

The Biological Outcome of CD40 Signaling Is Dependent on the Duration of CD40 Ligand Expression: Reciprocal Regulation by Interleukin (IL)-4 and IL-12

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Abstract

CD40 ligand (CD154) expression on activated T cells can be separated into an early TCR-dependent phase, which occurs between 0 and 24 h after activation, and a later extended phase, which occurs after 24 h and is reciprocally regulated by the cytokines IL-4 and IL-12. IL-4 represses, whereas IL-12 sustains CD154 expression. Consistent with this, Th1, but not Th2, cells express CD154 for extended periods. Differences in the duration of CD154 expression have important biological consequences because sustained, but not transient, expression of CD154 on activated T cells can prevent B cell terminal differentiation. Thus, the differential ability of Th cells to sustain CD154 expression is an important part of their helper function and should influence the activities of other CD40-expressing cell types.

Key words: CD154 • B lymphocyte • T helper lymphocyte • interferon- γ

Introduction

CD40 is constitutively expressed on B cells and other APCs (1) and is essential for the success of both cellular and humoral immune responses (2–4). In humoral immune responses, CD40 signaling induces B cell activation and facilitates T-dependent B cell proliferation (5–7), germinal center formation (3, 8, 9), and immunoglobulin isotype-switching (10–12). Several reports also suggest that CD40 signaling directly induces B cell terminal differentiation and antibody secretion (13–15), however, we and others showed that CD40 signaling actively prevents B cell terminal differentiation and antibody secretion (16–20). Interestingly, the major differences between the studies showing that CD40 signaling promotes antibody secretion and those showing that CD40 signaling inhibits antibody secretion are in the reagents used to trigger CD40 and the duration of the CD40 signal. This indicates that there are both qualitative and quantitative aspects of CD40 engagement that can dictate the biological outcome of CD40 signals in B lymphocytes. Since CD40 signaling *in vivo* is controlled by the availability of its ligand, CD154, the factors that regulate the level and duration of CD154 expression on the surface of T helper cells should therefore influence B cell differentiation and antibody secretion.

The initial studies of CD154 expression were performed with T cell clones (21–24) and suggested that CD154 expression is rapidly, but transiently, induced after T cell activation (21–23). One explanation for the transient nature of CD154 expression on APC-activated T cells is that CD154 is internalized upon encounter with CD40-expressing APCs (25). Consistent with the model of endosomal recycling, some memory T cells have preformed CD154 that can be rapidly expressed on the cell surface after TCR activation (26). Thus, T cells seem to have several mechanisms to ensure the rapid, yet transient, expression of CD154 on the cell surface. Although some cytokines, such as IFN- γ and TGF β , appear to reduce CD154 expression on some T cell clones (23), cytokines in general are not thought to regulate CD154 expression. Furthermore, because CD154 is rapidly expressed by both Th1 and Th2 cell lines within hours of stimulation (22, 23), differential CD154 expression is not thought to be a feature of Th cell polarization. Subsequent studies demonstrated that the rapid induction of CD154 expression on naive T cells occurs primarily through TCR stimulation, and although interactions between B7 and CD28 appear to augment CD154 expression (27–29), these interactions act by facilitating a more avid TCR engagement rather than directly inducing CD154 expression (30, 31).

In contrast to the prevailing opinion that CD154 expression is only transiently expressed for a few hours after T cell activation, there are scattered reports indicating that ac-

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tivated T cells can express CD154 for as long as 4 d (27, 29, 32). For example, the cytokines IL-2 and IL-15 appear to reinduce the expression of CD154 on effector T cells in the absence of TCR signaling (33). Additionally, IL-12 is reported to sustain CD154 expression on anti-CD3-activated T cells for up to 3 d (32). Furthermore, there are several reports of extended CD154 expression on T cells activated with anti-CD3, with or without anti-CD28 costimulation (27, 28, 30, 34, 35). Unfortunately, these studies are difficult to reconcile with those suggesting that CD154 is only transiently expressed on activated T cells, due to differences in the source of the responding T cells, methods of stimulation, and the times at which CD154 expression was examined. Since the kinetics and duration of CD154 expression likely control many APC effector functions, it is critical to better define the mechanisms that control expression of CD154 on antigen-activated T cells. Therefore, we set out to methodically determine the factors that control extended CD154 expression on activated T cells and to test whether sustained expression of CD154 can influence the antibody-secreting effector function of activated B cells.

Materials and Methods

Animals. B10.BR as well as AND TCR transgenic mice (36) on the B10.BR, C57BL/6, or C57BL/6-IL-4KO backgrounds were bred in the Trudeau Institute Animal Breeding Facility. B10.A mice were purchased from Jackson ImmunoResearch Laboratories. All procedures involving animals were approved by the Trudeau Institute Institutional Animal Care and Use Committee and were conducted according to the principles outlined by the National Research Council.

Cell Isolation. CD4⁺ T cells were purified from AND mice by positive selection. In brief, single cell suspensions were obtained from the spleens and LN of 4–6-wk-old mice and were incubated with FcR-blocking antibody (2.4G2) at 1 µg/10⁷ cells for 15 min on ice. Anti-CD4 magnetic beads (Miltenyi Biotec) were added at 25 µl/10⁸ total cells in 100 µl final volume for an additional 15 min on ice. Cells were washed, filtered, and passed over a type LS+ magnetic column (Miltenyi Biotec). After washing, the magnetically bound cells were eluted from the column with the supplied plunger. This procedure routinely resulted in 95–98% pure CD4⁺ T cells, of which ~90% expressed the TCR transgene. B cells were positively selected with anti-B220 beads using similar methods. The B cell lymphoma cell line, CH12, was maintained by weekly passage in B10.A mice as described (20, 37). Briefly, 10⁶ CH12 cells were injected into the peritoneal cavity of B10.A mice and ~5 × 10⁸ CH12 cells were recovered 7 d later by peritoneal lavage. The purity of the recovered cells was typically >95% and the cells were used without any additional purification.

(4-hydroxy-3-nitrophenyl)acetyl (NP)*-specific B cells were obtained from the spleens of B10.BR mice immunized 7 d previously with a combination of 50 µg NP-ovalbumin and 50 µg NP-pigeon cytochrome *c* adsorbed to alum. Splenocytes were stained with biotinylated anti-λ, NP-allophycocyanin, and anti-CD138. λ-Expressing cells were positively selected using strepta-

vidin-coated magnetic beads (25 µl/10⁸ total cells in 100 µl final volume) to bind cells to a type C magnetic column (Miltenyi Biotec). The selected cells were sorted for NP binding and the absence of CD138. The resulting cells were >95% pure.

