# Repeated stimulation of CD4 effector T cells can limit their protective function

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**Chronic infections often result in CD8 T-cell deletion or functional nonresponsiveness. However, to date no definitive studies have attempted to determine the impact of repeated T cell receptor stimulation on CD4 effector T cell generation. We have determined that when antigen presentation is limited to 2 d, optimum in vitro CD4 effector generation is achieved. Alternatively, repeated stimulation results in decreased CD4 effector expansion, decreased cytokine production, and altered migration. Similarly, functionally impaired effectors develop in vivo when antigen-pulsed antigen-presenting cells are replenished every 24 h during a primary immune response. CD4 effectors that are generated with repeated stimulation provide no protection during influenza infection, and have an impaired ability to provide cognate help to B cells. These results suggest that duration of antigen presentation dictates CD4 effector function, and repeated T cell receptor stimulation in vitro and in vivo that exceeds an optimal threshold results in effectors with impaired function.**

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Abbreviations used: Ag, antigen; AT×BM, adult thymectomized, lethally irradiated, bone marrow reconstituted; CFSE, carboxyfluorescein succinimidyl ester; ICCS, intracellular cytokine staining; NP, 4-hydroxy-3-nitrophenyl acetate; PCCF, pigeon cytochrome c peptide fragment; RS, repeated stimulation; Tg, TCR transgenic.

Efficient priming of naive T cells is essential for the generation of effective immunity to pathogens. The initiation of a protective adaptive immune response depends on successful interactions between T cells and APCs. When naive CD4 T cells successfully recognize antigen (Ag) that is presented by APCs, they expand into highly activated effectors that exhibit immediate cytokine-secreting function (1–3). We hypothesize that reaching an optimal threshold of TCR stimulation in the initial phases of a T cell response determines if an effector survives and acquires the functions that are necessary to provide cytokine or cognate help to other lymphocytes.

The progressive differentiation model that was proposed by Lanzavecchia and colleagues (4, 5) suggests that the level of TCR signal that is accumulated determines if T cells reach hierarchical thresholds for induction of proliferation and differentiation. In this model, random encounters of variable duration with APCs and cytokines result in the generation of effectors with different fates (6). The concept that TCR signal accumulation results in progressive differentiation of effectors is supported by the finding that the commitment of naive T cells to proliferate is reached in 6–12 h if they are stimulated by a high dose of Ag plus costimulation (7), but 40 h is required to generate an expanded effector population (8). Moreover, it has been demonstrated that multiple rounds of proliferation, and 2–4 d of culture, are required for naive T cells to expand into effectors that are capable of rapidly producing large amounts of cytokines (9, 10).

Less is known about the impact of repeated TCR stimulation on effector development, or the ability of the effectors that are generated to protect against infectious agents and provide help to other lymphocytes (11). The current body of literature primarily has focused on determining the minimal stimulation that is necessary to drive multiple rounds of T cell division (7, 12–14), rather than the optimal stimulation that is required for the generation of functional effectors.

The generation of effectors in vitro typically involves stimulating naive CD4 T cells with Ag-pulsed APCs, often with exogenous IL-2 and polarizing cytokines. Our previous studies demonstrated that under these conditions, the Ag-pulsed APCs disappear from culture within 48–60 h of culture initiation, and that cell division in the final 2–4 d of a 4-d culture is driven by cytokine (IL-2) stimulation. We define this set of conditions, where Ag-pulsed APCs are added only at the initiation of culture, as acute Ag stimulation. Alternatively, prolonging Ag stimulation beyond the first 2 d of culture resulted in reduced numbers of effectors (8). Therefore, we define

repeated TCR stimulation (RS) as that which results from the addition of Ag-pulsed APCs on the day of culture initiation and every 24 h for the duration of the 4 d culture. Here, we further investigate the consequences of acute versus repeated exposure to Ag-pulsed APCs on the generation and function of CD4 effector and memory T cells.

The results of this study reveal that highly activated, and well-polarized effectors can be generated under conditions of RS. However, RS leads to the generation of effectors that produce lower levels of cytokines in response to restimulation than acutely stimulated effectors. Moreover, repeatedly stimulated Th1 effectors fail to provide protection in mice that were lethally challenged with influenza virus. Additionally, Th2 polarized effectors that were generated with RS fail to provide cognate help to B cells. We also have investigated the effects of acute and RS in vivo, and have determined that RS profoundly affects the migration and function of the resulting effectors. We suggest that an initial 48–60 h of TCR stimulation during T cell priming results in an expanded population of optimally functional effectors that traffic efficiently through lymphoid organs, and are able to participate in antiviral responses and provide B cell help. We also suggest that longer durations of Ag stimulation result in T cells that exhibit dramatically reduced effector function, and fail to provide other lymphocytes with the cognate help that is necessary to stimulate vigorous immune responses.

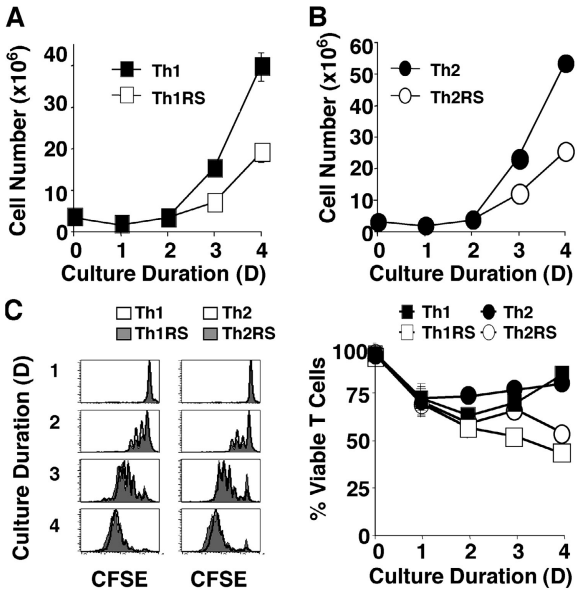


**Figure 1. Repeated Ag stimulation impairs effector T cell expansion.** Naive AND TCR Tg CD4 T cells, stimulated with acute or repeated Ag-pulsed APC stimulation under (A) Th1 or (B) Th2 polarizing conditions were enumerated, (C) analyzed for CFSE dye loss, and (D) cell viability. Data are means of triplicate cultures ± SD. Data shown in (C) is one representative of triplicate cultures. Data are representative of three to six independent experiments.

## RESULTS

### Repeated T cell receptor stimulation results in poor effector expansion

Naive and TCR transgenic (Tg) CD4 T cells were cultured with acute or repeated stimulation to generate Th1 and Th2 polarized effectors. By day 4, cell recovery in Th1 and Th2 cultures that were exposed to RS was 50% reduced, compared with acute stimulation (Fig. 1, A and B, respectively). Interestingly, carboxyfluorescein succinimidyl ester (CFSE) profile analysis showed that acutely and repeatedly stimulated effectors underwent equivalent rounds of division throughout the culture (Fig. 1 C). Analysis of cell viability indicated that the repeatedly stimulated effectors had fewer viable cells; this suggested that the decrease in effector recovery could be attributed to increased cell death compared with effectors that were generated with acute stimulation (Fig. 1 D).

