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

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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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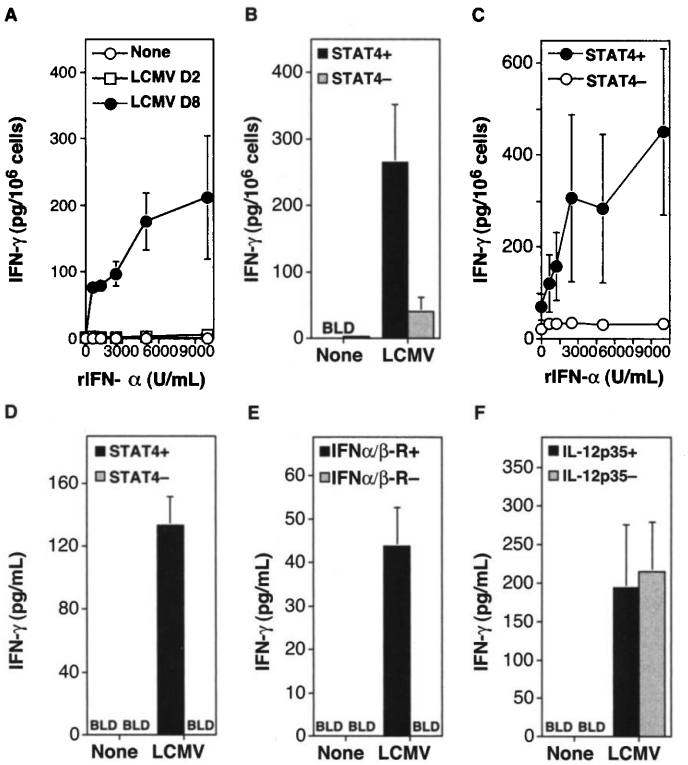

Fig. 1. Requirements for IFN-γ production by cells from uninfected or LCMV-infected mice in vitro and in vivo. (**A**) Splenic leukocyte IFN-γ production was examined after overnight stimulation in vitro with human recombinant IFN-α (rIFN-α) (that is, hybrid human IFN-α A/D), active on murine cells. Leukocytes were isolated from uninfected mice (open circles) or from mice on day 8 (solid circles) and day 2 (open squares) after infection. (**B**) STAT4 requirements for ex vivo spontaneous IFN-γ production by leukocytes after LCMV (day 8) infection were tested with cells with STAT4 (STAT4$^+$) (black bars) and with cells deficient in STAT4 (STAT4$^-$) (gray bars). (**C**) STAT4 requirements for rIFN-α–mediated induction of IFN-γ production by leukocytes after viral infection (day 8) were examined with cells from STAT4$^+$ (solid circles) and from STAT4$^-$ (open circles) mice. IFN-γ production was examined after overnight stimulation in vitro with rIFN-α (that is hybrid human IFN-α A/D). (**D** to **F**) Serum IFN-γ levels were determined in samples from mice on day 4 after infection or from uninfected mice that were immunocompetent: (D) STAT4$^-$, (E) deficient in IFN-α/βR (IFN-α/βR$^-$), or (F) deficient in the IL-12 p35 chain (IL-12p35$^-$). Immunocompetent mice are represented with black bars, and immunodeficient mice are represented with gray bars. IFN-γ values were measured by enzyme-linked immunosorbent assay. For all panels, the results are averages ± SEM of at least three mice per group. BLD, below limit of detection.