Generation of Polarized Effector or Memory T Cells. AND CD4⁺ effector cells were generated as previously described (38). In brief, purified CD4⁺ T cells from AND TCR Tg mice were cultured at 2 × 10⁵/ml with 5 µM PCCF and mitomycin C-treated DCEK-ICAM cells in the presence of polarizing cytokines and antibodies for 4 d. Th1 polarizing conditions included 100 ng/ml IL-2, 2 ng/ml IL-12, and 10 µg/ml anti-IL-4 (11B11). Th2 polarizing conditions included 100 ng/ml IL-2, 15 ng/ml IL-4, and 10 µg/ml anti-IFN-γ (XMG1.2). Polarized effector cells were used after 4 d of culture. To generate memory T cells, day 4 effectors were adoptively transferred into B10.BR mice that had been previously thymectomized, irradiated, and reconstituted with 2 × 10⁶ syngeneic T-depleted bone marrow (38, 39). Memory CD4⁺ T cells were recovered 5–10 wk after transfer.

Cell Culture and T and B Cell Activation. Cells were cultured in RPMI 1640 supplemented with vitamins, nonessential amino acid, glucose, sodium pyruvate, HEPES, penicillin, streptomycin, glutamine, and 10% FBS from GIBCO BRL. T cells were cultured in 48-well plates at 10⁶ cells/ml and were stimulated using plate bound anti-CD3ε at 10 µg/ml. In other experiments, AND T cells (10⁶ cells/ml) were cocultured with CH12 cells (10⁵ cells/ml) in 96-well round-bottom plates with combinations of cytokines and antibodies as indicated in the figures and figure legends. In some experiments, activated B cells were generated by stimulating purified splenic B cells with rabbit Fab'2 anti-IgM at 10 µg/ml and the supernatant of BAFF-transfected L cells.

Cytokines, Mitogens, and Antibodies Used in Culture. Purified, endotoxin-free CD154-blocking antibody, MR-1 (40), was obtained from Bio Express. IL-4-blocking antibody (11B11) and IFN-γ-blocking antibody (XMG1.2) were purified from culture supernatant. Recombinant IL-2, IL-4, and IL-10 were provided by Dr. Frances Lund, Trudeau Institute, Saranac Lake, NY. IFN-γ was obtained from culture supernatant of X63.Ag8-653 cells transfected with IFN-γ cDNA. IL-12 was provided by Dr. Stanley Wolf, Genetics Institute, Cambridge, MA. Other reagents purchased were as follows: LPS *Escherichia coli* O55:B5 (Difco), IL-5 (Sigma-Aldrich), IL-6 (Genzyme), and IL-13 (BD Biosciences).

Analysis of CD154 Expression. CD154 expression was assayed by staining cultured cells with biotinylated anti-CD154 (MR-1) followed by APC-streptavidin. Control staining for CD154 was achieved by adding excess unlabeled MR-1 to the standard staining procedure. Anti-Thy-1 was used to distinguish T cells from CH12 cells in cultures containing both cell types. All staining procedures were performed in the presence of FcR-blocking antibody (2.4G2). Flow cytometry was performed using a dual laser FACSCalibur™ (Becton Dickinson). The data were analyzed with FlowJo software (TreeStar).

Assay for Antibody Secretion. Cells were harvested from cultures containing both T cells and CH12 cells at 48 or 72 h and washed twice in PBS. Cells were stained with anti-Thy-1 to determine the relative numbers of T and B cells and equivalent numbers of B cells were recultured with fresh media and allowed to secrete antibody for 6 h without further stimulation. Supernatants were harvested and assayed for IgM secretion by ELISA. In brief, plates were coated with goat anti-κ, and IgM in culture supernatants was detected with HRP-labeled goat anti-mouse IgM. The purified mAb TEPC 183 was used as a standard. NP-specific

*Abbreviations used in this paper: NP, (4-hydroxy-3-nitrophenyl)acetyl; PCC, pigeon cytochrome *c*; PCCF, PCC fragment.

antibody was assayed by ELISA by coating plates with NP-BSA and developing with HRP conjugated anti-IgG.

Cytokine Detection Assays. IL-2 was quantitated by measuring the proliferation of the IL-2-dependent cell line, NK3, in the presence of the IL-4-blocking antibody 11B11 relative to a recombinant IL-2 standard. IL-4 and IFN- γ were quantitated by ELISA relative to recombinant IL-4 and IFN- γ standards.

Results

Two Phases of CD154 Expression on Activated Naive T Cells. To determine the kinetics of CD154 expression after T cell activation, purified naive CD4⁺ T cells from AND TCR transgenic mice were stimulated with plate-bound anti-CD3 in the absence of APCs. As shown in Fig. 1 A, CD154 expression was rapidly induced on a portion of the AND T cells after anti-CD3 stimulation. Surprisingly, high levels of CD154 were still observed on the majority of T cells between 24 and 72 h after stimulation. To determine whether CD154 was expressed with similar kinetics in a more physiological model of T cell activation, we cultured purified naive AND T cells, which recognize pigeon cytochrome *c* peptide 88–104 (PCCF) on I-E^k, with the I-E^k-expressing B cell lymphoma, CH12, in the presence or absence of PCCF. As seen in Fig. 1 B, a cognate interaction between AND T cells and PCCF-presenting CH12 cells resulted in expression of CD154 on about a third of the T cells as early as 6 h after activation. In contrast to our results using anti-CD3-activated AND T cells, we found that at 24 h, CD154 was almost undetectable on the surface of AND T cells stimulated in a cognate interaction. However, by 48 h, a second wave of CD154 expression was observed on essentially all cells, which then declined by 72 h after stimulation. In experiments not shown, we demonstrated that the temporary drop in CD154 expression at 24 h on T cells stimulated in a cognate interaction is due to the internalization of CD154 upon encounter

with CD40 on the APC. This is consistent with previous results (25).

Cytokines Control the Second Phase of CD154 Expression on Stimulated Naive Cells. To determine whether cytokines played a role in regulating the first or second phases of CD154 expression on activated T cells, purified naive AND T cells were stimulated with CH12 cells and PCCF in the presence of a variety of cytokines, and the kinetics of CD154 expression were followed over 72 h. We found that IL-2, IL-5, IL-6, IL-10, IL-13, and IFN- γ had no effect on either the levels or kinetics of CD154 expression (unpublished data). However, both IL-4 and IL-12 influenced the second phase of CD154 in the model of cognate T-B interaction (Fig. 2 A). Although IL-12 had minimal effect on the level of CD154 expression on peptide-stimulated T cells at 6 h, IL-4 slightly, but consistently, increased the percentage of cells expressing CD154 at this time (Fig. 2 A). Similar to what we observed in Fig. 1 B, there was a dramatic decrease in CD154 expression on T cells stimulated with peptide-presenting CH12 cells for 24 h, regardless of cytokine addition (Fig. 2 A). In striking contrast, however, the second phase of CD154 expression at 48 and 72 h was completely absent on T cells cultured with IL-4, whereas the second phase of CD154 expression remained elevated for extended periods on T cells cultured with IL-12 (Fig. 2 A). Furthermore, the inhibitory effects of IL-4 were dominant over the effects of IL-12 as T cells stimulated in the presence of both cytokines expressed CD154 in a pattern identical to that on T cells stimulated in the presence of IL-4 alone (Fig. 2 A). Similar results were seen using OTII transgenic T cells and with normal T cells (not depicted).

