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A Role for CD40 Expression on CD8$^+$ T Cells in the Generation of CD8$^+$ T Cell Memory

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The delivery of CD4 help to CD8$^+$ T cell responses requires interactions between CD40 and CD40 ligand and is thought to occur through antigen-presenting cell (APC) activation. Here we show that generation of memory CD8$^+$ T cells displaying an enhanced capacity for cell division and cytokine secretion required CD4 help but not CD40 expression by the APCs. Activated CD4$^+$ and CD8$^+$ T cells expressed CD40; and in the absence of this protein, CD8$^+$ T cells were unable to differentiate into memory cells or receive CD4 help. These results suggest that, like B cells, CD8$^+$ T cells receive CD4 help directly through CD40 and that this interaction is fundamental for CD8$^+$ T cell memory generation.

Interactions between CD40 and CD40 ligand (CD40L) play a major role in direct CD4$^+$ T cell–B cell collaboration, and the absence of these molecules results in a failure of germinal center formation, memory B cell activation, immunoglobulin class switching, and somatic hypermutation (*1*–*3*). The CD40 and CD40L deficiencies also impair CD8$^+$ T cell responses, suggesting an important role for these molecules in CD4$^+$ and CD8$^+$ T cell collaboration (*4*–*6*). This role has been confirmed by experiments showing that monoclonal antibodies (mAbs) to CD40 could substitute for the delivery of CD4 help to CD8$^+$ T cells in vivo (*7*, *8*). Because CD40 is expressed on antigen-presenting cells (APCs) (*9*), it has been assumed that CD4 help to CD8$^+$ T cells is indirect. Thus, CD4$^+$ CD40L$^+$ T cells would first activate APCs by engaging CD40, allowing activated APCs to become competent to drive CD8$^+$ T cell responses (*7*, *8*, *10*). Other experimental data, however, have suggested dissociation of APC activation from CD4-CD8$^+$ T cell collaboration. For example, CD8$^+$ T cells can activate APCs without CD4$^+$ T cells (*11*). In addition, CD4$^+$ T cells can activate APCs through a CD40-independent pathway (*12*),

