

Immune Response in Stat2 Knockout Mice

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Summary

Type I IFNs induce gene expression through Stat1 and Stat2, which can in turn associate either to form Stat1 homodimers or the transcription factor ISGF-3. Stat1 homodimers also transduce signals for IFN- γ . To explore the unique properties of Stat2 and ISGF-3 in type I IFN signaling, its gene was targeted for deletion. Stat2 null mice exhibit a number of defects in immune response. This includes an increased susceptibility to viral infection and the loss of a type I IFN autocrine/paracrine loop, which in turn regulates several aspects of immune response. Intriguingly, Stat2-deficient fibroblasts exhibit a more significant defect in their response to type I IFNs than macrophages, highlighting tissue-specific differences in the response to this family of ligands.

Introduction

Type I interferons (IFNs) mediate potent antiviral and antiproliferative activities on target cells through the activation of new genes. Characterization of their ability to induce these genes led to the elucidation of the JAK-STAT signaling pathway, where Janus kinases (JAKs) are receptor-associated tyrosine kinases and signal transducers and activators of transcription (STATs) are the transcription factors they activate. Subsequent studies have determined that most other members of the cytokine family transduce vital signals through this paradigm (reviewed in Ihle, 1995; Darnell, 1997; Schindler and Strehlow, 2000).

Type I IFN (e.g., IFN- α/β) signaling entails the rapid receptor-dependent activation of Stat1 and Stat2. Once activated, these two STATs can either heterodimerize, associate with the DNA binding protein IRF-9 (p48) and form ISGF-3 (IFN-stimulated gene factor-3), or they can form Stat1 homodimers (Darnell, 1997; Schindler and Strehlow, 2000). ISGF-3 promotes the activation of genes through the IFN-stimulated response element (ISRE). ISRE-driven genes include Ly-6C, the double-stranded RNA kinase (PKR), 2' to 5' oligoadenylate synthase (OAS), MX, and potentially MHC class I (Bothwell et al., 1988; Johnson and Pober, 1994; Stark et al., 1998; Lee et al., 1999). Stat1 homodimers, which also play a critical role in transducing signals for IFN- γ (Durbin et al., 1996; Meraz et al., 1996), promote the activation of

genes through members of the γ activation site (GAS) family of enhancers. GAS-driven genes including IRF-1, CIITA (an activator MHC class II), IGTP, MIG, and IP-10 (Decker et al., 1997; Taylor et al., 2000). Consistent with these observations, Stat1 knockout mice are defective in their response to both type I and type II IFNs (Durbin et al., 1996; Meraz et al., 1996). Thus, Stat2, an uncharacteristic member of the STAT family (Park et al., 1999), is unique to the biological response to type I IFNs.

Receptor gene targeting studies have provided important insight into the distinct biological responses to type I and II IFNs. Specifically, type I IFN receptor α chain (IFNAR1) null mice are exquisitely sensitive to infection with vesicular stomatitis virus (VSV), semliki forest virus (SFV), vaccinia virus (VV), and lymphocytic choriomeningitis virus (LCMV) but not to intracellular pathogens (Müller et al., 1994). In contrast, IFN- γ and IFN- γ receptor null mice are defective in their response to VV and LCMV but not VSV and SFV (Dalton et al., 1993; Huang et al., 1993; Lu et al., 1998). These mice are also uniquely sensitive to infection with obligate intracellular pathogens and exhibit notable perturbations in cellular immunity.

Although type I IFNs are well recognized for their role in promoting innate antiviral immunity, numerous studies have hinted at a more pervasive role in immune response (Biron, 1998). Recent studies have determined that a circulating dendritic cell precursor secretes large quantities of type I IFNs in response to infectious stimuli (Siegal et al., 1999). Type I IFNs have also recently been implicated in promoting the survival of memory T cells, especially from the CD8⁺ lineage (Tough et al., 1996; Marrack et al., 1999). This activity contrasts the suppressive effects ascribed to type I IFNs on earlier events in lymphopoiesis (Binder et al., 1997; Lin et al., 1998). The recent identification of limitin, a stromally secreted type I IFN that antagonizes lymphopoiesis, further implicates type I IFNs in the regulation of lymphoid development (Oritani et al., 2000).