To demonstrate that the cytokines were acting directly on T cells to influence CD154 expression, rather than on CH12 cells, purified T cells were stimulated with plate-bound anti-CD3 (without APCs) in the presence of either IL-4 or IL-12. As shown in Fig. 2 B, the expression of CD154 at 6 h on T cells stimulated through CD3 was somewhat decreased by the addition of IL-4 or IL-12, although in most experiments there was little change. However, the addition of IL-4 resulted in a strong reduction of CD154 surface expression at later times in anti-CD3 stimulated cultures (Fig. 2 B). In contrast, the addition of IL-12 resulted in a more robust and prolonged expression of CD154 at later points after anti-CD3 stimulation (Fig. 2 B). Therefore, we concluded that IL-4 and IL-12 were acting directly on the T cells to influence CD154 expression.

Because the surface expression of CD154 can be regulated by multiple mechanisms, we examined CD154 mRNA expression in T cells that had been stimulated with plate-bound anti-CD3 with or without IL-4 or IL-12. Surprisingly, we observed the highest levels of CD154 mRNA expression in naive, unstimulated AND T cells (Fig. 2 C), even though we could not detect any CD154 protein on the surface of these cells (not depicted). After anti-CD3 stimulation, CD154 mRNA expression in AND T cells began to decrease within 6 h and was reduced to near background levels by 24 h. CD154 expres-

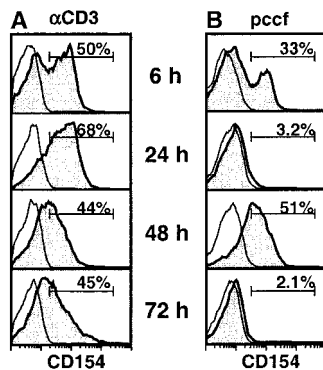


Figure 1. CD154 expression can be sustained for up to 72 h after T cell activation. (A) Purified naive AND T cells were stimulated with plate-bound anti-CD3. Cells were removed from culture at the times indicated and CD154 expression was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (B) Purified naive AND T cells were cultured with peptide bearing CH12 cells. Cells were removed from culture

at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. Control staining for CD154 was performed by adding excess unlabeled MR-1 before the standard staining procedure. The numbers in each panel represent the percentage of cells staining more brightly than controls. The data shown are representative of more than three independent experiments.

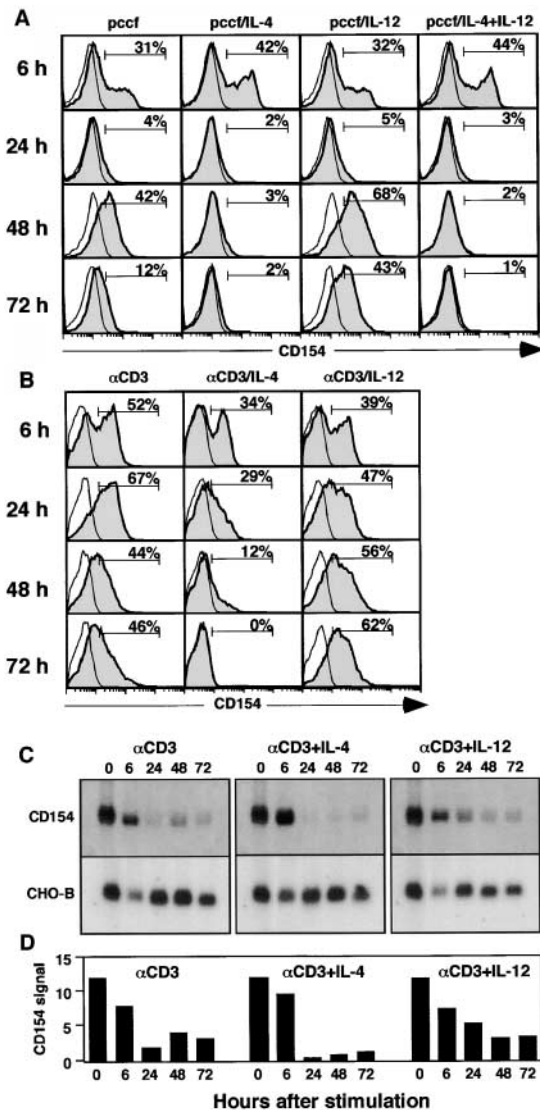


Figure 2. Cytokines control the second phase of CD154 expression. (A) Purified naive AND T cells were stimulated with peptide-bearing CH12 cells in the presence of the indicated cytokines. Cells were removed from culture at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (B) Purified naive AND T cells were cultured with plate-bound anti-CD3 in the presence of the indicated cytokines. Cells were removed from culture at the times indicated and CD154 expression was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (C) RNA was purified from AND T cells that had been stimulated by plate-bound anti-CD3 alone or with IL-4 or IL-12. 5 μg of purified total RNA was separated on a Northern blot, which was probed with the cDNA for CD154 and with the cDNA for a mitochondrial enzyme, CHO-B, as a loading control. (D) The amount of CD154 probe bound to the Northern blot was quantitated using a BioRad Molecular Imager FX PhosphorImager. The quantity shown represents the relative signal within the specific band minus the signal in an equivalent area adjacent to the specific band.

sion then increased slightly at 48 and 72 h after stimulation. The addition of IL-4 resulted in the maintenance of high levels of CD154 mRNA at 6 h, even though CD154 mRNA expression was dramatically lower at later times,

particularly at 24 and 48 h (Fig. 2 C). In contrast, the addition of IL-12 maintained CD154 mRNA expression throughout the course of the experiment (Fig. 2 C). These data are expressed quantitatively in Fig. 2 D. Together, these results demonstrate that although the late phase of CD154 surface expression is negatively regulated by IL-4, this regulation is only partly mediated by changes in CD154 mRNA expression.

The Second Phase of CD154 Expression Is Differentially Regulated on Polarized T Cell Subsets. Because IL-4 and IL-12 are the predominant cytokines that determine whether naive T cells develop into Th1 or Th2 effector cell types (39, 41), we examined whether T cells that had fully differentiated into Th1 or Th2 effector cells differed in their kinetics of CD154 expression after reactivation. We initially examined the expression of CD154 after the restimulation of day 4 Th1 and Th2 effector cells in a cognate interaction with peptide-bearing CH12 cells. Although CD154 expression was observed on both Th1 and Th2 effectors 6 h after restimulation, significantly more of the Th1 cells (40%) compared with the Th2 cells (11%) expressed CD154. As observed on stimulated naive T cells, CD154 expression declined by 24 h after stimulation (not depicted), however, it was impossible to examine CD154 expression at times later than 24 h because most of the restimulated effector T cells had already undergone activation-induced cell death (not depicted). Therefore, we next examined the expression of CD154 on restimulated polarized memory T cells, which are not susceptible to activation induced cell death.