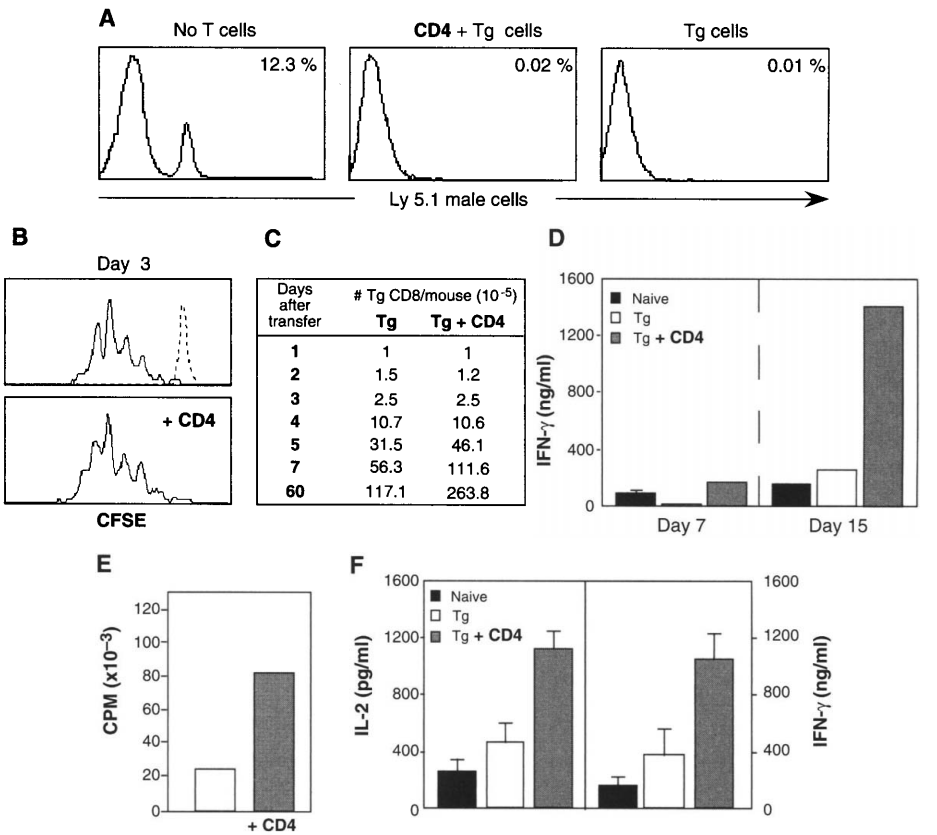

Fig. 1. The role of CD4 help in CD8$^+$ T cell responses. Mice carrying either Tg cells or Tg cells and CD4$^+$ T cells were immunized in vivo with male cells expressing the Ly5.1 marker (*16*). (**A**) Elimination of Ly5.1 male cells by Tg cells in the presence or absence of CD4$^+$ T cells, 2 months after priming. (**B**) Tg cells, labeled with 5- (and 6-) carboxyfluorescein diacetate succininyl ester (CFSE), 3 days after immunization with male cells. Dotted lines represent CFSE labeling of Tg cells in mice that were not immunized. By day 5 after immunization, all cells had lost CFSE labeling. (**C**) The number of Tg cells recovered at different times after immunization in one of three experiments. (**D**) Interferon-γ (IFN-γ) secretion by purified Tg cells (naïve, or recovered at different days after immunization). Purified Tg cells (naïve, or recovered 2 months after priming) were stimulated in vitro with male APCs: (**E**) optimal [^{3}H]thymidine incorporation and (**F**) cytokine secretion. CPM, counts per minute; IL-2, interleukin-2.

and CD40-deficient APCs retain their stimulatory capacity (*13*, *14*), even under conditions where $CD8^+$ T cell priming is disrupted (*14*).

To reconcile these findings, we used an experimental system in which each component of the response (i.e., $CD8^+$ T cells, $CD4^+$ T cells, and APCs) could be independently manipulated (*15*, *16*). Thus, $CD8^+$ T cells from female Rag2-deficient mice expressing a transgenic T cell receptor (TCR) specific for the male histocompatibility-Y (HY) antigen [transgenic (Tg) cells] were stimulated in an in vivo setting with male cells either in the presence or complete absence of $CD4^+$ T cells. To achieve this, we first transferred Tg cells to CD3ε-deficient female mice, which lack T cells and express the Ly5.2 congenic marker, with or without $CD4^+$ T cells from female mice. These mice were subsequently immunized with cells from Ly5.1 CD3ε-deficient male mice (see supporting text S1). In the presence of $CD4^+$ T cells, Tg cells eliminated these male cells, ultimately becoming efficient memory cells. In this respect, they displayed a significantly enhanced capacity for cell division and cytokine secretion, as compared with that of naïve Tg cells (*17*).

The absence of $CD4^+$ T cells did not prevent the elimination of male cells (Fig. 1A), indicating that high frequencies of $CD8^+$ T cells could kill target cells in the absence of help (*18*). Consistent with this, Tg cells stimulated by antigen in the presence or absence of $CD4^+$ T cells showed similar changes in the expression of CD69, CD44, L-selectin, interleukin-2R, and CD45 isoforms, as well as TCR and $CD8^+$ T cell levels (*19*). Additionally, up to day 4 after immunization, the presence of CD4 help did not modify proliferation (Fig. 1B); cell accumulation (Fig. 1C) and cytokine secretion remained at the level seen in naïve cells (*19*). However, after day 5 after immunization, recovery of Tg cells increased gradually in mice coinjected with $CD4^+$ T cells (Fig. 1C). In the presence of CD4 help, cytokine secretion also increased with time during the primary response, whereas it remained at a naïve cell level in the absence of help (Fig. 1D).