Type I IFNs have also been shown to function through an autocrine loop. Initial studies highlighted the ability of type I IFNs to antagonize the growth of several tumor lines through this loop (Resnitzky et al., 1986; Mattei et al., 1994; Kawabata et al., 1997). Subsequent biochemical and genetic studies have provided further and compelling evidence that this type I IFN autocrine loop is critical for the robust secretion of type I IFNs in response to viral infection and the antiviral activity of IFN- γ (Erlandsson et al., 1998; Wathlet et al., 1998; Takaoka et al., 2000). This may reflect the role of “early” type I IFNs as components of an autocrine loop that promotes the robust secretion of “late” type I IFNs (Erlandsson et al., 1998; Marie et al., 1998; Sato et al., 1998; Takaoka et al., 2000).

To address the unique properties of Stat2 in IFN signaling, the *Stat2* gene has been targeted for deletion by homologous recombination. As anticipated, Stat2 knockout mice are unresponsive to type I IFNs and exquisitely sensitive to viral infection. However, they also exhibit a number of additional significant defects, including the

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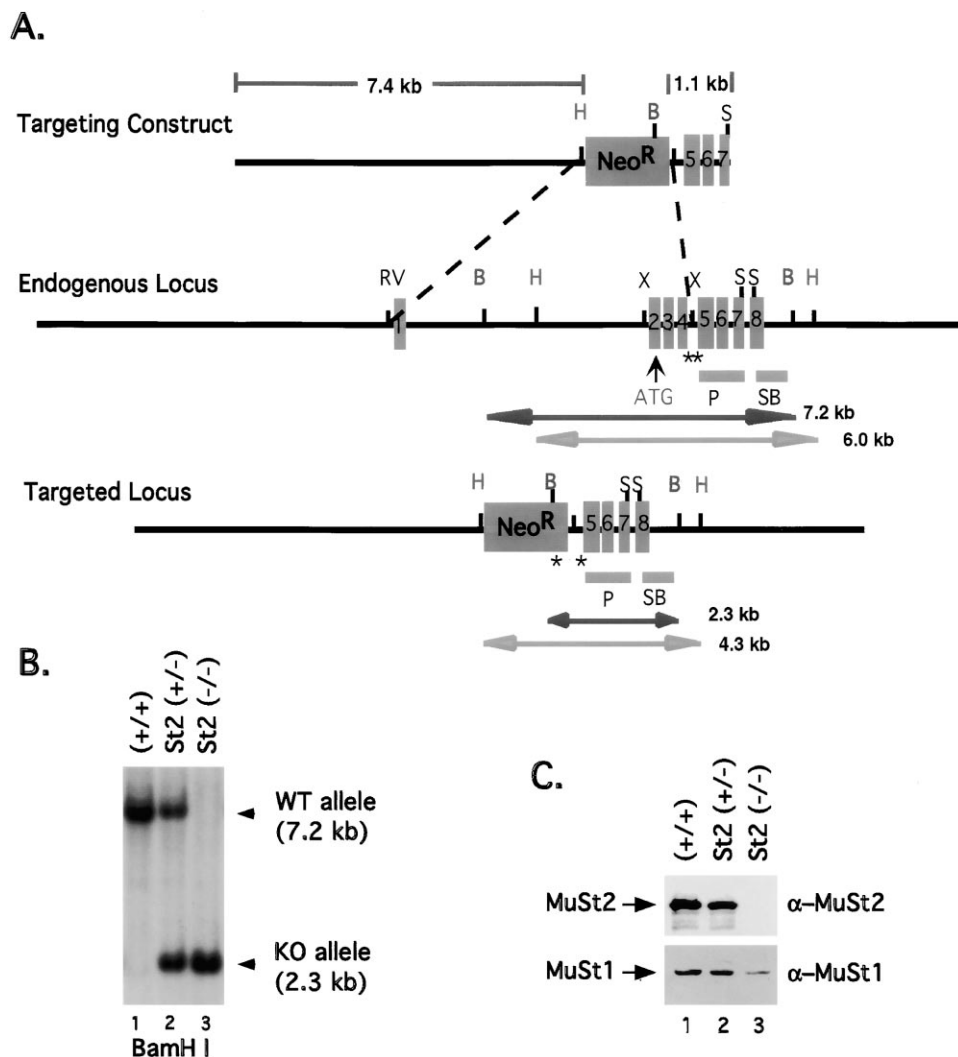