Memory Th1 and Th2 AND T cells were generated by “parking” day 4 polarized effectors in irradiated, thymectomized recipients (38). After 6 wk, the resulting resting memory Th1 and Th2 cells were purified from recipient mice and were restimulated with CH12 cells presenting PCCF. Unstimulated Th1 and Th2 memory cells did not express CD154 on their surface (not depicted). However, as shown in Fig. 3 A, restimulated Th1 memory cells expressed high levels of CD154 at 6 h, and although CD154 expression declined at later times, it was maintained at significant levels for longer than 72 h. Interestingly, the temporary drop in CD154 expression that typically occurs at 24 h after the stimulation of naive AND T cells with CH12 cells and PCCF was not observed with restimulated Th1 memory cells (Fig. 3 A). In contrast to the sustained expression of CD154 after stimulation of Th1 memory cells, restimulated Th2 memory cells expressed lower levels of CD154 at 6 h and expressed barely detectable levels of CD154 at 24, 48, and 72 h (Fig. 3 A). The differences in the kinetics of CD154 expression on restimulated Th1 and Th2 memory cells are summarized graphically in Fig. 3 B. Overall, the differential expression of CD154 on polarized effector and memory Th cells is consistent with the changes in CD154 expression caused by IL-4 and IL-12.

The Differential Expression of CD154 on Polarized T Cell Subsets Is Regulated by the Cytokines Produced by the T Cells. There were two possible explanations for the differences in CD154 expression in Th1 and Th2 memory

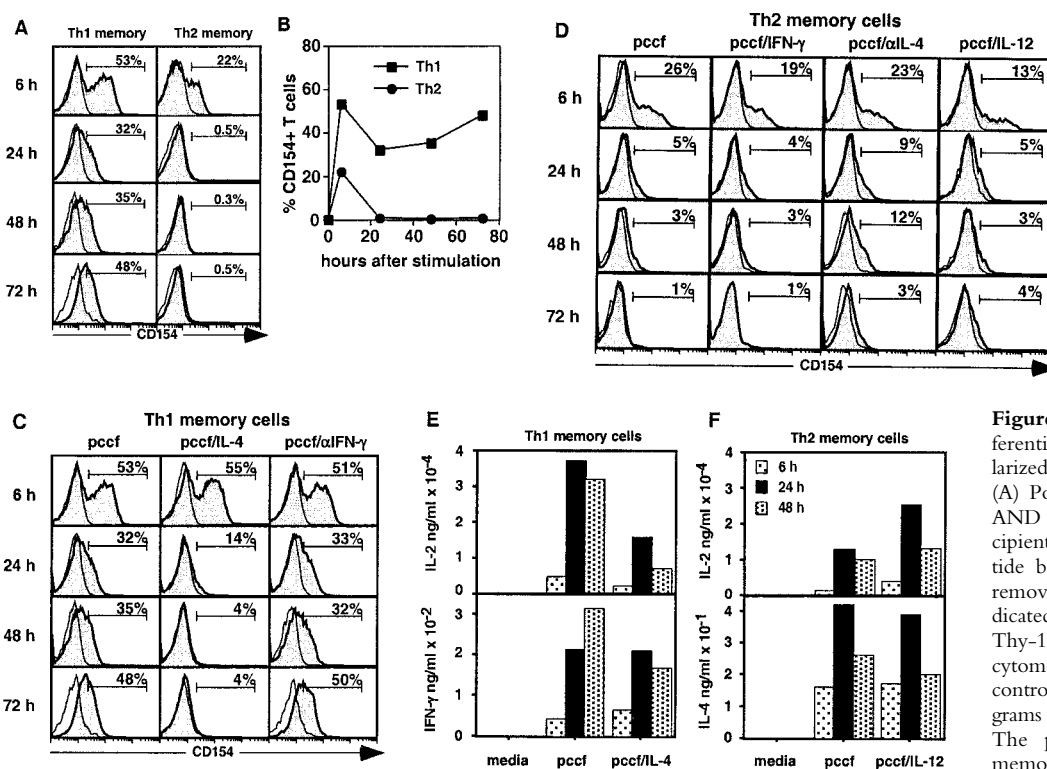


Figure 3. CD154 expression is differentially regulated on activated polarized Th1 and Th2 memory T cells. (A) Polarized Th1 and Th2 memory AND T cells were purified from recipient mice and stimulated with peptide bearing CH12 cells. Cells were removed from culture at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (B) The percentage of Th1 and Th2 memory AND T cells that expressed CD154 at the indicated times in A are

shown graphically. (C) Polarized Th1 memory AND T cells were purified from recipient mice and stimulated with peptide bearing CH12 cells in the presence of media alone, IL-4, or anti-IFN- γ . Cells were removed from culture at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (D) Polarized Th2 memory AND T cells were purified from recipient mice and stimulated with peptide bearing CH12 cells in the presence of media alone, IFN- γ , IL-12, or anti-IL-4. Cells were removed from culture at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (E) Supernatants from the experiment in C were collected at 6, 24, and 48 h and assayed for IL-2 and IFN- γ . (F) Supernatants from the experiment in D were collected at 6, 24, and 48 h and assayed for IL-2 and IL-4. Data shown are representative of multiple independent experiments.

cells. First, it was possible that CD154 expression is intrinsically polarized in Th1 and Th2 cells, much like the expression of IFN- γ and IL-4. Alternatively, differences in the pattern of CD154 expression observed in Th1 and Th2 cells could be a secondary effect of the polarized production of one or more cytokines, such as IL-4, that in turn regulate CD154 expression. To distinguish between these possibilities, Th1 memory cells were restimulated with CH12 cells and PCCF in the presence of either IL-4 or anti-IFN- γ . As shown in Fig. 3 C, the addition of IL-4 dramatically reduced the surface expression of CD154 on restimulated Th1 memory cells at 24, 48 and 72 h. In contrast, the addition of the IFN- γ -blocking antibody had no effect on either the level of CD154 or the percentage of CD154 positive T cells at any time (Fig. 3 C). The converse experiment was also performed, in which Th2 memory cells were restimulated with CH12 cells and PCCF in the presence of IFN- γ , IL-12 or anti-IL-4, as shown in Fig. 3 D. The addition of either IFN- γ or IL-12 did not restore CD154 expression at later times, suggesting that the expression of IL-4 by Th2 memory cells, but not in Th1 memory cells, was primarily responsible for the difference in expression of CD154 on these cell types. Consistent with this, the addition of IL-4-blocking antibody to cul-

tures of stimulated Th2 memory cells partially restored the second phase of CD154 expression, particularly at 48 h (Fig. 3 D).