We next studied the properties of memory T cells recovered 2 months after immunization. In response to male cells or to stimulation with antibody to CD3, Tg cells primed in the absence of help proliferated poorly (Fig. 1E), and cytokine secretion was similar to that seen in naïve Tg cells (Fig. 1F). Thus, whereas CD4 help did not modify the initial response of naïve $CD8^+$ T cells and did not prevent their differentiation into effector cells, it appeared to be fundamental for the generation of efficient memory cells, which was shown by their improved functional capacity relative to naïve cells (see supporting text S2 and S3).

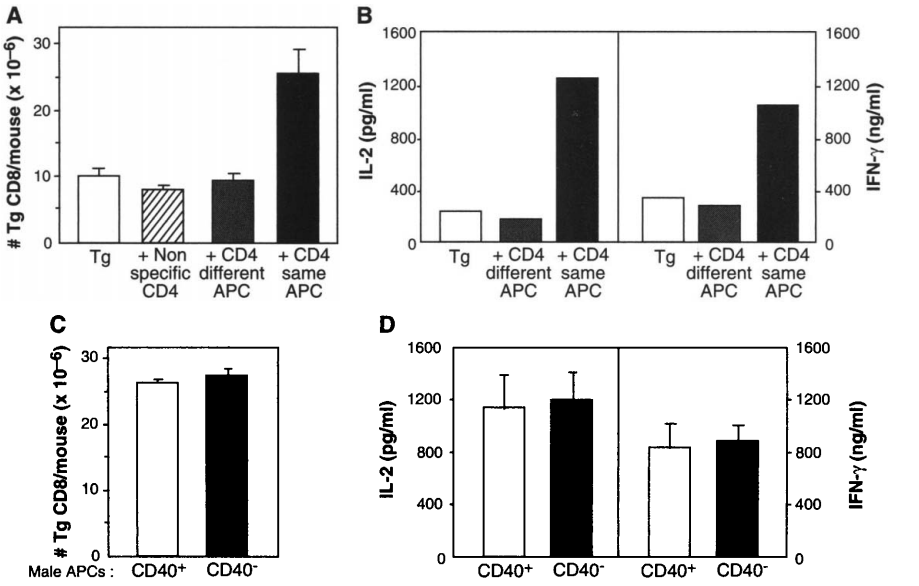

Fig. 2. The role of APCs in delivering CD4 help. (**A** and **C**) Number of Tg cells per mouse (mean ± SEM of three mice per group, in one of three experiments). (**B** and **D**) Optimal cytokine secretion of purified Tg cells (*16*) 2 months after in vivo immunization with male cells. In (A) and (B), Tg cells were primed without help (open bars) or in the presence of $CD4^+$ T cells in three different regimes: with $CD4^+$ T cells depleted of male-specific cells [hatched bar in (A)], when $CD4^+$ and Tg cells recognized antigen on different APCs (gray bars), or when $CD4^+$ and Tg cells recognized antigen on the same APCs [right-hand black bar in (A) and black bars in (B)]. In (C) and (D), Tg and $CD4^+$ cells were transferred to $CD40^+$ female mice and immunized with $CD40^+$ male cells (open bars) or were transferred to $CD40^{-/-}$ female mice and immunized with $CD40^{-/-}$ male cells (solid bars).