Figure 1. Generation of Stat2 Null Mice

(A) Schematic representation of the Stat2 targeting construct and ~14 kb of the endogenous murine Stat2 gene. The positions of exons one through eight are indicated along with informative endonuclease restriction sites (H, HindIII; B, BamHI; S, Sall; X, XbaI; and P, PstI). Double-sided arrows indicate the position of diagnostic restriction fragments used in genotyping with probes P and SB. Asterisks indicate the location of primers employed for PCR genotyping.

(B) Representative Southern blot of BamHI restricted DNA prepared from wild-type (+/+), Stat2^{+/-}, and Stat2^{-/-} tail DNA and then hybridized with SB. The relative position of the wild-type (WT) and Stat2 null (KO) allele is indicated in the right margin.

(C) Representative Stat2 and Stat1 immunoblot of whole-cell extracts prepared from wild-type (+/+), Stat2^{+/-}, and Stat2^{-/-} PMEFs. The relative positions of murine Stat1 (MuStat1) and murine Stat2 (MuStat2) are indicated in the left margin.

loss of the type I IFN autocrine loop and unique defects in macrophage and T cell responses. Characterization of Stat2^{-/-} cell types highlights important tissue-specific differences Stat2 plays in mediating the biological response to IFN- α . These observations indicate that Stat2 is critical to the biological response of type I IFNs and demonstrate that this subfamily of IFNs plays a more pervasive role in immune response than had been anticipated.

Results

Targeting Stat2 for Deletion

A targeting construct designed to replace the first four exons of the Stat2 gene (7 kb) with a pGK-neomycin

resistance cassette (Figure 1A) was introduced into ES cells. Heterozygous Stat2 knockout mice were generated from successfully targeted embryonic stem (ES) cells (Figure 1B). Interbreeding of heterozygous mice yielded Stat2 null mice at the expected Mendelian ratio. The Stat2 null mice developed and bred normally in a specific pathogen free (SPF) environment. Consistent with the genotyping data (Figure 1B), extracts prepared from Stat2 null primary embryonic fibroblasts (PMEFs) did not express Stat2 protein (Figure 1C). Moreover, heterozygous (i.e., Stat2^{+/-}) PMEFs exhibit an ~50% reduction in Stat2 protein level (Figure 1C), suggesting a gene dosage effect. Notably, there was also ~50% reduction in the level of Stat1 expression in Stat2 null cells, indicating that the basal level of Stat1 in PMEFs