### Repeated stimulation impairs effector cytokine production

Because the protective immune functions of T cells are mediated, in large part, by the effector cytokines that they produce (15, 16), we examined the effects of acute versus RS on the cytokine production by Th1 and Th2 effectors. Acutely and repeatedly stimulated Th1 and Th2 effectors had the capacity to secrete polarized cytokines in response to plate-bound anti-CD3 in a dose dependent manner (Fig. 2, A and B, respectively). However, repeatedly stimulated effectors produced significantly reduced amounts of IFNγ (Th1) and IL-4 (Th2) when compared with effectors that were generated with acute stimulation (Fig. 2, A and B). To determine if the reduction in cytokine production by repeatedly stimulated effectors was a result of fewer effectors that were capable of producing cytokines, or if the effectors were producing less cytokine on a per cell basis, intracellular cytokine staining (ICCS) was performed on viable effectors that were restimulated for 24 h with optimal concentrations of Ag-pulsed APCs. Fig. 2, C and D show that fewer effectors that were generated with RS were capable of producing cytokines, and those that were producing cytokines were making less cytokine on a per cell basis.

### Impaired cytokine production persists in memory T cells

Several groups have reported that, following primary stimulation, T cells can enter a state of transient unresponsiveness (17, 18). This nonresponsive state is of limited duration, and lasts only 6 d for CD4 T cells and 2 weeks for CD8 T cells (17, 18). Additionally, the repeatedly stimulated effectors were tested for cytokine production immediately following their last encounter with Ag-pulsed APCs, whereas the acutely stimulated effectors were tested for cytokine production following 2 d of "rest" without Ag-pulsed APC stimulation. Therefore, to determine if RS during CD4 effector generation is inducing a temporary state of nonresponsiveness, we adoptively transferred 4 d Th1 and Th2 polarized effectors, generated with acute or RS, into adult thymectomized, lethally irradiated, bone marrow reconstituted (AT×BM) host mice and waited for 3 wk for the progression to resting memory (9). Fig. 3 A indicates that when equal numbers of viable 4-d Th1 and Th1RS effectors were

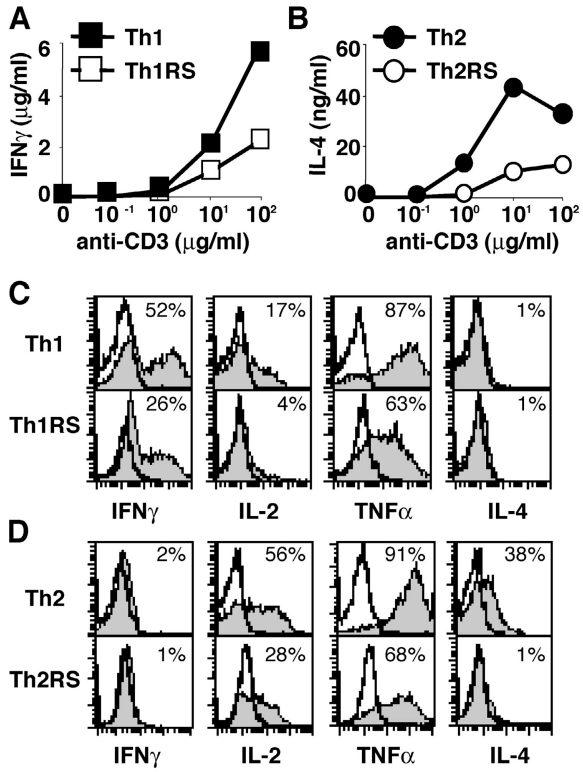


**Figure 2. Repeated Ag stimulation impairs effector cytokine production.** Viable AND TCR Tg (A) Th1 and (B) Th2 polarized 4 -d effectors were restimulated for 24 h with plate-bound anti-CD3 and supernatants were analyzed by ELISA. Four-d (C) Th1 and (D) Th2 effectors were cultured for 24 h without (open histograms) or with 10 μg/ml of plate-bound anti-CD3 restimulation (gray filled histograms) and analyzed by ICCS. Numbers within the histograms are % of cytokine-producing cells in the restimulated cultures. Data in (A and B) are means of triplicate cultures ± SD. Data in (C and D) are representative of triplicate cultures. Data shown are representative of three independent experiments.

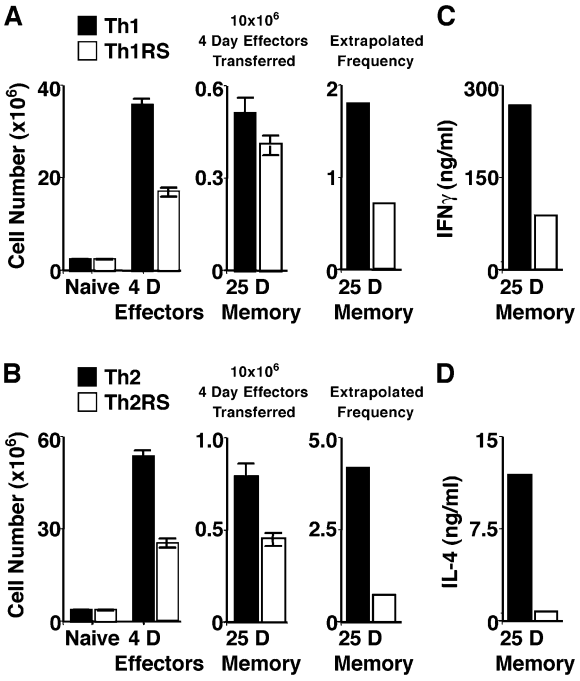


**Figure 3. Impaired cytokine production persists in memory T cells.** AND TCR Tg CD4 T cells stimulated with acute or RS under (A) Th1 or (B) Th2 polarizing conditions were enumerated at days 0 and 4 of in vitro culture. 10 × 10⁶ effectors were transferred into AT×BM hosts and recovered from lymph nodes and spleens 21 d later. Twenty-five-d memory T cells were enumerated, and extrapolated memory frequencies were determined by multiplying the number of memory cells recovered by the fraction of 4-d effectors that were generated in vitro. (C) Th1 and (D) Th2 polarized memory cells were restimulated for 24 h with Ag-pulsed APCs and supernatants were analyzed by ELISA. Data (A and B) are means of triplicate cultures ± SD. Data (C and D) are representative of duplicate cultures. Data are representative of three independent experiments.

transferred into AT×BM hosts there was no significant difference in the number of memory T cells that were recovered (center graph, 25 d memory). However, when we extrapolate from the total number of effectors that originally were generated in the primary cultures (~36 × 10⁶ Th1 and ~19 × 10⁶ Th1RS), the total number of Th1RS memory cells that are generated as a result of RS would be reduced compared with Th1 memory cells (right graph). Fig. 3 B indicates that when equal numbers of viable 4-d Th2 and Th2RS effectors were transferred into AT×BM hosts, there was a 25% reduction in the ability of Th2RS effectors that were generated with RS to progress to memory (center graph). Even more striking was the ~75% reduction in the number of Th2RS memory cells that were generated as a result of RS that would have resulted if the total number of effectors that were generated in primary culture (~55 × 10⁶ Th2 and ~25 × 10⁶ Th2RS) had been transferred into the AT×BM hosts (right graph). We then restimulated equal numbers of memory cells with Ag-pulsed APCs for 24 h to determine if the transition to a memory-like state could restore the cytokine-producing function of repeatedly stimulated T cells. Fig. 3, C and D indicate that the Th1 and Th2 memory cells that were generated from repeatedly stimulated effectors still produced lower levels of IFNγ and IL-4, respectively, compared with memory cells that were generated from acutely stimulated effectors.