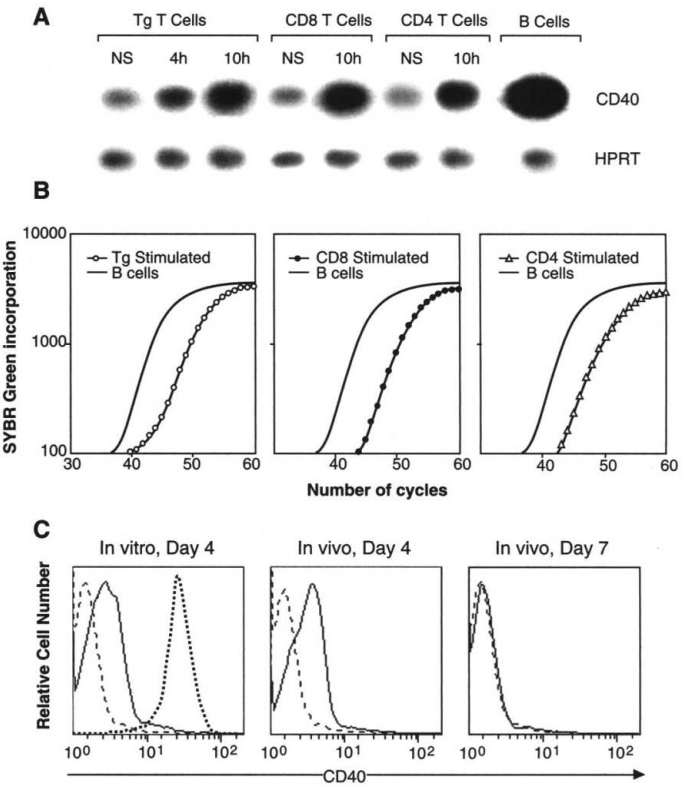

Fig. 3. Expression of CD40 on T cells. C57BL/6 B cells, Tg cells, and $CD8^+$ and $CD4^+$ T cells, either naïve or stimulated in vitro by mAbs to CD3 were sorted. (**A**) cDNA was amplified with specific CD40 primers. Results are the Southern blots of PCR products hybridized with ^{33}P-labeled CD40-specific probes. CD40 cDNA, 10^4 cells; hypoxanthine-guanine phosphoribosyl-transferase (HPRT), 10^3 cells. (**B**) CD40 mRNA quantitation was determined by SYBR-Green incorporation in real-time RT-PCR (*16*) in 500 naïve B cells and 10 hours in in vitro activated T cells. (**C**) CD40 cell surface expression on naïve B cells (dotted line) and on naïve Tg cells (dashed line) or primed Tg cells (solid line) at different time points after antigen stimulation. Labeling with an isotype control was at the same level in naïve and activated cells (*19*).

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To establish the role of the APCs on $CD8^+$ memory T cells generation, we first examined whether delivery of CD4 help to $CD8^+$ T cells required the formation of a three-cell cluster, in which antigen-specific $CD4^+$ and $CD8^+$ T cells interact with antigen presented by the same APCs (*20*, *21*). Help did require the presence of antigen-specific $CD4^+$ T cells, because depletion of these lymphocytes resulted in a poor response by Tg cells (see supporting text S4) (Fig. 2A). We then examined the requirement for CD4 help, when $CD4^+$ and Tg $CD8^+$ T cells were forced to recognize antigen presented by different APCs. $CD4^+$ and Tg $CD8^+$ T cells were transferred into major histocompatibility complex (MHC) class $II^{-/-}$ mice and immunized with two types of male cells: those from MHC class $II^{-/-}$ mice (which could only interact with $CD8^+$ T cells through MHC class I) and those from MHC class I $Db^{-/-}$ mice (which could only interact with $CD4^+$ T cells) (see supporting text S5). In this case, help was absent, demonstrating that efficient $CD8^+$ Tg responses required antigen to be recognized by $CD8^+$ and $CD4^+$ T cells on the same APCs (Fig. 2, A and B). We next tested the potential role of CD40 expression by the APCs in facilitating CD4-$CD8^+$ T cell collaboration. When APCs were derived from $CD40^{-/-}$ mice, the helper effect remained unaffected (Fig. 2, C and D). These results demonstrate that CD4-$CD8^+$ T cell collaboration requires the formation of a CD4–$CD8^+$ T cell–APC cluster but is independent of the CD40 expression by the APCs.