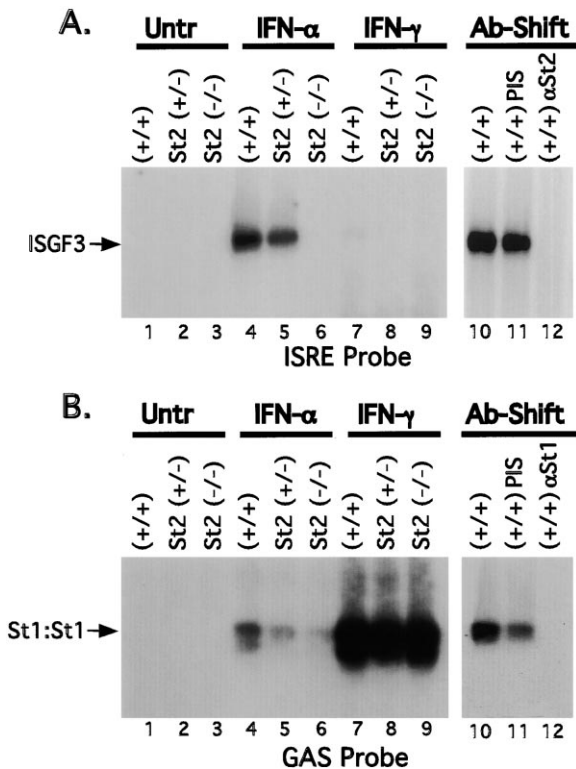


Figure 2. IFN-Dependent Activation of STAT Transcription Factors in Stat2 Null PMEFs

(A) IFN-dependent activation of ISGF-3. Whole-cell extracts were prepared from wild-type (+/+), *Stat2*^{+/-}, and *Stat2*^{-/-} PMEFs, both before and after stimulation with IFN- α (1000 U/ml; 20 min) or IFN- γ (66 U/ml; 20 min). The extracts were then evaluated by EMSA with an OAS ISRE probe. In lanes 11 and 12, wild-type (+/+) extracts were supershifted by the addition of either preimmune serum (PIS) or a Stat2-specific antibody (α Stat2), 30 min prior to incubation with probe. The mobility of ISGF-3 is indicated in the left margin.

(B) IFN-dependent activation of Stat1 homodimers. Whole-cell extracts from (A) were evaluated by EMSA with an IRF-1 GAS probe. In lanes 11 and 12, wild-type (+/+) extracts were supershifted by the addition of either preimmune serum (PIS) or a Stat1-specific antibody (α Stat1), 30 min prior to incubation with probe. The mobility of Stat1 homodimers is indicated in the left margin.

is dependent on Stat2 (see below). In sum, these studies demonstrated that the Stat2 locus had been effectively targeted.

Evaluation of STAT Activation

Type I IFNs transduce signals through the activation of ISGF-3. To evaluate ISGF-3 activation, extracts were prepared from PMEFs before and after stimulation with IFN- α and examined by electrophoretic mobility shift assay (EMSA) with an ISRE probe (Figure 2A). As anticipated, stimulation with IFN- α led to a robust induction of ISGF-3 in wild-type cells, which could in turn be “supershifted” with a murine Stat2-specific antibody (Park et al., 1999). However, this complex was absent in Stat2 null cells. Consistent with a decrease in the levels of Stat2 and Stat1, ISGF-3 formation was also modestly reduced in heterozygous Stat2 null cells. As anticipated, stimulation with IFN- γ did not lead to the formation of notable levels of ISGF-3 activity. These DNA binding

activities correlated directly with robust Stat1 and Stat2 tyrosine phosphorylation (data not shown).

Type I IFNs also promote the formation of Stat1 homodimers, albeit at modest levels. To evaluate this response, PMEFL extracts were evaluated with a GAS probe. As anticipated, IFN- γ stimulated a robust GAS binding activity (i.e., Stat1 homodimers), which could be supershifted with a Stat1-specific antibody (Figure 2B). No significant differences were noted in extracts prepared from knockout (i.e., *Stat2*^{+/-} and *Stat2*^{-/-}) cells after stimulation with a standard amount of IFN- γ (Meraz et al., 1996). Slightly faster migrating Stat1 β homodimers were also readily apparent in the IFN- γ -stimulated samples. The GAS binding activity recovered from IFN- α -stimulated cells was, however, considerably more modest. It was even further reduced in the *Stat2*^{+/-} and *Stat2*^{-/-} cells, suggesting that Stat2 contributes to the IFN- α -dependent activation of Stat1, as had recently been observed in a Stat2-deficient tumor line (Leung et al., 1995; Farrar et al., 2000).