Given that the polarized expression of cytokines by Th1 and Th2 cells is thought to be stabilized by permanent changes in the chromatin structure of cytokine genes (41), it was surprising that IL-4 could reverse the extended expression of CD154 on memory Th1 cells. To ensure that the Th1 and Th2 memory cells were indeed activated and remained polarized, we measured the production of IL-2 and IFN- γ by Th1 cells that had been stimulated with or without IL-4, and we measured the production of IL-2 and IL-4 by Th2 cells that had been stimulated with or without IL-12. Although the addition of IL-4 completely blocked the expression of CD154 at later times on stimulated Th1 memory cells (Fig. 3 C), these cells still made significant amounts of IFN- γ (Fig. 3 E). Furthermore, Th1 memory cells stimulated in the presence of IL-4 made levels of IFN- γ similar to those made by Th1 memory cells stimulated in the absence of IL-4 (Fig. 3 E). However, the addition of IL-4 did consistently reduce the amount of IL-2 produced by stimulated Th1 memory cells at 6, 24, and 48 h (Fig. 3 E). In the converse experiment, peptide-stimulated Th2 memory cells produced nearly identical levels of IL-4 and

IL-2 as those stimulated in the presence of IL-12 (Fig. 3 F). Thus, despite the ability of IL-4 to profoundly alter CD154 expression on activated Th1 memory cells, both Th1 and Th2 memory cells remained polarized when activated in the presence of opposing cytokines.

To more convincingly demonstrate that IL-4 was directly responsible for the lowered expression of CD154 at later times on activated Th2 memory cells, Th2 memory cells were generated from AND-IL-4KO mice and the kinetics of CD154 expression on these cells was compared with the kinetics of CD154 expression on WT AND Th2 memory cells after activation. When restimulated with anti-CD3, the IL-4KO Th2 memory cells expressed levels of CD154 similar to WT Th2 memory cells at 6 h (Fig. 4, A and B). However, although the expression of CD154 rapidly declined on WT Th2 memory cells after 6 h, the expression of CD154 on activated IL-4KO Th2 memory cells remained consistently higher (Fig. 4 A) and was sustained longer (Fig. 4 B). Although the inability to make IL-4 clearly influenced CD154 expression, the typical cytokines made by restimulated Th2 memory cells, such as IL-5 and IL-6, were produced in similar quantities from both AND-WT as well as AND-IL-4KO (not depicted) demonstrating that the cells were still Th2 cells. These data are consistent with those in Fig. 3 and suggest that IL-4 is the predominant cytokine controlling the second phase of CD154 expression on activated T cells.

Sustained CD154 Expression on Activated T Cells Can Inhibit Antibody Production by CH12 Cells. In previous (20) studies, we had observed that B cell terminal differentiation and antibody secretion were specifically blocked by the interaction of B cells with cells that constitutively expressed CD154 (20). Given these results, we hypothesized that the sustained CD154 expression observed on T cells stimulated in the presence of IL-12 could prevent antibody secretion by B cells in a model of cognate T-B interaction. To test this, the ability of CH12 cells to differentiate into antibody-secreting cells was examined when they were used as

APCs to present PCCF to naive AND T cells in the presence of IL-4 or IL-12. Since naive T cells do not initially produce enough cytokines to induce CH12 cell differentiation, LPS was added to the cultures to trigger the differentiation of CH12 cells into IgM-secreting cells. After 72 h of culture, equivalent numbers of B cells were washed and recultured for an additional 6 h. Supernatants from the 6-h culture were collected to determine the amount of antibody secreted by the B cells.

As seen in Fig. 5 A, LPS-stimulated CH12 cells secreted antibody in the absence of T cells (Fig. 5 A, white bars) regardless of the addition of peptide, cytokines or antibodies. Furthermore, the addition of T cells had no effect on antibody secretion in the absence of peptide (not depicted). However, consistent with our previous report (20), we found that a cognate encounter between naive T cells and peptide-bearing CH12 cells could inhibit antibody secretion at 72 h (Fig. 5 A, PCCF black bar). This inhibition was demonstrated to be CD154 dependent, as the addition of the CD154-blocking antibody, MR-1, reversed the effect (Fig. 5 A, PCCF/MR-1 black bar). The differentiation of LPS-activated CH12 cells was similarly inhibited when the duration of CD154 expression on T cells was augmented with IL-12 (Fig. 5 A, PCCF/IL-12 black bar). Again, the block in antibody secretion was shown to be CD154 dependent using MR-1 (Fig. 5 A, PCCF/IL-12/MR-1 black bar). However, antibody secretion was not inhibited when IL-4 was included in the cultures (Fig. 5 A, PCCF/IL-4 black bar), and was unaltered on addition of MR-1 (Fig. 5 A, PCCF/IL-4/MR-1 black bar). Because IL-4 does not induce differentiation of CH12 cells by itself (20, 37) and cannot overcome the CD40-mediated block of antibody secretion even in combination with LPS (20), IL-4 must allow antibody secretion in this assay by inhibiting the sustained expression of CD154 on the activated T cells.

MR-1 is often used to block T cell activation in vivo (42, 43), presumably by blocking APC activation. However, because CH12 cells are constitutively activated and express high levels of costimulatory molecules, MR-1 does not block T cell activation in our experiments. This is demonstrated in Fig. 5 B, in which AND T cells that had been stimulated for 48 h under the conditions of the previous experiment expressed similarly enhanced levels of the activation markers CD25, CD44, and CD69 relative to their resting counterparts. Furthermore, these T cells produced similar levels of IL-2 at this time under all conditions tested (not depicted). Therefore, naive AND T cells stimulated with peptide-presenting CH12 cells become equivalently activated, regardless of cytokine or MR-1 addition. Thus, the primary effect on antibody secretion must be mediated through changes in the duration of CD154 expression and not by differential T cell activation.

These results predicted that the ability to inhibit antibody secretion through sustained CD154 expression would be a property of Th1 memory cells, but not Th2 memory cells. Therefore, cultures were set up containing either Th1 or Th2 memory AND T cells and CH12 cells

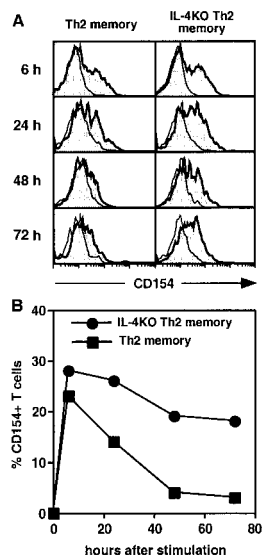


Figure 4. IL-4-deficient memory Th2 cells can express CD154 with extended kinetics. (A) Polarized Th2 memory AND T cells were generated from AND mice on the C57BL/6 background or from AND mice on the C57BL/6-IL-4KO background. Memory T cells were purified from recipients and were stimulated with plate-bound anti-CD3. Cells were removed from culture at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (B) The percentage of T cells staining more brightly than controls was calculated by electronic gating at each time. This experiment was performed twice with similar results.