It has previously been shown that antibodies to CD40 can substitute for CD4 help in optimizing $CD8^+$ T cell responses. One interpretation for reconciling these findings with our results is that T cells might also express CD40. Consistent with this, $CD4^+$ and $CD8^+$ T cells expressed very low levels of CD40 mRNA, and these transcripts were up-regulated after activation (Fig. 3A). Quantification of CD40 mRNA expression by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) in 500 activated $CD4^+$ and $CD8^+$ T cells showed that the number of mRNA molecules was similar in each type of activated T cell and, in these activated cells, was 1/50 to 1/100 times that of B lymphocytes (Fig. 3B). Naïve and memory Tg cells lacked CD40 cell surface expression, although induction of this protein was evident after activation but was again lower than that of B cells (Fig. 3C). These results reveal transient expression of CD40 by both $CD4^+$ and $CD8^+$ T lymphocytes during immune responses. These cells also express CD40L under these conditions (*19*, *22*, *23*) [see supporting text S6 (*16*)].

To determine whether CD40 expression by $CD8^+$ T cells was required to receive $CD4^+$ T cell help, we studied monoclonal CD40-deficient TCR-Tg mice. The CD40 deficiency did not modify the naïve Tg cell response to antigen in vitro, because proliferation (Fig. 4A) and cytokine secretion (*19*) were analogous to those of naïve $CD40^+$ Tg cells. Early cell division, measured in vivo during the primary immune response, was also not affected (Fig. 4B). In contrast, CD40 deficiency had a major impact on the properties of primed T cells generated during the primary immune response. $CD40^+$ Tg cells in mice primed in the presence of $CD4^+$ T cells and challenged again by antigen in the presence of $CD4^+$ T cells showed stronger proliferation than did naïve cells (compare Fig. 4B, top, with Fig. 4C, top) and secreted higher levels of cytokines (Fig. 4D). In contrast, $CD40^{-/-}$ Tg cells, stimulated in vivo in the presence of help, proliferated poorly (Fig. 4C). Cytokine secretion after antigen restimulation remained similar to that of naïve cells throughout the primary immune responses (*19*) and in the memory phase (Fig. 4D). We conclude that without CD40 expression on Tg cells, these cells could not acquire the capacity for rapid proliferation or high levels of cytokine secretion, even in the presence of CD4 help.

To determine whether CD40 expression by $CD8^+$ T cells was necessary and sufficient to allow $CD8^+$ T cells to receive helper signals specifically from $CD4^+$ T cells, we primed $CD40^{-/-}$ Tg cells in the presence or absence of $CD4^+$ T cells and tested them after antigen restimulation, in vitro. The presence of CD4 help did not modify the behavior of $CD40^{-/-}$ Tg cells (Fig. 4E), demonstrating