Evaluation of STAT-Dependent Gene Expression

Type I IFNs mediate their biological effects through the induction of new genes. This predominately includes genes whose expression is dependent on ISGF-3 (i.e., ISRE-driven genes). The response of GAS-driven genes (i.e., Stat1 homodimer dependent) has been reported to be considerably more modest. To evaluate IFN-dependent gene expression, RNA was prepared from PMEFs before or after stimulation with a standard dose of IFN- α . This RNA was fractionated and then sequentially hybridized with probes for known ISRE-driven (e.g., MxA, OAS, and PKR; Stark et al., 1998; Schindler and Strehlow, 2000) and GAS-driven genes (e.g., IRF-1 and IGTP; Pine et al., 1994; Taylor et al., 2000). Stat1 and GAPDH probes were included as controls. As anticipated, IFN- α failed to induce the expression of ISGF-3 target genes (i.e., Mx, OAS, and PKR) in Stat2 null cells (Figure 3A). Two of these genes (i.e., Mx and OAS) demonstrated a significantly reduced level of expression in *Stat2*^{+/-} cells, consistent with their reduced levels of ISGF-3 found in those cells (Figure 2). The pattern of Stat1 expression paralleled that of an ISRE-driven gene. This observation, along with evidence supporting the existence of a type I IFN autocrine loop (Erlandsson et al., 1998; Sato et al., 1998; Takaoka et al., 2000), provides a potential explanation for the lower level of Stat1 found in *Stat2*^{-/-} fibroblasts.

When GAS-driven genes were examined, a different pattern emerged. As anticipated, IFN- γ potently induced IGTP and IRF-1 (Figure 3B). IFN- γ -dependent expression was robust in *Stat2*^{+/-} and *Stat2*^{-/-} cells as well, suggesting that the reduced levels of Stat1 were not significant for this pathway. In contrast, IFN- α -dependent induction of these genes was modest in wild-type PMEFs and absent in the *Stat2*^{-/-} cells. This paralleled the lower levels of Stat1 homodimer activity recovered from these cells and suggested that IFN- α -stimulated activation of Stat1 may be Stat2-dependent in fibroblasts. A similar conclusion has been reached in a Stat2-deficient human tumor fibroblast line (Leung et al., 1995; Farrar et al., 2000).