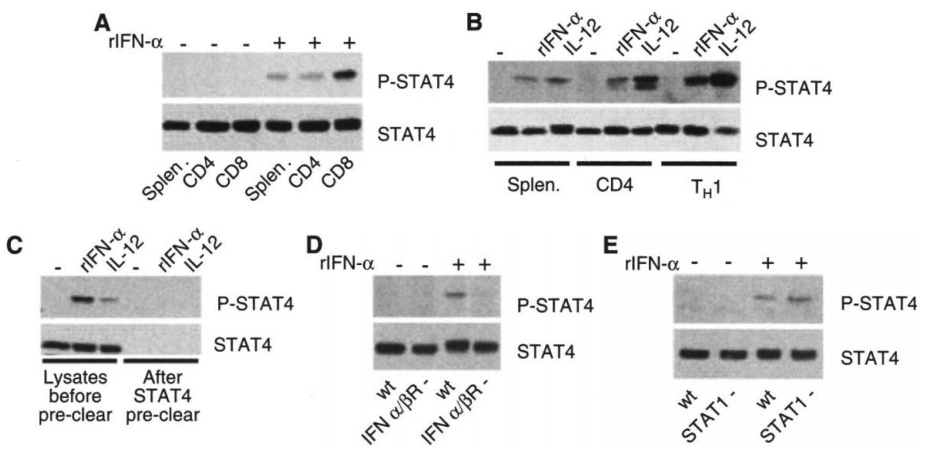

Fig. 2. IFN-α activates STAT4 phosphorylation in murine leukocytes. (**A**) Splenic leukocytes were fractionated into CD4 and CD8 cells and stimulated for 20 min with 5000 U/ml of murine rIFN-α. Cell lysates were analyzed by Western blotting with an antibody specific to P-STAT4 or STAT4. (**B**) Naïve (CD62L$^+$) CD4 T cells were isolated and stimulated with plate-bound antibody to CD3 and antibody to CD28 for 5 days in the presence of IL-2, IL-12, and antibody to IL-4 to promote T_H1 differentiation, followed by 2 days of culture in IL-2 alone. Cells were rested for 6 hours before 20-min stimulation with 10,000 U/ml of rIFN-α (murine) or 50 ng/ml of rmIL-12 (rm, recombinant murine). Fresh total and CD4 T cell splenic populations were prepared on day 7 for comparison. Cell lysates were analyzed. (**C**) A total of 5×10^7 leukocytes were stimulated with 10,000 U/ml of rIFN-α (murine) or 50 ng/ml of IL-12 for 20 min, and cell lysates were prepared. Three rounds of immunoprecipitation with a STAT4 antibody were used to preclear the total STAT4. Lysates before and after STAT4 preclearance were analyzed for STAT4 activation as above. (**D**) Cell lysates, prepared from leukocytes isolated from wild-type (wt) or IFN-α/βR$^-$ mice and stimulated for 90 min with rIFN-α A/D, were analyzed. (**E**) Cell lysates, prepared from leukocytes isolated from wild-type or STAT1$^-$ mice and stimulated for 90 min with or without rIFN-α A/D, were analyzed.

ducers and activators of transcription) for IFN-γ gene expression (*12*, *13*). To reconcile the divergent observations, we revisited the ability of type 1 IFNs to induce T cell IFN-γ during viral infections in mice and explored the mechanisms underlying this regulation.

Responses to lymphocytic choriomeningitis virus (LCMV) infections in mice include prominent innate IFN-α/β production and adaptive CD8 T cell expansion with IFN-γ production (*8*, *14*–*16*). Work from our laboratories has demonstrated that type 1 IFNs facilitate the CD8 T cell IFN-γ production (*8*), with peak endogenous CD8 T cell IFN-γ responses occurring on days 4 to 6 after infection (*17*). However, IFN-α/β fail to induce IFN-γ production in vitro by cells isolated from uninfected mice or from mice on day 2 after infection (*6*, *18*) (Fig. 1A). To evaluate the responsiveness of cells after initiation of the adaptive immune responses, we examined IFN-γ induction by type 1 IFNs in culture with cells isolated on day 8 after LCMV challenge (Fig. 1A). In contrast to the lack of IFN-γ production by populations from uninfected mice or from mice on day 2 after infection, splenic leukocytes from mice on day 8 after infection produced IFN-γ when stimulated with type 1 IFNs. The induction was IL-12–independent because it also was elicited with cells from mice on day 8 after infection, but not from uninfected mice, deficient in IL-12 (*17*).