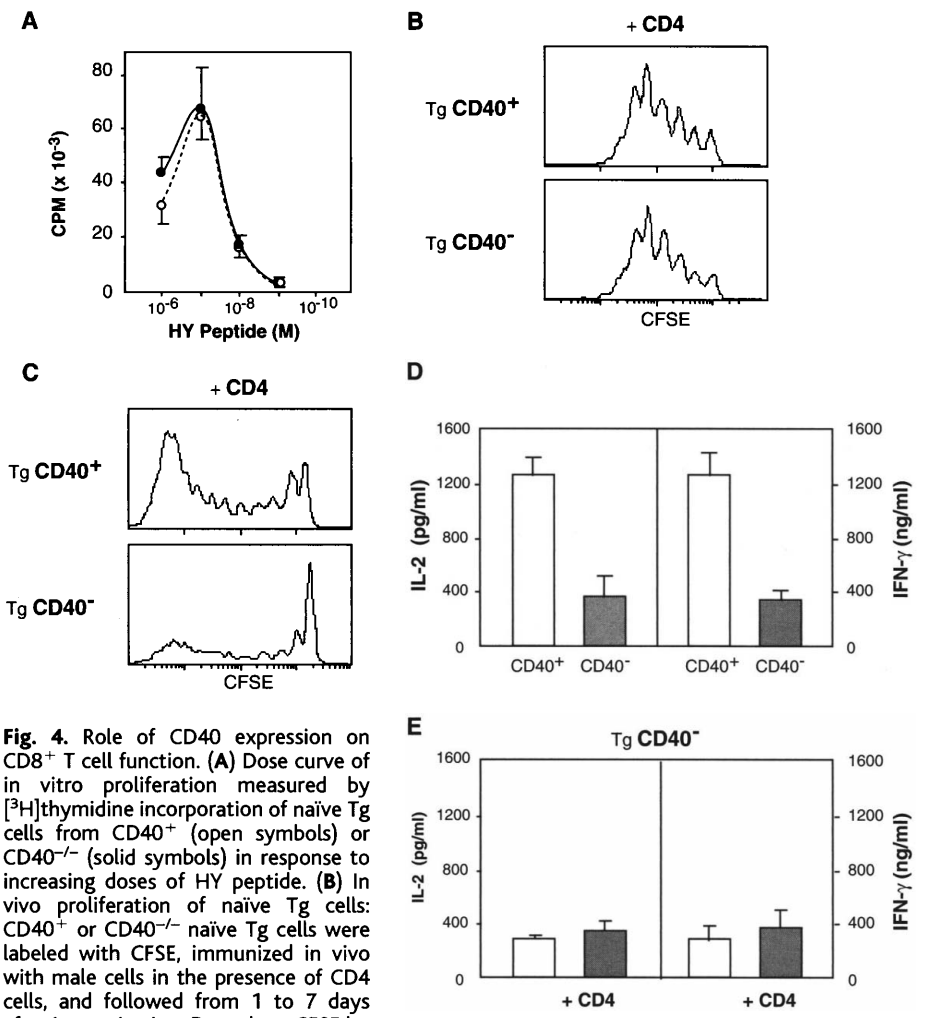

Fig. 4. Role of CD40 expression on $CD8^+$ T cell function. **(A)** Dose curve of in vitro proliferation measured by [^{3}H]thymidine incorporation of naïve Tg cells from $CD40^+$ (open symbols) or $CD40^{-/-}$ (solid symbols) in response to increasing doses of HY peptide. **(B)** In vivo proliferation of naïve Tg cells: $CD40^+$ or $CD40^{-/-}$ naïve Tg cells were labeled with CFSE, immunized in vivo with male cells in the presence of CD4 cells, and followed from 1 to 7 days after immunization. Data show CFSE labeling on day 3. **(C and D)** $CD40^+$ or $CD40^{-/-}$ Tg cells, along with $CD4^+$ T cells, were immunized with male cells and isolated 3 months after immunization. In (C), these Tg cells were labeled with CFSE and restimulated in vivo with antigen in the presence of $CD4^+$ T cells. Data show CFSE-labeled cells on day 3 after immunization. $CD40^+$ memory cells lost CFSE labeling by day 4 after immunization, whereas $CD40^{-/-}$-primed cells were CFSE-negative by day 7. In (D), these Tg cells were restimulated in vitro with male cells, and optimal cytokine secretion was determined. **(E)** CD4 help to Tg $CD40^{-/-}$ cells. $CD40^{-/-}$ Tg cells were primed in vivo with male cells (with or without CD4 cells) and recovered 3 months after immunization. Results show optimal cytokine secretion after antigen restimulation.

that CD4 helper effects were mediated through interactions with CD40 expressed by $CD8^+$ T cells.

Our data indicate that $CD4^+$ help to $CD8^+$ responses does not require CD40 expression by the APCs but depends instead on the expression of CD40 by $CD8^+$ T cells. These data suggest a reevaluation of the cellular interactions involved in CD4-$CD8^+$ T cell collaboration. The CD40L signals required by $CD8^+$ T cells do not appear to originate from an autologous source, as CD40L-deficient $CD8^+$ T cells maintain the capacity to receive CD40 signals (*24*). Because murine APCs do not express CD40L (*24–26*), it is likely that CD40L signals to $CD8^+$ T cells originate from activated $CD4^+$ T cells, with help involving a direct CD4-$CD8^+$ T cell interaction. This direct interaction explains previous data suggesting a dispensable role of CD40 interaction on APC activation in CD4 helper activity (*11–14*, *24*). It is also compatible with the capacity of mAbs to CD40 to substitute for CD4 help in vivo (*7*, *8*) but suggests that the mAb in these experiments acted directly on $CD8^+$ T cells. Other reports have shown that activated APCs result in the detection of cytolytic T lymphocyte (CTL) activity in the absence of CD4 help (*10*). These activated APCs, however, could have influenced $CD8^+$ T cell responses by an independent mechanism, different from that used by $CD4^+$ T cells, because it has been demonstrated that differentiation of $CD8^+$ T cells into CTL does not require CD4 help (*18*), (see supporting text S7).