To explore the potential effect of a loss of Stat2 on IFN

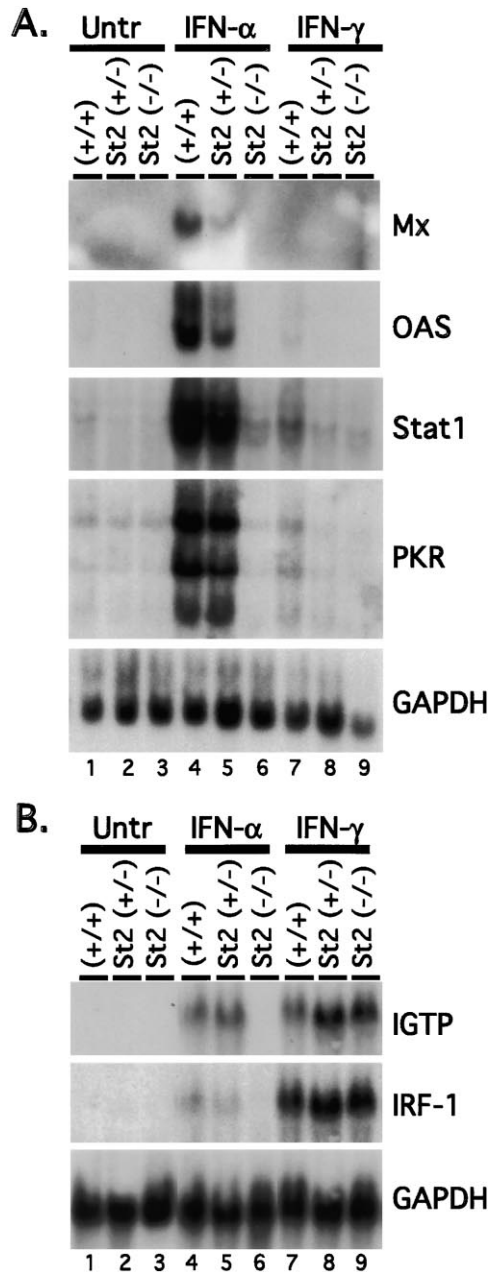


Figure 3. Evaluation of IFN-Dependent Gene Expression by Northern Blotting

(A) Twenty micrograms of total RNA was prepared from wild-type (+/+), *Stat2*^{+/-}, and *Stat2*^{-/-} PMEFs, both before and after stimulation with IFN- α (1000 U/ml; 6 hr) or IFN- γ (66 U/ml; 2 hr). The blot was then fractionated and sequentially hybridized with probes for MxA, OAS, Stat1, PKR, and GAPDH.

(B) Twenty micrograms of total RNA was prepared from wild-type (+/+), *Stat2*^{+/-}, and *Stat2*^{-/-} PMEFs, both before and after stimulation with IFN- α (1000 U/ml; 2 hr) or IFN- γ (66 U/ml; 2 hr). The blot was then fractionated and sequentially hybridized with probes for IGTP, IRF-1, and GAPDH.

signaling in hematopoietic lineages, the IFN-dependent expression of two cell surface proteins was examined. MHC class I expression was evaluated first, because it provided an opportunity to compare responses between macrophages and fibroblasts. Although regulation of

this locus appears to involve three enhancers (i.e., enhancer A, the IFN consensus site, and site- α), at least one of these sites is ISRE like, and Stat1 has been shown to be required for the response to IFNs (Johnson and Pober, 1994; Meraz et al., 1996; Lee et al., 1999). As anticipated, stimulation of wild-type PMEFs with IFN- α or IFN- γ led to a robust increase in MHC class I expression (Figure 4A, left). The response to IFN- α was, however, reduced in *Stat2*^{+/-} PMEFs and absent in *Stat2*^{-/-} PMEFs. This paralleled the pattern observed with ISRE-driven genes in our Northern blotting analysis (Figure 3A). The response to IFN- γ was, however, unaffected by the loss of Stat2. When MHC I expression was evaluated in peritoneal macrophages, a different pattern emerged. Again, IFN- α and IFN- γ both stimulated a robust increase in MHC I cell surface expression in wild-type cells (Figure 4A, middle). Remarkably, this pattern did not change in heterozygous and homozygous null cells, indicating that both IFN- α and IFN- γ will upregulate MHC I expression in a Stat2-independent manner in macrophages.

Struck by the difference in IFN- α -dependent regulation of MHC class I observed in primary fibroblasts and macrophages, a number of additional studies were carried out. We focused our attention on Stat1, because previous studies had suggested that IRF-1, a GAS-driven gene, may mediate the IFN- α -dependent regulation of MHC class I (Johnson and Pober, 1994). First, MHC class I expression was evaluated in macrophages collected from Stat1/Stat2 double knockout mice (Figure 4B, left). These cells were unable to significantly upregulate MHC class I expression in response to either IFN- α or IFN- γ , suggesting that Stat1 is the critical regulator. Since studies in *Stat2*^{-/-} PMEFs had determined that Stat1 levels were diminished and that this may have contributed to a defect in the regulation of GAS-driven genes by IFN- α , Stat1 protein levels were evaluated in macrophages. In striking contrast to PMEFs, *Stat2*^{-/-} macrophages exhibited normal levels of Stat1 protein (Figure 4B, middle). Finally, the ability of IFN- α to directly regulate GAS-driven genes was evaluated in macrophages. Due to the intrinsic limitations in recovering RNA from this tissue, an RT-PCR analysis was performed (Figure 4B, right). The IFN- α -stimulated expression of three GAS-driven genes (i.e., IRF-1, IP-10, and MIG) was normal in *Stat2*^{-/-} macrophages but not in *Stat1*^{-/-} or *Stat1/Stat2* double knockout (dko) macrophages. These observations highlight an important intrinsic difference between macrophages and fibroblasts regarding the IFN- α -dependent regulation of GAS-driven genes (which in turn regulate MHC class I expression) (Johnson and Pober, 1994; Martin et al., 1997).

