fully activated, responding CD4 T cells must be segregated from Ag-bearing APC. In these studies, which use highly purified naive CD4 T cells and APC populations, autocrine IL-2 and exogenous IL-4 are the only IL-2Rγ chain-binding, growth-promoting cytokines available. It is likely that other IL-2Rγ chain-binding cytokines, or other growth-promoting cytokines, may drive in vivo responses (48, 49). Thus, it will be important to determine whether other cytokines can promote this Ag-independent phase in vivo and to determine what regulates their availability.

By supplementing T cell cultures with more prolonged Ag presentation, either in the form of Ag/APC or plate-bound anti-TCR Ab, we found that Ag presentation beyond day 2 leads to the disappearance and death of responding CD4 T cells, thus resulting in recovery of fewer effectors at day 4. Moreover cytokine production by surviving effectors is decreased by the prolonged Ag exposure (Figs. 4 and 5). Many responding CD4 T cells, which continue to encounter Ag/APC, apparently undergo AICD, which results in the selection of a population of cells which produce less cytokine, perhaps reflecting a selection for the least differentiated cohorts of cells. This may be a means to prevent uncontrolled effector expansion, which could lead to detrimental inflammatory responses. These results augment many previous in vitro studies whereby restimulated effector T cells undergo cell deletion via AICD (17, 50). We have recently confirmed that CD4 effectors transferred to adoptive hosts also disappear when hosts are immunized with Ag (E. Roman, G. E. Huston, and S. L. Swain, unpublished observations). As discussed below, we suggest that these findings may indicate that productive in vivo responses must involve a process which ensures responding CD4 T cells are not reexposed to Ag after 2 days.

The results presented in these studies also have important implications for the popular in vitro model system in which TCR stimulation is provided through plate-bound anti-TCR Ab. These studies are commonly performed by incubating T cells in tissue culture plates coated with anti-CD3 Ab, anti-TCR Ab, or tetramers for the entire duration of the experiment, which typically extends to 4–7 days of culture. These results predict that prolonged TCR stimulation, beyond the first 2 days of culture for naive T cells, may select for a subpopulation of less functional effectors and preclude recovery of the most active populations of effectors. These results also predict that extended TCR stimulation would promote the selection of a subpopulation of effectors resistant to cell death. These results present a need to evaluate the functional and phenotypic qualities of effectors generated using this particular in vitro model system for T cell stimulation.

Since optimal CD4 effector generation and function is dependent on an initial 1–2 days of Ag presentation followed by the absence of Ag presentation during an IL-2-driven cell expansion phase lasting an additional 2 days (Fig. 6), it is important to consider the possibility that in vivo CD4 T cell responses may be compartmentalized to achieve this set of conditions. Several published reports support the concept of chronologically and anatomically separate phases in CD4 T cell responses. Ingulli et al. (18) have shown that Ag (OVA)-specific CD4 T cells and Ag-pulsed

FIGURE 6. Cytokine dependence during Ag-independent phase of effector generation. Naive CD4 AND T cells were cultured at 3 × 10^5 T cells/culture with 1.5 × 10^3 PCCF peptide-pulsed DCEK-ICAM under Th2-polarizing conditions for 2 days. To synchronize the CFSE labeling, the cultured cells were then washed to remove exogenous and secreted IL-2, CFSE labeled, and cultured an additional 2 days with either their original culture supernatants added back, T cell media plus only exogenous IL-2, or T cell media plus anti-IL-2Rα-blocking Ab. a, Live Vβ3^+ (AND)/CD4^+ effector recoveries were determined at day 4 of culture by direct cell counts. Cell death was determined by FSC × SSC FACS profiling and was 17, 14, and 25%, respectively. b, FACS analysis of CFSE dye loss of live Vβ3^+ (AND)/CD4^+ effectors was used to measure cell division during the last 2 days of culture. c, ICCS for IL-2 and IL-4 was measured on live CFSE^−Vβ3^+(AND)/CD4^+ effectors restimulated for 4 h with PMA + ionomycin. Data are representative of three independent experiments.
dendritic cells (DC) accumulate to a maximal level in the T cell-rich paracortical regions of spleen and lymph nodes within 24 h of i.v. injection of the Ag-pulsed DC and that the accumulation of DC:T cell clusters then declined by 48 h (18). This dissociation could have been due to limitations of Ag or instead an active process associated with the CD4 T cell response. By 48 h, Ag-pulsed DC rapidly disappeared from the lymph nodes, either because they migrate out of the lymph node or are killed by the responding T cells. Two days after immunization, both Ag-specific T cells and B cells moved toward each other from their separate starting locations and cognate T cell-B cell interactions could be visualized at the edge of the B cell-rich follicles at the border between the follicles and the T cell areas in the spleen (33). By days 3 and 4, the Ag-specific T cells are no longer found near the Ag-presenting B cells (18, 33). Perhaps those T cells destined to expand and produce large populations of effectors relocate as well to a separate location rich in growth and differentiation-promoting factors. Indeed, a recent study by Vasseur et al. (32) suggests that Ag-specific T cell accumulation in the lymph nodes is very transient (1–2 days), and proliferating cells recirculate in the blood rapidly after Ag presentation and T cell activation (32). Thus, the concept that the period where Ag is presented to CD4 T cells is of limited duration seems to be mirrored by the in vivo migratory patterns of responding CD4 T cells and APC. Together, these studies support a model in which CD4 T cells are activated in the first 1–2 days of an immune response by Ag-presenting DC and begin division. Then the T cells migrate to either an area in the lymphoid compartment where they can interact with Ag-presenting B cells or they may migrate away from the Ag/APC environments altogether. Whether there is a defined site of additional CD4 T cell expansion and differentiation is unclear (18, 32, 33, 51, 52). However, the need for responding CD4 T cells to encounter IL-2 or in an in vivo surrogate for full expansion and effector development would suggest that they may do so in a defined niche.

References