### Repeated T cell receptor stimulation impairs polyclonal CD4 T cell responses

The examination of TCR Tg CD4 T cells did not enable us to observe the evolution of a normal polyclonal response, and it was possible that the repeated Ag stimulation of polyclonal CD4 T cells could inhibit the functions of dominant T cell clones while permitting the expansion of subdominant clones. Therefore, it was important to demonstrate that the effects of repeated Ag stimulation were not confined to the responses of TCR Tg CD4 T cells. Naive polyclonal BALB/c CD4 T cells were cultured under acute and RS conditions with plate-bound anti-CD3 to generate Th1 and

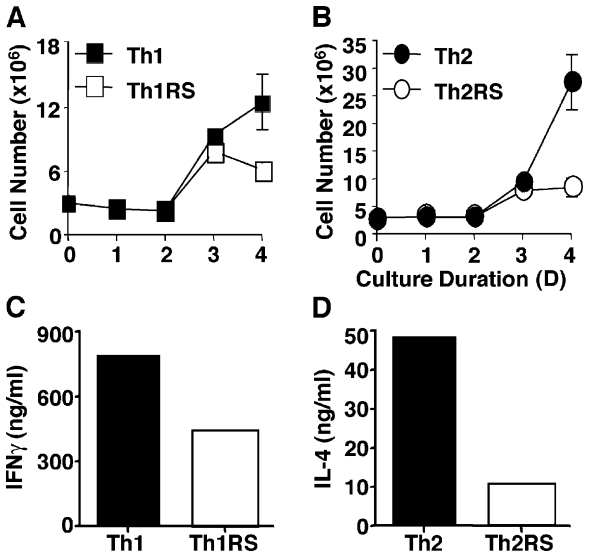


**Figure 4. Repeated Ag stimulation impairs polyclonal CD4 T cell responses.** Naive BALB/c polyclonal CD4 T cells that were cultured with acute or repeated plate-bound anti-CD3 stimulation under (A) Th1 or (B) Th2 polarizing conditions were enumerated. Four-d (C) Th1 and (D) Th2 effectors were restimulated for 24 h with 10 μg/ml plate-bound anti-CD3 and supernatants were analyzed by ELISA. Data (A and B) are means of triplicate cultures ± SD. Data (C and D) are one representative of triplicate cultures. Data are representative of two independent experiments.

Th2 polarized effectors. Cell recovery in 4-d Th1 and Th2 cultures that were exposed to RS was reduced by at least 50%, compared with acute stimulation (Fig. 4, A and B, respectively). We next examined the effects of acute versus RS on the cytokine-producing potential of Th1 and Th2 effectors. As observed with the TCR Tg CD4 effectors, repeatedly stimulated polyclonal effectors produced significantly reduced amounts of IFNγ (Th1) and IL-4 (Th2) when compared with effectors that were generated with acute stimulation (Fig. 4, C and D), although they maintained their respective polarized phenotypes.

### Repeated Ag stimulation does not result in cytokine unresponsiveness

Strong TCR stimulation or Ag persistence can induce a state of permanent unresponsiveness, or anergy (1, 19–21). This hypo-responsive state often is defined as the maintenance of T cells with a reduced ability to proliferate to IL-2 and/or produce effector cytokines. To explore further the functional potential of acutely versus repeatedly stimulated effectors, we determined whether effectors that were generated with RS were able to proliferate in response to IL-2 or other cytokines that stimulate T cells through the common γ-chain (19). Therefore, we CFSE-labeled 4-d Th1, Th1RS, Th2, and Th2RS effectors and recultured them in fresh T cell medium using optimal concentrations of common γ-chain binding cytokines. The acutely and repeatedly stimulated effectors divided in response to IL-2, IL-4, IL-7, and IL-15 (Fig. 5 A). Interestingly, the repeatedly stimulated effectors expanded when recultured with IL-2 and IL-7, whereas the acutely stimulated effectors simply were maintained during this reculture period (Fig. 5 B).

The 4-d primary effectors that were generated with RS had the potential to have down-regulated TCR expression as a result of excessive TCR stimulation during the primary culture. Lower levels of TCR expression could have a dramatic impact on the ability to restimulate the effectors for cytokine production. Despite the fact that 4-d Th2 effectors that were stimulated repeatedly (Fig. 5 C-Th2RS) had a slight down-regulation of their TCR (Vα11 shown, Vβ3-unpublished observation), these were the effectors that were most capable of expanding in response to an excess of TCR and IL-2 stimulation (Fig. 5 D).

### Repeatedly stimulated effectors have unique phenotypic characteristics

CD69 is one of the most prominently cited markers of recent Ag stimulation, and CD62L is one of the only molecules that has been clearly established as playing an important role in lymphoid homing and T cell trafficking in vivo; therefore, we focused on the expression of these two molecules on acutely and repeatedly stimulated effectors. Under Th1 and Th2 polarizing conditions, RS resulted in a more pronounced elevated level of expression of CD69 by 4-d effectors (Fig. 6, A and B). This indicated that RS is capable of maintaining a high level of TCR signaling throughout the 4-d culture period. Additionally, repeatedly stimulated effectors exhibited more extensive down-modulation of CD62L under Th1 and Th2 polarizing conditions when compared with effectors that were generated with acute stimulation. This pattern of expression indicated that repeatedly stimulated effectors underwent higher levels of activation and differentiation compared with T cells that were generated with acute stimulation.

Because expression of these markers has been shown to be transient, a static view of 4 d-effectors did not allow us to determine if there were differences in the kinetics of expression of these markers during effector generation. Therefore, we labeled naive CD4 T cells with CSFE and analyzed the T cells every 24 h during effector generation. These data are presented as flow cytometry dot plot overlays of days 1 (red), 2 (blue), 3 (green), and 4 (orange). Th1 (Fig. 6 C) and Th2 (Fig. 6 D) effectors exhibited a progressive differentiation to a $CD62L^{lo}$ phenotype, which is more dramatic with RS. Transient expression of CD69 was observed when effectors were generated with acute stimulation; up-regulated expression lasted only for the first 48 h of culture, whereas CD69 expression was maintained throughout the entire 4 d of effector generation when stimulated with RS (Fig. 6, C and D). We also analyzed effectors that were stimulated acutely and repeatedly for expression of a variety of other activation markers (CD25, and CD44, unpublished data and references 3, 22); adhesion markers (CD43, CD49d, and CD162; references 3, 22–24); and chemokine receptors CCR5 (unpub-