Because of the documented importance of STAT4 in regulating IFN-γ production, we next tested the responsiveness of cells to type 1 IFNs in the absence of functional STAT4. Spontaneous release, without the addition of exogenous factors, demonstrated that cells from infected STAT4-deficient mice were unable to produce IFN-γ (Fig. 1B). This effect was observed despite comparable induction of CD8 T cell expansion (fig. S1). Likewise, type 1 IFN induction of IFN-γ in culture was inhibited in STAT4-deficient cells (Fig. 1C). STAT4-deficient mice were also dramatically inhibited in their endogenous IFN-γ response to LCMV (Fig. 1D). The response was dependent on type 1 IFNs, because IFN-α/βR (R, receptor)–deficient mice lacked IFN-γ production (Fig. 1E) in the presence of CD8 T cell expansion (*8*). Although LCMV is not a potent inducer of biologically active IL-12 (*4*, *19*), we evaluated a possible requirement for this cytokine, because of its potent ability to induce IFN-γ through STAT4 (*1*, *2*, *20*). Mice deficient in the IL-12 p35 chain displayed IFN-γ responses comparable to those of controls (Fig. 1F), and similar results were obtained with mice deficient in the IL-12 p40 chain (*17*). Thus, IFN-γ responses to LCMV infection were dependent on IFN-α/β and STAT4 but not on IL-12.

Although these data suggest that IFN-α/β elicit STAT4 activation for IFN-γ induction, the ability of IFN-α to access STAT4 has

been reported to be absent in certain mouse cell populations (*9*, *11*, *13*). We tested whether primary leukocytes, stimulated in vitro with IFN-α, could induce STAT4 activation. As shown in Fig. 2A, IFN-α elicited STAT4 tyrosine phosphorylation in total leukocytes and in both CD4 and CD8 T cells, but STAT4 tyrosine phosphorylation was most strongly elicited in CD8 T cells. Although IL-12 was stronger than IFN-α in mediating STAT4 activation in polarized T_H1 cells, IFN-α induced STAT4 phosphorylation in this subset as well (Fig. 2B). Loss of the phosphorylated protein detected with phosphospecific STAT4 (Fig. 2C) or antibodies to phosphotyrosine (fig. S2) by preclearance of total STAT4 confirmed that the induced protein was phosphorylated STAT4 (P-STAT4). The response was the result of type 1 IFN stimulation because P-STAT4 was not detected in the absence of a functional receptor (Fig. 2D). It was, however, readily apparent in STAT1-deficient cells (Fig. 2E). The possibility that IL-12 was acting as an intermediary was formally excluded by the finding that IFN-α induced STAT4 phosphorylation in leukocytes deficient in the IL-12 p35 chain (*17*). Together, these data show that there is a murine IL-12/STAT1–independent pathway for type 1 IFN activation of STAT4.

A direct role of STAT4 in IFN-γ gene regulation has not been established. There is evidence, however, that it can bind the IFN-γ gene (*21*), and IL-12 is thought to directly induce IFN-γ production through this pathway. To confirm the functional relevance of type 1 IFN–driven STAT4 activation, with respect to IFN-γ gene regulation, we used chromatin immunoprecipitation (Fig. 3). STAT4 was not bound to the proximal IFN-γ promoter in the absence of stimulation (Fig. 3, lanes 7 through 9) but was present after LCMV infection (Fig. 3, lanes 25 through 27). Moreover, stimulation of leukocytes in vitro with IFN-α induced STAT4 binding to the IFN-γ gene (Fig. 3, lanes 16 through 18). As expected, IL-12 also induced STAT4 binding (*17*). In contrast to STAT4, STAT1 can negatively regulate certain downstream consequences of signal transduction and may act as a negative regulator of IFN-γ (*6*, *22*–*25*). We hypothesized, therefore, that a changing ability of type 1 IFNs to activate STAT4 and/or modifications in STAT1 levels might contribute to mechanisms supporting the differential effects on IFN-γ induction during infection (Fig. 1A). To evaluate this, we isolated cells from uninfected mice and from mice on days 2, 5, and 8 after LCMV infection and analyzed them for overall STAT levels as well as for responsiveness to IFN-α. As shown in Fig. 4A, although the STAT4 levels did not change dramatically during the course of infection (lanes 1 through 4 and 5 through 8), the ability of IFN-α to induce STAT4 phosphorylation did. High levels of P-STAT4 were elicited in response to IFN-α stimulation in populations isolated from uninfected mice (lane 5) or mice on day 8 (lane 8) but not day 2 (lane 6) or day 5 (lane 7) after infection (Fig. 4A). CD8 T cell proportions increased during the course of infection, but the P-STAT4 responses of enriched CD8 T cells were similar with populations from uninfected mice or mice on day 8 after infection (*17*). The levels of STAT1 were also dynamically altered after viral challenge. They were very low in leukocytes from uninfected mice (lanes 1 and 5), but initially increased (lanes 2 and 3, and 6 and 7) and then decreased (lanes 4 and 8) after infection (Fig. 4A). Thus, viral infection appears to induce a dynamic regulation of STAT1 expression and STAT4 phosphorylation through type 1 IFNs.