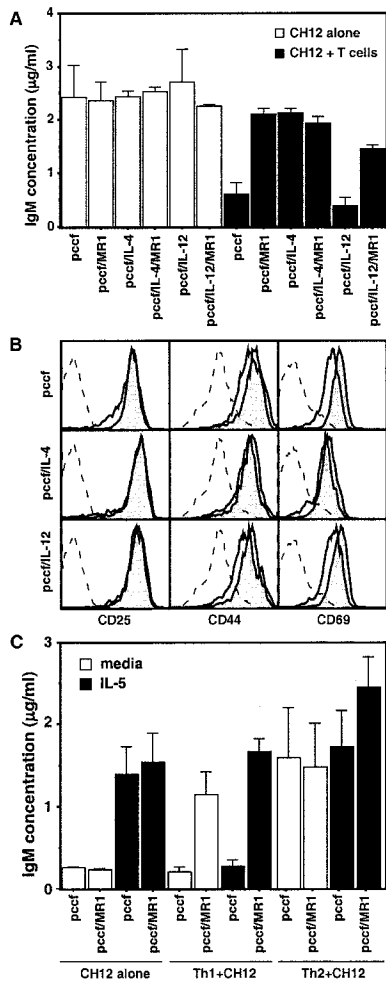


Figure 5. Sustained CD154 expression inhibits antibody secretion by CH12 cells during a cognate T-B interaction. (A) CH12 cells were cultured alone (white bars) or with naive AND T cells (black bars) in the presence of the indicated combinations of cytokines, MR-1 and PCCF. All cultures included LPS to induce the differentiation of CH12 cells. After 72 h, cells were removed from culture, counted, and an aliquot of cells was stained with anti-Thy-1 to determine relative B and T cell numbers. The remaining cells were washed in PBS and recultured at equivalent B cell concentrations in fresh media without any additional stimulation for another 6 h. Antibody secretion was measured in the 6-h supernatants by ELISA. The data shown are representative of three independent experiments. (B) In a parallel experiment, purified naive AND T cells were cultured with CH12 cells in the presence of the indicated combinations of PCCF, IL-4, and IL-12 for 48 h with MR-1 (open histograms) or without MR-1 (shaded histograms). Cells were stained with anti-Thy-1 to identify T cells and with anti-CD25, anti-CD44, or anti-CD69 to measure the levels of these activation markers. The data shown are gated on Thy-1⁺ cells. The levels of activation markers on unstimulated naive AND T cells are shown (dotted lines). (C) PCCF-bearing CH12 cells were cultured alone with AND Th1 memory cells or with AND Th2 memory cells in the presence or absence of MR-1 as indicated. To induce CH12 differentiation, IL-5 was included in some cultures (black bars), and excluded in others (white bars). After 48 h, cells were removed from culture, counted, and an aliquot of cells was stained with anti-Thy-1 to determine relative B and T cell numbers. The remaining cells were washed in PBS and recultured at equivalent B cell concentrations in fresh media for another 6 h. Antibody secretion was measured in the 6-h supernatants by ELISA. CD154 expression in this experiment was similar to that in Fig. 3. The data shown are representative of three independent experiments.

presenting PCCF, with or without exogenous IL-5 to trigger CH12 cell differentiation. After 48 h of culture, equivalent numbers of B cells were washed and recultured to measure antibody secretion. As predicted, activated Th1 memory cells inhibited CH12 cell differentiation and antibody secretion (Fig. 5 C, Th1 PCCF white bar), even when exogenous IL-5, a potent inducer of CH12 terminal differentiation, was added to the cultures (Fig. 5 C, Th1 PCCF black bar). Furthermore, this inhibition was CD154 dependent because the addition of MR-1 restored antibody secretion to levels seen from CH12 cells stimulated with IL-5, in the absence of T cells. However, activated Th2 memory cells were unable to inhibit antibody secretion (Fig. 5 C, Th2 PCCF white or black bars). The expression of CD154 was monitored in parallel cultures at 6, 24, 48, and 72 h and was found to be similar to that in Fig. 3, in which sustained CD154 expression was observed on Th1 memory cells and transient CD154 expression was observed on Th2 memory cells (not depicted). Therefore, the sustained expression of CD154 after the activation of either naive T cells or memory Th1 cells, but not memory Th2 cells, was sufficient to block B cell terminal differentiation and antibody secretion.

An alternative explanation for the lack of IgM secretion from CH12 cells cultured with Th1 cells was that the T cells were inducing isotype switching in CH12 cells. However, we obtained similar results when we performed ELISAs specific for κ light chain rather than IgM, suggesting that isotypes other than IgM do not make up any significant fraction of the secreted antibody in these experiments (not depicted). Furthermore, we have found little evidence for isotype switching in the CH12 cell line that we are using under any conditions (unpublished data).

Sustained CD154 Expression on Activated T Cells Can Inhibit the Terminal Differentiation of Normal Activated B Cells. Although CH12 is a useful cell line that can be easily manipulated to dissect the molecular events that occur in the late stages of B cell differentiation (37, 44), it does not necessarily reflect the activity of normal B cells. Therefore, we wished to confirm our observations using activated normal B cells to present antigen to AND T cells. Because resting B cells are not efficient APCs, we activated purified splenic B cells with anti- μ and the cytokine BAFF for 24 h. We used anti- μ as a surrogate antigen to stimulate B cell activation and BAFF as a non-T cell-derived survival/proliferation factor for B cells (45). This combination was sufficient to induce the expression of both B7.1 and B7.2 on B cells and to make them efficient APCs (not depicted). These activated B cells were then cultured for 72 h with naive AND T cells in the presence of LPS or the combination of IL-2, IL-5, and IL-10 to induce antibody secretion, PCCF to induce T cell activation, and either IL-4 or IL-12 to modulate CD154 expression. The CD154-blocking antibody, MR-1, was added to parallel cultures to block CD154-CD40 interactions. As shown in Fig. 6 A, the AND T cells expressed CD154 within 6 h regardless of IL-4 or IL-12 addition. However, CD154 expression was suppressed in the IL-4-stimulated cultures at 24, 48, and

72 h, whereas CD154 expression was observed at these times in the IL-12-stimulated cultures.