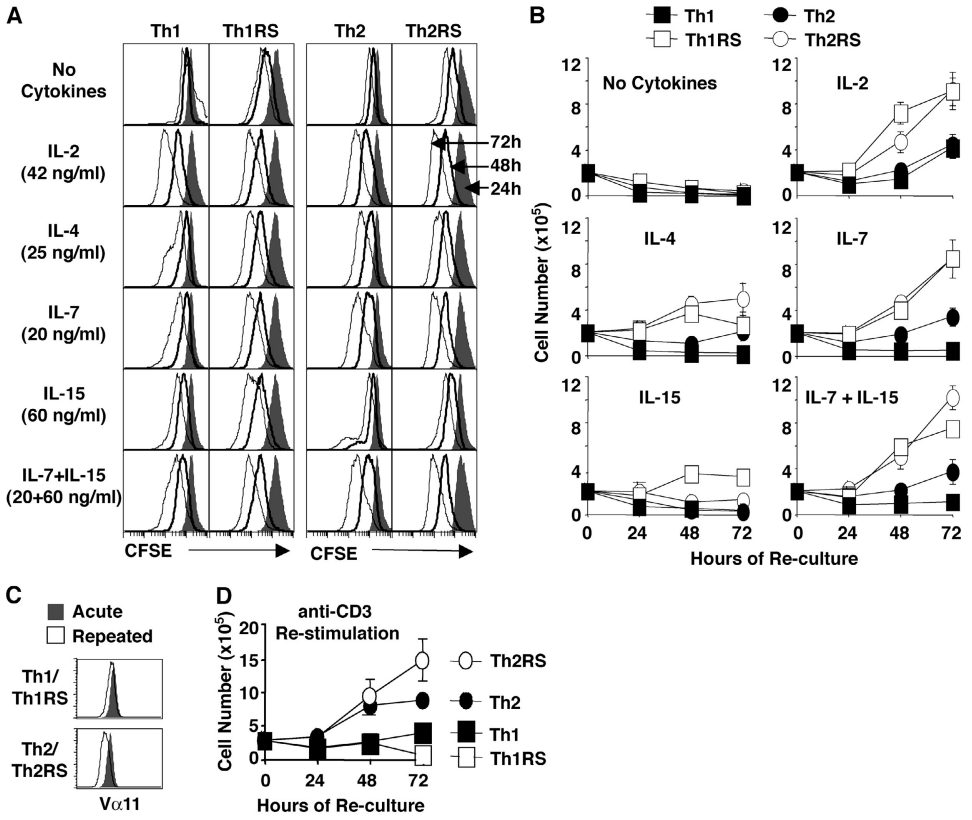


**Figure 5. Repeatedly stimulated effectors are responsive to re-stimulation.** Four-d AND TCR Tg effectors were CFSE labeled, recultured with common γ-chain binding cytokines, and analyzed every 24 h for (A) CFSE dye loss (filled histograms = 24 h, thick lines = 48 h, thin lines = 72 h), and (B) were enumerated. (C) TCR expression was determined by anti-Vα11 staining. (D) Effectors were restimulated with 10 μg/ml plate-bound anti-CD3 and IL-2 and enumerated every 24 h of reculture. Histograms (A and C) are representative of triplicate cultures. Data (B and D) are means of triplicate cultures ± SD. Data are representative of three independent experiments.

lished data) and CXCR3 (25, 26). Overall, repeatedly stimulated effectors exhibited extensive up-regulation of activation markers, and an interesting blockade of the modulation of all adhesion markers, except for CD62L (Fig. 6, E and F).

### Repeated stimulation reduces the ability of Th1 effectors to protect mice from lethal influenza challenge

Previous studies showed that IFNγ production by Th1 and Tc1 effectors is critical to clearance of influenza virus (3, 27–29). Therefore, we tested the ability of adoptively transferred acutely and repeatedly stimulated influenza-specific Th1 and Th1RS effectors to protect mice that were challenged lethally with influenza virus. Fig. 7 A shows that all of the mice that were infected with a $2LD_{50}$ of influenza virus survived when $5 \times 10^6$ Th1 or Th1RS effectors were transferred. When the number of effectors was reduced to $2.5 \times 10^6$ per mouse, Th1 effectors remained 100% protective (Fig. 7 A-middle graph), but only 25% of the mice that received Th1CS effectors were protected. Reducing the number of effectors to $0.5 \times 10^6$ per mouse resulted in neither the Th1 nor the Th1RS effector being able to protect against a $2LD_{50}$ of influenza virus (Fig. 7 A-bottom graph). The protection against lethal influenza challenge, afforded by sufficient numbers of Th1 polarized effectors, correlated with clearance of live virus from the lungs of the mice (unpublished data). These data indicate that it may be the quantity of IFNγ which is produced by the CD4 effectors that determines their ability to protect against lethal influenza challenge. Interestingly, despite the unique phenotypic differences between Th1 and Th1RS effectors (Fig. 6), the repeatedly stimulated effectors were recovered from the infected mice in numbers similar to, or even slightly greater than, the acutely stimulated effectors (Fig. 7 B). Furthermore, host CD4 and CD8 numbers were similarly recruited to the lungs, draining lymph nodes, and spleens of infected mice 4 d after receiving Th1 or Th1RS therapy (Fig. 6 D).

### Repeated stimulation impairs the ability of Th2 effectors to provide cognate help

Following immunization, CD4 T cells provide helper functions that are necessary for the generation of B cell–rich germinal centers (30). To examine if repeatedly stimulated effectors could provide cognate help to B cells, we adoptively transferred 4-d acutely and repeatedly stimulated Th2 effec-

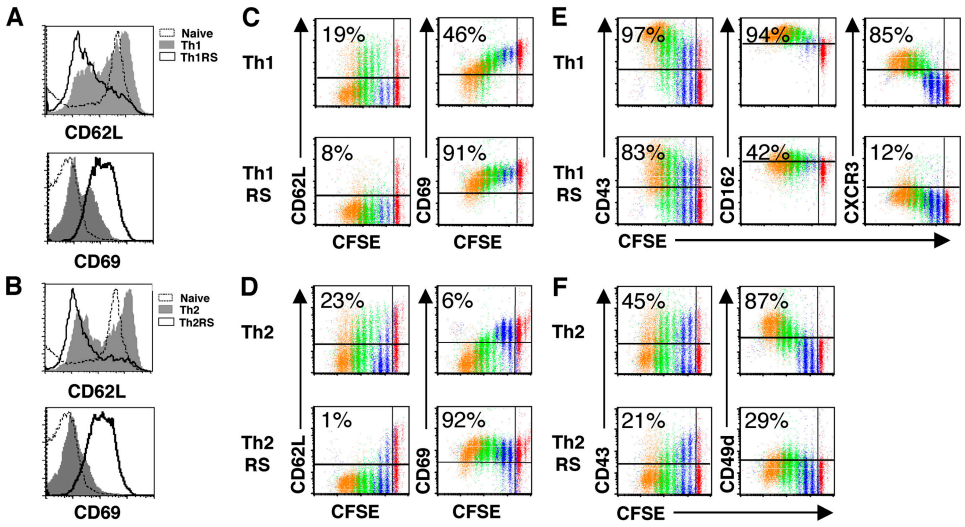


**Figure 6. Repeated Ag stimulation generates effectors with a more activated phenotype.** CD62L and CD69 surface expression on 4 d (A) Th1 and (B) Th2 effectors (acute stimulation = filled histograms, RS = solid lines) were compared with naive (dotted line) AND TCR Tg CD4 T cells. (C) Th1 and (D) Th2 polarized T cells were analyzed every 24 h for surface CD62L or CD69 expression versus CFSE loss. (E) Th1 and (F) Th2 polarized T cells were analyzed every 24 h for surface CD43, CD162, CXCR3, or CD49d expression versus CFSE loss. Red = day 1, blue = day 2, green = day 3, and orange = day 4. Numbers represent percentage of day 4 T cells in the upper left quadrant. Data are representative of three to five independent experiments.

tors [OT-II TCR Tg, specific for a peptide of ovalbumin (OVA)] into CD4 KO hosts (31). Analysis of host 4-hydroxy-3-nitrophenyl acetate (NP)-binding B cells 16 d following immunization revealed that almost three times as many NP-specific B cells differentiated to a germinal center phenotype ($CD38^{lo}PNA^{hi}$) in the presence of $10^7$ Th2 effectors that were generated with acute stimulation, compared with Th2 effectors that were generated with RS (Fig. 8 A). Fig. 8 B indicates that the transfer of $10^6$ or $10^7$ Th2 effectors generated a significant expansion of NP-specific, germinal center phenotype B cells, whereas Th2RS were unable to generate an expanded population of NP-specific, germinal center phenotype B cells. Additionally, significantly more NP-specific IgG1 was detected in the serum of mice when $10^7$ Th2 effectors were transferred into NP-OVA immunized hosts, compared with the transfer of $10^7$ Th2RS effectors (Fig. 8 C). We also assayed for the production of NP-specific IgM, IgG2a, and IgG2b and found no significant production of these antibodies (unpublished data).