Our data suggest that the differentiation of $CD8^+$ T lymphocytes into efficient primed cells is analogous to isotype switching and somatic hypermutation during memory B cell generation. Both phenomena are apparent relatively late in the primary immune response, requiring CD4 help and CD40 expression by the target cell. The requirement for this type of direct CD4-$CD8^+$ T cell interaction suggests that a reevaluation of strategies of vaccination to induce optimal $CD8^+$ T cell response might be appropriate. Although activated APCs may not be sufficient to substitute for CD4 help, our data suggest that mAbs to CD40, which operate directly on $CD8^+$ T cells, might have therapeutic value in correcting $CD8^+$ T cell dysfunction associated with CD4 lymphopenia, as found in HIV infection. The expression of both CD40 and CD40L on activated $CD4^+$ and $CD8^+$ T cells and the expression of CD40 on the APCs suggest that although help can be received by $CD8^+$ T cells directly, complex cross talk between these three populations may nevertheless exist.

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26. We could not formally show that CD40L expression on CD4 cells was responsible for the helper effect, because $CD4^+$ $CD40L^{-/-}$ T cells proliferate very poorly in vivo (*25*). Experiments are therefore difficult to interpret in these conditions because the absence of help may be due to a low frequency of antigen-specific CD4 cells, rather than being a direct consequence of the absence of CD40L signals to $CD8^+$ T cells. We cannot therefore exclude the existence of another type of CD40L, which would be able to trigger CD40 on $CD8^+$ T cells.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/297/5589/2060/DC1
Materials and Methods
Supporting Text S1 to S7
References

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Critical Role for STAT4 Activation by Type 1 Interferons in the Interferon-γ Response to Viral Infection

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Interferons (IFNs) are essential for host defense. Although the antiviral effects of the type 1 IFNs IFN-α and IFN-β (IFN-α/β) have been established, their immunoregulatory functions, especially their ability to regulate IFN-γ production, are poorly understood. Here we show that IFN-α/β activate STAT4 directly (STAT, signal transducers and activators of transcription) and that this is required for IFN-γ production during viral infections of mice, in concert with T cell receptor–derived signals. In contrast, STAT1 appears to negatively regulate IFN-α/β induction of IFN-γ. Thus, type 1 IFNs, in addition to interleukin-12, provide pathways for innate regulation of adaptive immunity, and their immunoregulatory functions are controlled by modulating the activity of individual STATs.

Type 1 interferons (IFNs), including IFN-α and IFN-β (IFN-α/β), are critical in innate immunity, and their antiviral effects have been established. The type 2 interferon IFN-γ is also a pivotal cytokine required for defense against infections from a wide range of agents. The innate cytokine interleukin-12 (IL-12) is a potent signal for IFN-γ production and regulates adaptive immunity by promoting differentiation of naïve CD4 T cells to the T helper cell 1 (T_H1) subset (*1*, *2*). Type 1 IFNs can be induced to high levels during viral infections and may provide a unique alternative innate pathway for shaping adaptive immune responses, but their effects on IFN-γ expression have been paradoxical and controversial. IFN-α/β can inhibit IL-12 induction and block its downstream effects (*3–7*). Conversely, type 1 IFNs can enhance T cell IFN-γ responses in humans and during viral infections in mice (*8–10*). In contrast to human cell responses, however, the cytokines do not support T_H1 differentiation of murine cells (*9*, *11*), and this has been attributed to a failure to recruit STAT4 (STAT, signal trans-

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