To determine whether the modulation of CD154 expression on T cells by IL-4 or IL-12 controlled the ability of activated B cells to differentiate into antibody-secreting cells, we assayed antibody secretion at 72 h. Neither IL-4 nor IL-12 influenced antibody secretion in cultures that did not contain T cells (not depicted). However, as shown in Fig. 6 B, activated B cells cultured with AND T cells in the presence of IL-4 secreted antibody (Fig. 6 B, white bars). Although B cells stimulated with the combination of IL-2, IL-5, and IL-10 secreted more antibody than those stimulated with LPS in the IL-4-containing cultures (Fig. 6 B, white bars), it was important to note that the addition of MR-1 did not influence antibody secretion in either case. In contrast, activated B cells cultured with AND T cells in the presence of IL-12 secreted very little antibody regardless of whether the B cells were stimulated with LPS or the combination of IL-2, IL-5 and IL-10 (Fig. 6 B, black bars). Furthermore, antibody secretion was substantially increased in IL-12-treated cultures by the addition of MR-1, demonstrating that CD154 expression was responsible for the

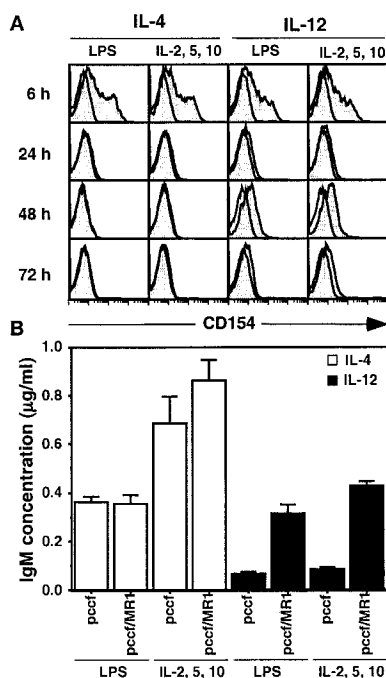


Figure 6. Sustained CD154 expression inhibits antibody secretion by activated splenic B cells during a cognate T–B interaction. (A) Purified splenic B cells were stimulated with rabbit Fab'2 anti-IgM and supernatants from BAFF-transfected L cells for 24 h. These activated B cells were then cultured with PCCF and purified naive AND T cells in the presence of LPS or a combination of IL-2, IL-5, and IL-10 to stimulate antibody production by the B cells and either IL-4 or IL-12 to modulate CD154 expression on T cells. MR-1 was included in parallel cultures to block CD154–CD40 interactions. Cells were removed from culture at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (B) Cells were harvested at 72 h, washed, and recultured at equivalent B cell concentrations for an additional 6 h. Secreted antibody was measured by ELISA.

reduction of antibody secretion in cultures containing IL-12. Although secreted IgG made up only a minor fraction of total antibody in this experiment (not depicted), similar results were observed when the secretion of total IgG was evaluated. Thus, the reduction in IgM secretion by cells cultured with IL-12 could not be explained by preferential switching to IgG.

To test whether sustained CD154 expression altered the antibody-secreting ability of B cells that had been activated in a more physiological setting, we purified antigen-specific B cells from mice that had been immunized 7 d previously with NP-conjugated pigeon cytochrome *c* (NP-PCC). NP-binding, CD138[−] cells (NP-specific, nonplasma cells) were sorted (Fig. 7 A) and cultured alone or with Th1 or Th2 memory AND T cells for 72 h in the presence of LPS and PCCF, with or without MR-1. As shown in Fig. 7 B, both Th1 and Th2 memory cells were activated by peptide-presenting B cells to express CD154 by 6 h. However, although Th1 memory cells activated by PPC-presenting NP-specific B cells maintained CD154 expression at later times, Th2 memory cells did not (Fig. 7 B). To determine whether antibody secretion was influenced by CD154 expression patterns on Th1 or Th2 memory cells, we measured NP-specific IgG in culture supernatants after 72 h. NP-specific IgG was produced by the LPS-stimulated B cells in the absence of T cells, regardless of MR-1 addition (Fig. 7 C, white bars). Similar levels of IgG were produced by B cells cultured with Th2 AND memory T cells in the

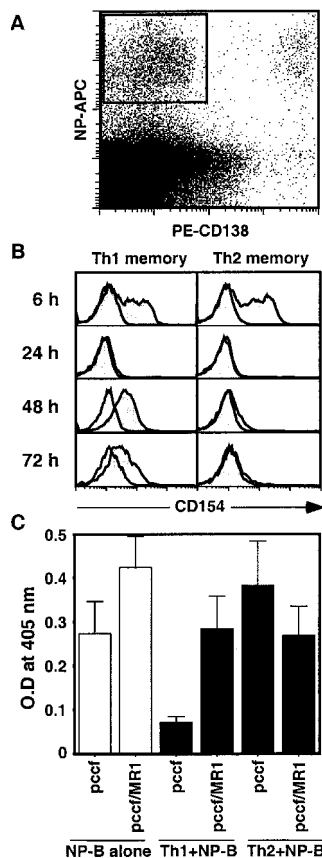


Figure 7. Sustained CD154 expression inhibits antibody secretion by NP-specific germinal center B cells. (A) B10.BR mice were immunized with NP-PCC and NP-OVA and NP-specific B cells were purified 7 d later by a combination of positive selection and cell sorting. The NP-specific cells were identified by their ability to bind NP-allophycocyanin and antibody-secreting cells were excluded by the expression of CD138 (syndecan). (B) Sorted NP-specific B cells were cultured with PCCF, LPS, and either Th1 AND memory T cells or Th2 AND memory T cells. MR-1 was included in parallel cultures to block CD154–CD40 interactions. Cells were removed from culture at the times indicated and CD154 expression on Thy-1⁺ T cells was analyzed by flow cytometry. Open histograms represent control staining, whereas shaded histograms represent CD154 expression. (C) Cells were harvested at 72 h, washed, and recultured at equivalent B cell concentrations for an additional 6 h. Secreted NP-specific antibody was measured by ELISA.

presence or absence of MR-1. However, we observed a dramatic drop in IgG production by B cells that had been cultured with Th1 AND memory T cells. As expected, antibody production in Th1 cultures was restored by the addition of MR-1 (Fig. 7 C), demonstrating again that the sustained expression of CD154 leads to the prevention of antibody secretion by B cells. Unlike the previous experiment, the antibody secreted under these conditions consisted almost entirely of IgG, with nearly undetectable levels of IgM (not depicted). This is most likely due to the fact that most of the germinal center B cells had already switched to IgG before they were isolated.

Discussion

CD154 Expression Can Be Sustained for Extended Periods on Activated CD4⁺ T Cells. The initial studies of CD154 expression on activated T cells concluded that CD154 was expressed only transiently after activation (46, 47). Thus, many subsequent studies examined CD154 expression only at early times after activation, reinforcing the concept that CD154 was expressed only transiently (23, 30). Contrary to this prevailing dogma, the results presented here demonstrate that CD154 expression can easily be sustained on activated T cells for at least 72 h. This sustained expression of CD154 could be observed on either anti-CD3 (without anti-CD28) or APC/peptide-stimulated T cells (Figs. 1 and 2) and was most easily observed on cells stimulated under Th1-inducing conditions (Figs. 2 and 3). Moreover, our results suggest that CD154 expression is not simply sustained, but that there are two discreet phases of CD154 expression that are regulated by different mechanisms. A distinction between the first and second phases of CD154 expression is most easily discerned on T cells that have been stimulated with antigen bearing APCs, as these cells appear to internalize CD154 at 24 h after stimulation, before the reappearance of CD154 at 48 h. This is consistent with previous results demonstrating that CD154 is internalized after contact with its receptor CD40 (25). Thus, studies that followed the kinetics of CD154 expression for only 24 h would find that CD154 expression peaked within 6–8 h after activation and dropped to nearly background levels by 24 h. Those studies that did examine CD154 expression at later times often reported robust CD154 expression, but concluded that the observed expression was due to a novel aspect of that experiment, such as the addition of costimulation, cytokines, or the source of T cells (27, 29, 32). Our results agree with those that reported that CD154 expression can be sustained under some circumstances, particularly under Th1-inducing conditions.