### Effectors generated with repeated stimulation in vivo have profound functional deficiencies

To reproduce conditions of RS in vivo we used an adoptive transfer model that involves the cotransfer of Ag-pulsed DCs with naive Ag-specific CD4 T cells into intact hosts. The cotransfer of Ag-pulsed DCs with Ag-specific CD4 T cells was necessary to optimize the primary stimulation of naive CD4 T cells in vivo, and enabled us to visualize the transferred T cells in sufficient numbers to perform phenotypic and functional analysis. Previous studies suggested that Ag-bearing APCs would be deleted rapidly by CTLs (13, 32–34), and, in fact, Ag-pulsed DCs were undetectable within 2 d of transfer (R.A. Kemp, unpublished data). To overcome this, we cotransferred Ag-pulsed DCs with naive T cells at day 0, followed by transfer of Ag-pulsed DCs on days 1, 2, 3, and 4 of a 5-d in vivo immune response to model RS. We modeled acute stimulation by cotransferring Ag-pulsed DCs with naive T cells at day 0, followed by transfer of nonpulsed DCs on days 1, 2, 3, and 4.

On day 5, following naive T cell transfer, we recovered and analyzed donor T cells from peripheral lymph nodes, spleens, lungs, and livers of host mice. Effectors that were generated in vivo with acute stimulation migrated primarily to the peripheral lymph nodes and spleens; few cells migrated to tertiary sites (e.g., lungs, liver; Fig. 9 A). Conversely, we found increased migration of effectors that were generated in vivo with RS to the spleen, lung, and liver (Fig. 9 A). This was not unexpected because phenotypic analysis of the in vitro generated effectors revealed that repeatedly stimulated effectors had a largely $CD62L^{lo}$ phenotype, which has been shown to promote T cell migration out of lymph nodes (22). In fact, effectors that were generated in vivo with acute stimulation had down-regulated CD62L to a lower extent when compared with the highly activated phenotype of repeatedly stimulated Th1 effectors (Fig. 9 B). This was true particularly for those effectors that had migrated to the lymph nodes and spleen, because the effectors that migrated to the lungs and liver exhibited a more highly activated phenotype, regardless of the conditions under which they were stimulated (Fig. 9 B).

Effectors that were generated in vivo with acute stimulation resulted in an expanded population of Th1-like effec-

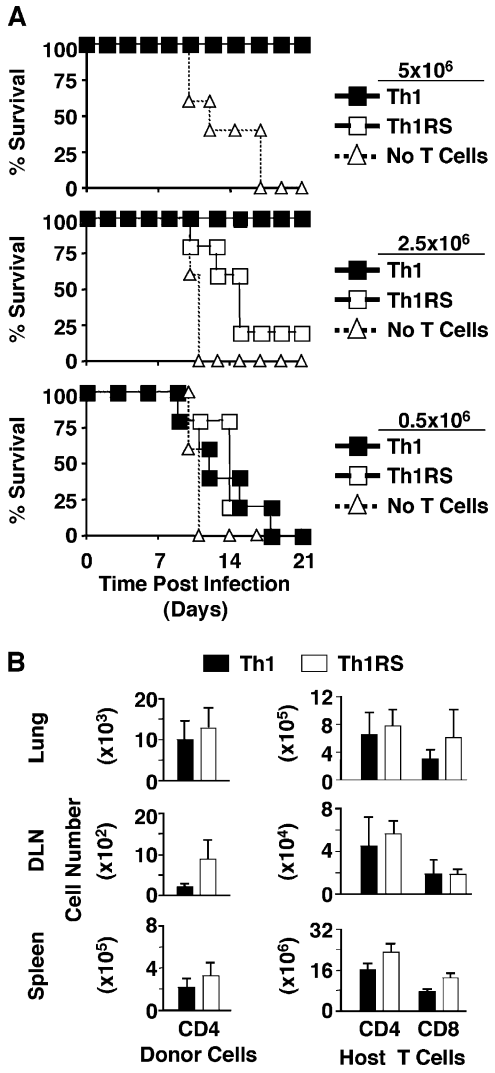


**Figure 7. Repeatedly stimulated Th1 effectors fail to protect against lethal influenza infection.** $5 \times 10^6$, $2.5 \times 10^6$, and $0.5 \times 10^6$ 4-d HNT TCR Tg Th1 or Th1RS effectors were transferred into mice that were lethally infected with influenza virus. (A) Host survival was monitored, and (B) donor CD4 and host CD4 and CD8 T cells were enumerated 4 d following transfer. Data (A) have a starting population of five mice per group. Data (B) are means of three mice per group ± SD. Data are representative of two to three independent experiments.

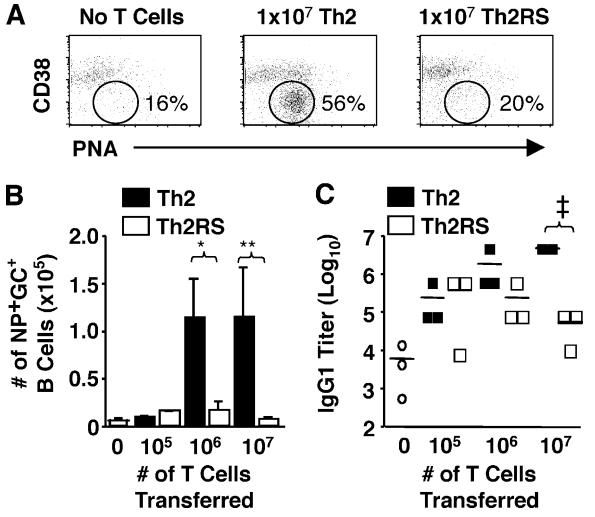


**Figure 8. Th2RS effectors provide minimal cognate B cell help in vivo.** Four-d Th2 or Th2RS effectors, or PBS alone, were transferred into CD4 KO hosts that were immunized with NP-OVA in alum. Sixteen d following immunization (A) $NP^+$ B cells were harvested from spleens and analyzed for germinal center ($GC^+$) phenotype ($CD38^-/PNA^+$). (B) total numbers of $NP^+GC^+$ B cells were determined (*P = 0.006, **P = 0.007), and (C) serum was analyzed for NP-specific IgG1 (‡P = 0.00006). Data (A) is one representative of triplicate mice. Data (B and C) are means ± SD of triplicate mice. Data are representative of three independent experiments.

tors with the capacity to produce high levels of IFNγ (Fig. 9, C and D). In contrast, effectors that were generated in vivo with RS failed to expand into a large population of IFNγ–producing effectors (Fig. 9 D). This is despite the fact that RS in vivo resulted in only a modest increase in total numbers of effectors (Fig. 9 D). Repeatedly stimulated effectors that were generated in vivo also produced less IL-2 when compared with the acutely generated effectors; neither population produced any detectable IL-4 (unpublished data).

Because RS of T cells in vivo generated effectors of similar functional deficiencies as those that were generated in vitro, we determined if the repeatedly stimulated effectors that were generated in vivo could protect against lethal influenza challenge. However, we needed to optimize the conditions of in vivo stimulation of adoptively transferred HNT TCR Tg CD4 T cells because it was demonstrated that large numbers if IFNγ–producing T cells would be necessary to protect mice against lethal challenge. Careful titration of naive HNT TCR Tg CD4 T cells and HNT-pulsed DCs enabled us to generate enough acutely stimulated effectors to protect 80% of the mice from lethal challenge (Fig. 9 E), whereas only 40% of the mice survived when the T cells were stimulated with repeated injections of HNT-pulsed DCs.