However, the data presented in Figs. 1 and 4A indicate that low STAT1 levels and high IFN-α–induced STAT4 phosphorylation are not sufficient for IFN-γ induction (that is, uninfected responses as compared to day 8 responses). CD8 T cells are particularly sensitive to IFN-α–induced STAT4 phosphorylation (Fig. 2A) and are the major populations primed to make

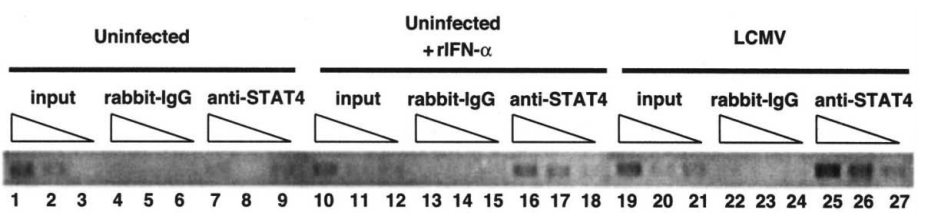

Fig. 3. IFN-α drives STAT4 binding to the IFN-γ gene. Splenic leukocytes were obtained from uninfected mice (lanes 1 through 18) or from mice on day 7 after LCMV infection (lanes 19 through 27); lanes 10 through 18 show cells stimulated in vitro with IFN-α for 90 min. Proteins were cross-linked with formaldehyde and immunoprecipitated with antibody to STAT4 (anti-STAT4) or control antibody [rabbit immunoglobulin G (rabbit-IgG)]. After reversing the cross-linking, polymerase chain reaction (PCR) for the IFN-γ gene was performed on fourfold serially diluted samples. Input represents PCR amplification of the total sample. Results are representative of two independent experiments.

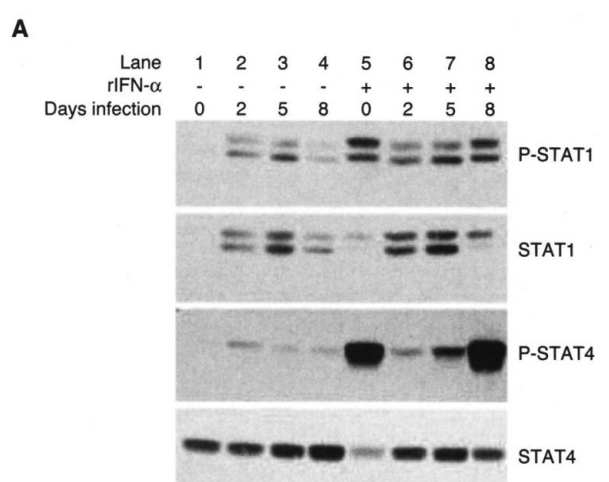

Fig. 4. Dynamic regulation of responsiveness to IFN-α for STAT4 phosphorylation, overall STAT1 expression, and synergy between IFN-α and TCR stimulation for IFN-γ production, during viral infection. (**A**) Splenic leukocytes isolated from uninfected mice or from mice infected with LCMV for 2, 5, or 8 days were stimulated with or without rIFN-α A/D for 90 min. Cell lysates were analyzed for basal and activation levels of STAT1 and STAT4 by Western blotting. (**B**) Leukocytes from mice on day 8 after LCMV infection were stimulated, for IFN-γ production, overnight in culture with increasing concentrations of rIFN-α A/D and no (open symbols) or increasing concentrations (increasing solid symbol sizes indicate 24, 98, 1563, and 6250 pg/ml) of the LCMV immunodominant peptides for CD8 T cell responses NP396 (left) or GP33 (right).