The Second Phase of CD154 Expression Is Regulated by the Polarizing Cytokines IL-12 and IL-4. Not surprisingly, the kinetics of CD154 expression on T cells after activation was found to be dependent on the activating stimulus, the presence of cytokines and the differentiation state of the responding T cells. The first phase of CD154 expression appears to be induced by TCR engagement alone, which is consistent with results showing that a more avid TCR sig-

nal increases CD154 expression between 2 and 8 h after activation (30), but that neither costimulation nor cytokine addition had significant effects (30). However, the second phase of CD154 expression is under more complex control. IL-4 negatively regulates CD154 expression during the second phase, whereas the absence of IL-4 allows sustained CD154 expression at these later times. Interestingly, there is a bimodal distribution of CD154 on T cells activated for only 6 h (Figs. 1–4), which resolves into a unimodal expression pattern at later times. This raises the possibility that IL-12 or IL-4 preferentially expand the cells that were initially either CD154⁺ or CD154⁻, and that the subsequent effector populations simply continue to express CD154 (or not). However, sorting experiments demonstrated that both initial populations have the ability to express CD154 at later times (not depicted) and suggested that cytokine signaling, rather than selective outgrowth, controls CD154 expression on effector T cells. Consistent with the idea that IL-4 controls CD154 expression, CD154 is only transiently expressed on activated Th2 memory cells, whereas its expression is sustained on activated Th1 memory cells that are programmed not to produce any IL-4. Thus, the second phase of CD154 expression appears to be regulated according to the Th1–Th2 paradigm. However, unlike IFN- γ production (41), the sustained expression of CD154 on activated Th1 cells is not a fixed property of these cells because the addition of IL-4 can block extended expression of CD154 on activated Th1 memory cells, but not the production of IFN- γ . Therefore, it seems that extended CD154 expression is a secondary effect of polarized cytokine production, and that the inability of Th1 cells to produce IL-4 allows extended CD154 expression. This is an important observation in two respects. First, it demonstrates functional IL-4 receptor signaling on polarized Th1 memory cells, even though IL-4 receptor signaling is thought to be impaired in polarized Th1 effectors (48). Second, it suggests that the ability of Th1 cells to induce CD40-dependent inflammatory responses can be tempered by Th2 cytokines, which may have important therapeutic benefits in diseases where Type 1 cells are pathogenic (49).

Sustained Expression of CD154 Can Alter T Cell Effector Functions. CD40 signaling has been implicated in the activation/differentiation of all APCs, including B cells (31), macrophages (50, 51) and dendritic cells (52), and therefore, plays a central role in almost all immunological processes. Thus, it is reasonable to predict that factors affecting the ability of T cells to express CD154 would have a profound impact on the ability of T cells to influence APC activation or differentiation. A major effector function of CD4⁺ T cells is to provide B cell “help” and, not surprisingly, CD40 signaling is a critical component of T cell-dependent B cell help as CD40 signaling on B cells is necessary for Ig isotype-switching (3, 12, 53), affinity maturation (11), and germinal center formation (2, 3, 9). Here, we show that the ability of Th cells to promote B cell terminal differentiation and antibody secretion is, in part, dependent on their ability to limit the duration of CD154 ex-

pression. Activated Th1 memory cells express CD154 for longer than 3 d and suppress antibody secretion from activated B cells during this time. Conversely, activated Th2 memory cells express CD154 for <12 h and allow antibody secretion to proceed.

Despite these differences, we believe that Th1 cells are still capable of providing B cell help and promoting robust antibody production *in vivo*. Although there is a persistent notion that Th1 cells are poor at inducing humoral immune responses, both Th1 and Th2 cells can provide B cell help *in vivo* (54). Additionally, our current results clearly show that Th1 cells have the ability to promote antibody secretion by B cells, as long as CD40 signaling is checked (Fig. 7). Furthermore, previous results from several groups demonstrated that although sustained CD40 signaling could inhibit antibody secretion by B cells, it maintained B cell activation and proliferation, and once B cells were removed from the source of CD154 they were capable of secreting antibody normally (16, 20). Therefore, it is probably more accurate to suggest that Th1 and Th2 cells provide different kinds of B cell help. Interactions with Th1 cells might favor more extensive B cell proliferation, switching to isotypes, such as IgG2a, and delayed antibody secretion due to extended CD40 engagement. On the other hand, interactions with Th2 cells might favor less B cell proliferation and more rapid terminal differentiation and antibody secretion due to a limited CD40 engagement.

Such a situation is easy to visualize *in vivo*. Antigen-activated B cells might interact with CD154-expressing Th cells for several days, thus promoting B cell activation, proliferation and isotype-switching, but preventing B cell terminal differentiation and antibody secretion. Such interactions might occur in germinal centers, which are known to be dependent on CD40 signaling (9). Subsequently, some of the activated B may migrate to locations devoid of CD154-expressing T cells, which would effectively end CD40 signaling and allow B cell terminal differentiation. Thus, the ability of sustained CD40 signaling by Th1 cells to temporarily block antibody secretion does not preclude an important role for sustained CD40 signaling in facilitating robust IgG2a antibody responses.

Although Th1 cells can play an important role in humoral immunity, they are most often associated with cell-mediated immunity. It is intriguing that we find that IL-12 sustains the expression of CD154 on activated T cells (Fig. 2) because it also appears that CD40 signaling can promote the production of IL-12 and other inflammatory cytokines by APCs (55–58). In addition, CD40 signaling has been shown to be an important regulator of the inflammatory process and the development of Th1 cells (59, 60). Thus, it is tempting to speculate that signals generated in T cells by inflammatory cytokines and signals generated in APCs through CD40 are involved in a positive feedback loop that generates and maintains an inflammatory type 1 response. Consistent with this, CD40 signaling is necessary for the clearance of microorganisms that require a type 1 response (61–63). However, it is not clear if CD40 signaling is simply necessary to promote the initial polarization of

type 1 responses, or if sustained CD154 expression contributes to Th1 effector function. Such a contribution may be the synergism of IFN- γ and sustained CD40 signaling leading to macrophage activation (62). This hypothesis is currently being investigated.

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