## DISCUSSION

Repeated stimulation of CD4 T cells during primary culture led to the generation of effectors that produced strikingly low levels of polarized cytokines, without changing their overall polarization profiles. Repeated stimulation did not induce anergy, as defined by the ability of effectors to proliferate to γ-chain binding cytokines, restimulation with Ag, or their ability to persist in vivo. The repeatedly stimulated effectors also were inefficient in protection against influenza virus and were inhibitory to cognate help.

We have determined that RS in vitro, whether achieved by adding Ag-pulsed APCs every 24 h of culture or continual culture on plate-bound anti-CD3 (8) compromises primary CD4 T cell expansion and differentiation into cytokine-producing effectors. This finding has significant implications regarding the quality of the effectors that are generated with the widely used 4–7 d plate-bound anti-

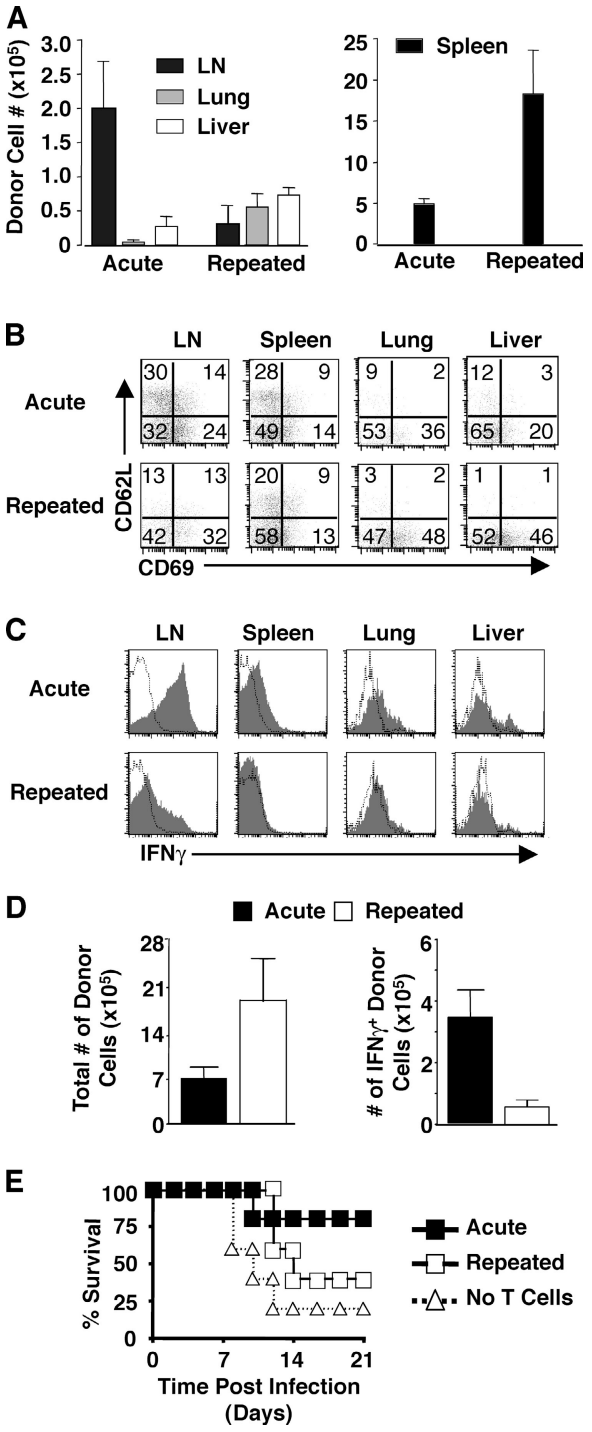


**Figure 9. Repeated Ag stimulation in vivo leads to the generation of functionally impaired effectors.** Naive AND TCR Tg × GFP CD4 T cells were mixed with PCCF-pulsed DCs and transferred into intact B10.Br mice. Subsequently, empty DCs (acute stimulation) or PCCF-pulsed DCs (RS) were injected every 24 h. On day 5, donor T cells ($GFP^+CD4^+$) were (A) enumerated, and assayed for expression of (B) CD69 versus CD62L, and (C) IFNγ by ICCS with (filled histograms) or without (dotted lines) PMA/ionomycin restimulation. (D) Total numbers of donor T cells recovered from all organs and total numbers of $IFN\gamma^+$ donor T cells were calculated. (E) On day 5 of in vivo HNT TCR Tg effector generation, host mice were lethally infected with influenza virus and survival was monitored. Donor cell frequencies in (A) and (D) are means ± SD of triplicate mice. FACS analysis data (B and C) are one representative of triplicate mice. Data (E) have a starting population of five mice per group. Data are representative of two to four independent experiments.

CD3/anti-CD28 continual stimulation technique. Typically this technique is used to stimulate polyclonal CD4 and CD8 T cells in vitro, which are then examined for effector responsiveness in a variety of model systems. A previous study indicated that mitomycin-c–treated, Ag-pulsed APCs normally disappear from in vitro culture systems between 48–60 h of culture with Ag-specific CD4 T cells, and that the late phases of T cell differentiation are driven by growth-promoting cytokines like IL-2 (8). We would suggest that plate-bound anti-CD3 stimulation can only mimic Ag-pulsed APC stimulation conditions if T cells are removed from the plate-bound anti-CD3 stimulation between 48–60 h of culture and transferred, without washing, into uncoated tissue culture containers for the remainder of the culture period. Otherwise, repeatedly stimulated effectors will be generated, which produce lower levels of cytokines upon restimulation, are unable to provide protection against lethal influenza infection, and are inhibitory to cognate help.

The acutely and repeatedly stimulated effectors that were generated in vitro were able to secrete polarized cytokines in a dose dependent manner in response to plate-bound anti-CD3 restimulation, and were able to proliferate in response to common γ-chain cytokines and Ag restimulation; this suggested that the repeatedly stimulated effectors were not anergic. The ability of repeatedly stimulated effectors to proliferate in response to cytokines like IL-7—known to be an important CD4 T cell survival factor (35)—could help to explain how repeatedly stimulated effectors persisted as memory T cells following adoptive transfer, but died during the in vitro cultures. The availability of common γ-chain cytokines in vivo could have provided survival or expansion signals to repeatedly stimulated effectors. However, the repeatedly stimulated effectors remained poorly functional as memory T cells, even though they were allowed to rest from TCR stimulation for 21 d before being restimulated. The implications of a persisting population of poorly functional memory T cells has been discussed by many groups (36–39). As individuals age, thymic output decreases and T cell repertoire diversity contracts; this makes it even more important that any persisting memory T cells have optimal functional capacities. The persistence of a large population of poorly functional memory T cells could compromise severely the ability to establish and maintain a diverse T cell repertoire of functional effector and memory T cells.

The repeatedly stimulated effectors that were generated in vitro inhibited the up-regulation of several surface molecules that had been implicated in promoting T cell migration to the spleen and tertiary sites; however, when the repeatedly stimulated effectors were generated in vivo, they did migrate in greater numbers to the spleen and tertiary sites.