IFN-γ on day 8 of infection (*8*, *14*, *15*). We therefore asked whether IFN-α enhances the stimulation of CD8 T cells isolated from infected mice through the receptor for antigen [the T cell receptor (TCR)]. As shown in Fig. 4B, IFN-α had synergistic effects with the major LCMV immunodominant epitopes recognized through the CD8 TCRs [that is, NP396–404 and GP33–41 (NP, nucleoprotein; GP, glycoprotein)]. Thus, type 1 IFNs do elicit production of IFN-γ by normal cells isolated from mice on day 8 after infection, but they act in concert with CD8 T cell epitopes to drive high levels of IFN-γ release.

Our data indicate that STAT4 activation is a critical intermediary in the induction of mouse IFN-γ by type 1 IFNs. In this capacity, it is rapidly activated and binds to the proximal IFN-γ promoter, and STAT4 deficiency dramatically impairs IFN-α/β–dependent induction of IFN-γ during viral infection. If STAT4 activation by type 1 IFNs is important in the mouse, why was it overlooked and reported not to occur? Previous studies were limited to CD4 T cells (*9*, *11*), genetic constructs in artificial cells (*13*), use of low concentrations of IFN-α (*9*, *11*, *13*), and examination of a mouse strain (*9*, *11*) with reduced STAT4 (*26*). Although there may be an additional mechanism leading to STAT4 recruitment for activation by type 1 IFNs in humans [that is, a STAT2-dependent pathway (*12*)], our results indicate that this is not the sole mechanism and that mice and humans do not fundamentally differ in type 1 IFN–mediated regulation of STAT4. Moreover, as STAT1 can have negative effects on responses in both humans and mice (*6*, *22*–*25*), our observations suggest that major biological consequences of type 1 IFN exposure may be the same in both species if STAT1 effects are dominant. STAT4 activation by type 1 IFNs does not appear to be sufficient for peak IFN-γ induction by CD8 T cells, but this is similar to other stimuli; for example, IL-12 is not sufficient to induce IFN-γ production by resting T cells, because naïve T cells must be activated to up-regulate the IL-12 receptor (*2*). Likewise, the transcription factor T-bet is an important regulator of IFN-γ production in CD4 T cells, and it too is induced by TCR occupancy but not by type 1 IFNs (*27*).

Our findings establish a means by which the innate immune response may govern adaptive immunity. More important, however, they provide conceptual insights into mechanisms for plasticity of responses to one cytokine. Together with our earlier work, these findings demonstrate that type 1 IFNs can attenuate or enhance IFN-γ production as a result of differential expression of signaling molecules.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/297/5589/2063/DC1

Materials and Methods

Figs. S1 and S2

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Evolution of Autoantibody Responses via Somatic Hypermutation Outside of Germinal Centers

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Somatically mutated high-affinity autoantibodies are a hallmark of some autoimmune diseases, including systemic lupus erythematosus. It has long been presumed that germinal centers (GCs) are critical in autoantibody production, because they are the only sites currently believed to sustain a high rate of somatic hypermutation. Contrary to this idea, we found that splenic autoreactive B cells in autoimmune MRL.Fas^{lpr} mice proliferated and underwent active somatic hypermutation at the T zone–red pulp border rather than in GCs. Our results implicate this region as an important site for hypermutation and the loss of B cell self-tolerance.

The activation of autoreactive B cells is pivotal for the development of systemic autoimmune diseases, because these lymphocytes secrete pathogenic autoantibodies and promote the activation of pathogenic autoreactive T cells (*1*). Because affinity-enhancing somatic mutations are prevalent in these autoantibodies, it has long been hypothesized that the GC provides the critical signals for B cell activation in auto-immune disease. This theory, however, has never been proven, and the steps leading to the production of autoantibodies in systemic autoimmune diseases remain elusive.

The rheumatoid factor (RF) autoantibody response was studied by crossing onto the MRL.Fas^{lpr} (MRL/lpr) lupus-prone mouse strain a transgene (Tg) encoding an immunoglobulin (Ig) Vh (H chain) that is derived from an RF monoclonal autoantibody (AM14) specific for IgG2a of the "a" allotype (IgG2a^a) (*2*, *3*). The resulting mice have a diverse endogenous light chain repertoire along with an increased precursor frequency of an IgG2a^a-specific auto-

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