These findings seemed to be inconsistent, until we reconsidered the modulation of CD62L to be not only a measure of the level of activation of T cells, but also a potent modulator of CD4 T cell migration between lymph nodes and tertiary sites. Of the adhesion markers that we analyzed, CD62L is the most thoroughly published as being able to dictate the migration of T cells in and out of lymphoid compartments (22), and it is with respect to CD62L expression that our in vitro phenotype data and our in vivo migration data are most consistent. Unfortunately, although the modulation of adhesion molecules and chemokine receptors has been established as being relevant for the trafficking and tissue localization of activated T cells during immune responses (23), no study has revealed conclusively the definitive combination of surface molecule expression that is required to promote optimal CD4 T cell migration between the lymph nodes, spleen, and tertiary sites. The only conclusion that we have drawn from this study is that repeated Ag stimulation in vivo led to the generation of effectors that preferentially migrate to the spleen and tertiary sites, rather than the lymph nodes, and that repeated exposure to Ag stimulation has a dramatic impact on the migratory potential of primary effectors. Further studies are necessary to assess the role of individual surface molecules—particularly those that failed to become up-regulated in response to RS—on the migration and interaction of CD4 effectors with other lymphocytes in vivo.

The finding that large numbers of repeatedly stimulated effectors failed to provide, or directly impaired, efficient cognate help to B cells also had interesting implications, particularly with regards to immunization strategies. The poor helper function was not surprising, considering that repeatedly stimulated Th2 effectors had a marked reduction in their ability to produce IL-2 and IL-4. In fact, Grabstein et al. (40) demonstrated that CD4 T cell–derived cytokines were essential for contact-dependent T cell help for Ag-specific primary antibody responses. Specifically, the induction of polyclonal Ig secretion required IL-4 and IL-5, and optimal Ag-specific antibody formation required IL-2. Because repeatedly stimulated Th2 polarized effectors produced low levels of IL-2 and IL-4, it was not surprising that they failed to provide optimum levels of cognate help to B cells. However, because the deficit in T cell help was most pronounced when a high number of effectors were transferred, these results also would be consistent with the induction of an inhibitory mechanism at high T cell numbers. In fact, Eaton et al. (31) recently reported that the transfer of too few or too many naive CD4 T cells resulted in reduced cognate helper function. Additionally, Erickson et al. (41) reported that although CD40L ligation of CD40 on B cells has been used as a potent immune adjuvant for short-lived humoral-mediated immunity, increasing the magnitude of T cell help ablated germinal center formation and prematurely terminated the humoral immune response. In fact, the highly activated phenotype that was characteristic of repeatedly stimulated effectors included a dramatic up-regulation of CD40L surface expression (unpublished data).

Several studies have focused on the impact of varied durations of Ag stimulation on CD4 T cell activation and development of effector functions. However, the central question of the studies that have published to date was to determine the minimal duration of Ag stimulation that was necessary to promote CD4 T cell activation, division, and development of a cytokine-producing phenotype. The general consensus of these studies was that longer durations of TCR stimulation—from 8 to 20 h—were required to induce IL-2 and IFN$\gamma$ production when Ag dose or affinity was low, and that high Ag dose or affinity could drive more progressive differentiation of proliferating T cells with as little as 4–6 h of TCR stimulation (12, 42, 43). These studies suggested that the quantity of TCR stimulation augmented T cell activation, at least in part, by reducing the time that is required for cytokine secretion. However, in most of the protocols, TCR stimulation and/or T cell culture was terminated by 48 h, and hence, did not determine the consequences of prolonged Ag stimulation on the peak of effector responses, which typically is determined at 4–7 d of a primary in vitro immune response. Even studies that had attempted to establish if cell division could determine the development of cytokine production by effectors used in vitro APC populations, which have been established to be eliminated from in vitro cultures within the first 48–60 h, and hence, would be unable to stimulate repeated Ag stimulation (8, 10, 44). This study is the first to determine the direct impact of repeated Ag presentation on primary CD4 effector responses.

Generally, it had been accepted that different pathogens pose unique challenges to the immune system, and variations in pathogen load and Ag persistence may affect primary T cell responses dramatically. Recent studies revealed that Ag-bearing DCs were highly efficient at recruiting and stably capturing T cells with high avidity interactions for long periods of time (6). The ability to introduce Ag-bearing DCs repeatedly to induce effectors with lower IFN$\gamma$ production suggested that persistent infections might induce poor CD4 T cell responses as a result of repeated Ag stimulation, particularly when CTLs are not able to eliminate Ag-bearing APCs.

Studies of CD4 T cell responses to chronic or persistent pathogens like HIV, *Mycobacterium tuberculosis*, EBV would support the hypothesis that Ag persistence leads to T cell death and functional nonresponsiveness of surviving CD4 T cells. Following infection with HIV, *M. tuberculosis*, and EBV, the frequency of Ag-specific CD4 T cells that is recovered from individuals during the latent phase of the infection is significantly lower than that detected during primary infection, rather than maintaining high frequencies of Ag-specific CD4 T cells throughout the infection (45–48). Alternatively, failed attempts to vaccinate against diseases, like leishmaniasis and malaria, suggested that the maintenance of protective CD4 T cell–mediated immunity would require the continued presence of Ag stimulation (49, 50). These infections induce strong CD4 effector T cell responses, and because of their persistent nature, may drive all of the T cells to terminal exhaustion; this leaves no partially

primed T cells to mount a secondary response. Recent attempts at vaccination of mice with *Leishmania major* parasites that only survived for a short time revealed that the T cells that were generated with this vaccination strategy were partially primed T cells and were protective to an extent. However, the most efficient protection against these pathogens requires the maintenance of large numbers of highly activated Th1 effectors, and that resting memory T cells were not present in sufficient numbers to afford a high level of protection against rechallenge (49, 50). The findings regarding chronic pathogen infections all have a common theme; CD4 T cells that were generated in response to chronic pathogen infections had qualitative deficits in their functional capacities during the chronic phase of the infection. Our study revealed that chronic Ag stimulation by Ag-bearing APCs was too much of a good thing and may be the critical factor that is involved in inducing a functionally nonresponsive state in Ag-specific T cells. These findings have important implications for vaccine design and may lead to a better understanding of how to combat persistent infections.

## MATERIALS AND METHODS

**Animals.** AND (B10.Br) TCR Tg, AND TCR Tg × GFP, Thy1.1$^+$HNT (BALB/c ByJ) TCR Tg, OT-II (C57BL/6) TCR Tg, and BALB/c ByJ mice were used as sources of naive CD4 T cells at 4–6 wk of age, and were bred in the animal breeding facilities at Trudeau Institute. B10.Br, BALB/c ByJ, and C57BL/6 × CD4 KO mice were used at 2–4 mo of age, and were bred in the animal facilities at Trudeau Institute or purchased from Jackson ImmunoResearch Laboratories. Experimental procedures were approved by Trudeau Institute's Institutional Animal Care and Use Committee.

**Preparation of APCs.** All cells were cultured in cell culture medium (RPMI 1640 supplemented with penicillin [200 μg/ml; Sigma-Aldrich], streptomycin [200 μg/ml; Sigma-Aldrich], glutamine [4 mM; Sigma-Aldrich], 2-ME [50 μM; Sigma-Aldrich], Hepes [10 mM; Sigma-Aldrich], and 8% FBS [Intergen]). T-depleted B cells were prepared by isolating lymphocytes from spleens of intact mice in cell wash buffer [RPMI 1640 supplemented with Hepes and 1% FBS], and depleting T cells using antibody and complement as described previously (8). The T-depleted spleen cells were stimulated for 2–3 d with LPS and dextran sulfate (Sigma-Aldrich) as described previously (8). Bone marrow–derived DCs were prepared by isolating cells from bone marrow of B10.Br or BALB/c ByJ mice, and culturing at $4 \times 10^5$ cells/ml with 20 ng/ml GM-CSF (Preprotech) and 20 ng/ml IL-4 for 6–8 d, with 25 ng/ml LPS (Sigma-Aldrich) added for the last 18–24 h. The DCs were loaded with 5 μM pigeon cytochrome c peptide fragment (PCCF) or HNT peptide. B cells were treated with 100 μg/ml of mitomycin-c (Sigma-Aldrich) before use, whereas DCs that were used in vivo were not treated.

**Naive CD4 T cell isolation.** The enrichment of naive CD4 T cells from pooled spleen and lymph node lymphocytes was performed as described previously (8). In brief, lymphocytes were passed through nylon wool, treated with MHC class II and CD8 depletion antibodies followed by complement and DNase I (Sigma-Aldrich) treatment, and discontinuous Percoll (Sigma-Aldrich) gradient separation. Cells at the 63%/80% Percoll interface were used for T cell assays. The purified cell populations were >85% CD4$^+$ cells, 90–95% of which had a naive phenotype (CD45RB$^{hi}$, CD62L$^{hi}$, CD44$^{low}$, CD25$^{low}$).

**In vitro culture of CD4 T cells and memory generation.** Naive TCR Tg CD4 T cells ($3 \times 10^5$/ml) were labeled with CFSE (Molecular Probes Inc.) as previously described (8), and cultured with T-depleted splenic APC blasts ($3 \times 10^5$/ml) pulsed with 5 μM peptide Ag (AND TCR Tg T cells were stimulated with B10.Br APC + PCCF peptide; HNT TCR Tg T cells were stimulated with BALB/c APC + HNT peptide; and OT-II TCR Tg T cells were stimulated with C57BL/6 APC + OVA peptide). All peptides were purchased from New England Peptide. Th1 effectors were generated by culture with IL-2, IL-12, and anti–IL-4, and Th2 effectors were generated by culture with IL-2, IL-4, and anti-IFNγ as described previously (8).

Acutely stimulated CD4 effectors (Th1 and Th2) were generated in vitro by adding peptide-pulsed APC at day 0, followed by the addition of fresh nonpulsed APCs every 24 h. Repeatedly stimulated CD4 effectors (Th1RS and Th2RS) were generated by adding peptide-pulsed APCs at day 0, followed by addition of fresh peptide-pulsed APCs every 24 h. Acutely stimulated polyclonal effectors were generated by culturing naive polyclonal BALB/c ByJ CD4 T cells for 48 h on plate-bound anti-CD3 (10 μg/ml) with soluble anti-CD28 (5 μg/ml), and then removing the T cells without washing to uncoated flasks for the last 48 h of culture (8). Repeatedly stimulated polyclonal effectors were generated by culturing on the plate-bound anti-CD3 for the entire 96 h of culture.

$10 \times 10^6$ 4-d effector T cells were transferred i.v. into AT×BM mice as previously described (9), and allowed to return to a resting state for 21 d. 25-d memory T cells were harvested from spleens and peripheral lymph nodes, enumerated, and assayed by ELISA for cytokine production.

**Analysis of cytokine production.** T cells ($5 \times 10^5$/ml) were restimulated for 24 h with 10 μg/ml plate-bound anti-CD3, unless indicated otherwise, and culture supernatants were analyzed by ELISA (8). Alternatively, T cells ($5 \times 10^5$/ml) were restimulated for 24 h with T-depleted, spleen-derived APC blasts ($5 \times 10^5$/ml) that were pulsed with peptide Ag (10 μg/ml), followed by ELISA or ICCS as described previously (8).

**Flow cytometry.** Lymphocytes were stained with saturating concentrations of fluorochrome-labeled mAbs as described previously (8). All surface-staining reagents were purchased from BD Biosciences. PE-labeled anti-Vβ3 (AND TCR Tg), anti-Vβ8.3 (HNT TCR Tg), anti-Vβ5 (OT-II TCR Tg), allophycocyanin or peridium chlorophyll protein–labeled anti-CD4 and peridium chlorophyll protein–labeled anti-Thy1.1 were used to identify donor CD4 T cells in all FACS analysis. Flow cytometry was performed using FACScalibur flow cytometers (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star, Inc.).

**Restimulation of T cells with common γ-chain–binding cytokines.** 4-d effector T cells were washed extensively, labeled with CFSE, and cultured at $2 \times 10^5$/ml with the indicated concentration of common γ-chain binding cytokines (Preprotech).

**Virus infections.** A/PR/8/34 (PR8) strain of influenza A virus was grown in the allantoic fluid of 10-d-old embryonated chicken eggs, from an aliquot that originally was obtained from D. Morgan (The Scripps Research Institute, La Jolla, CA). Mice were inoculated intranasally during light isoflurane anesthesia with $50 \times 10^3$ PFU/ml ($2LD_{50}$) of virus in 100 μl PBS. $5 \times 10^6$, $2.5 \times 10^6$, or $0.5 \times 10^6$ 4-d effectors that were generated in vitro from Thy1.1 HNT TCR Tg CD4 T cells, or 200 μl of PBS alone, were transferred i.v. into infected mice. Donor and host T cells were isolated for analysis 4 d following transfer from draining lymph nodes (parathymic and mediastinal), spleens, and perfused lungs as previously described (3).

**Analysis of B cell help.** C57BL/6 × CD4 KO mice were immunized by i.p. injection of 200 μg 4-hydroxy-3-nitrophenyl acetyl (Sigma-Aldrich) conjugated OVA (NP-OVA) or PBS in 100 μl alum (Sigma-Aldrich). $10^7$, $10^6$, or $10^5$ 4-d effectors that were generated in vitro from OT-II TCR Tg CD4 T cells, or 200 μl of PBS alone, were transferred i.v. into immunized mice. Host B cells were isolated from spleens 16 d following transfer, and serum was harvested from blood. The total number of host NP-specific, germinal center phenotype (CD38$^{lo}$/PNA$^{hi}$) B cells were calculated by as-

sessing the percentage of NP-APC$^{+}$/CD38-PE$^{lo}$/PNA-FITC$^{hi}$ host B cells, multiplied by the total number of live lymphocytes that were recovered from the spleen. Serum samples were analyzed by ELISA for the presence of NP-specific IgG1.

**In vivo effector generation.** $10^6$ naive AND TCR Tg × GFP CD4 T cells were mixed with $2 \times 10^5$ PCCF peptide-pulsed DCs and transferred i.v. into intact B10.Br mice. Subsequently, $2 \times 10^5$ DCs without PCCF peptide (acute stimulation) or $2 \times 10^5$ PCCF peptide-pulsed DCs (RS) were transferred on days 1–4. Alternatively, $2 \times 10^6$ naive HNT TCR Tg CD4 T cells were mixed with $2 \times 10^6$ HNT peptide-pulsed DCs and transferred i.v. into intact B10.Br mice. Subsequently, $2 \times 10^6$ DCs without HNT peptide (acute stimulation) or $2 \times 10^6$ HNT peptide-pulsed DCs (RS) were transferred on days 1–4. The numbers of naive CD4 T cells and peptide pulsed DCs were titrated to ensure optimal CD4 effector responses under conditions of acute stimulation. The in vivo generated effectors were harvested 5 d following transfer.

This work was supported by National Institutes of Health grants (AI26887, AI4650, and HL3925 to S.L. Swain and AG21054 to L. Haynes) and by the Trudeau Institute.

The authors have no conflicting financial interests.

**Submitted: 7 September 2004**
**Accepted: 1 February 2005**

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