Lymphocytes were the final primary tissue in which IFN- α -dependent gene expression was evaluated. For this study, Ly-6C, a T cell-specific marker whose expression is known to be upregulated by type I IFNs, was evaluated (Bothwell et al., 1988; Lin et al., 1998). Wild-type T cells exhibited a bimodal pattern of Ly-6C expression, suggesting a significant level of basal expression (Figure 4A, right). As anticipated, the level of Ly-6C increased with IFN- α treatment. In contrast, both basal and IFN- α -dependent Ly-6C expression was reduced in *Stat2*^{+/-} T cells and absent in *Stat2*^{-/-} T cells. This gene dosage effect was reminiscent of what had been observed in fibroblasts and indicated that Stat2 regulates

Table 1. Viral Yield from VSV-Infected Murine Embryonic Fibroblasts

Experiment 1			
Genotype	Unstim.	IFN- α	IFN- γ
wt	1	1.1×10^{-3}	2.1×10^{-4}
<i>Stat2</i> ^{+/-}	10.9	1.1×10^{-3}	2.2×10^{-2}
<i>Stat2</i> ^{-/-}	38.9	45.3	1.1
Experiment 2			
Genotype	Relative Titer		
wt	1		
<i>Stat2</i> ^{+/-}	18.3		
<i>Stat2</i> ^{-/-}	83.5		
<i>IFNAR1</i> ^{-/-}	45.7		
Experiment 3			
Genotype	Relative Titer		
wt	1		
<i>Stat2</i> ^{+/-}	12		
<i>Stat2</i> ^{-/-}	26.2		
<i>Stat2</i> ^{-/-} + <i>Stat2</i> ²	6.92		
<i>Stat2</i> ^{-/-} + <i>Stat2</i> ¹	10.85		
<i>Stat1</i> ^{-/-}	8.31		

Murine embryonic fibroblasts (2×10^4 /well) were stimulated for 24 hr with IFN (IFN- α_{AD} at 1000 U/ml or IFN- γ at 66 U/ml), prior to infection with VSV (multiplicity of infection [MOI] = 1×10^{-6} ; 2 hr). Twenty-four hours postinfection, supernatants were collected and titered on HeLa cells. Results are presented as the relative titer, compared to that obtained with wild-type embryonic fibroblasts. The fibroblasts employed in experiments one and two were primary, whereas those in experiment three were immortalized (i.e., by crisis). Each of the results are representative of three independent experiments.

istence of a type I IFN autocrine loop. This suggested that *Stat2*^{-/-} cells should be inherently more sensitive to viral infection. Consistent with this prediction, unstimulated *Stat2*^{-/-} PMEFs produced on average ~60-fold more virus than wild-type PMEFs (Table 1, experiments 1 and 2). Providing independent support for the existence of a type I IFN autocrine loop, *IFNAR1*^{-/-} PMEFs exhibited an analogous pattern of increased viral production. Consistent with the gene dosage effect observed in DNA binding and gene expression studies, the *Stat2*^{+/-} cells exhibited an intermediate phenotype. Similar observations were made with immortalized murine embryonic fibroblasts (i.e., MEFs; see Table 1, experiment 3).

To confirm that defects found in *Stat2* knockout cells were solely due to the loss of *Stat2*, *Stat2* expression was reestablished in *Stat2*^{-/-} MEFs through stable transfection. Cells stably reexpressing *Stat2* were selected by their ability to upregulate expression of MHC I in response to IFN- α . Two pools of "rescued" cells (i.e., *Stat2*^{-/-} + *Stat2*¹ and *Stat2*^{-/-} + *Stat2*²) were collected and evaluated for their biological response to IFN- α . The ability of these cells to form ISGF-3 and activate target genes was effectively restored (data not shown). Consistent with a good but incomplete restoration in IFN- α signaling, these cells exhibited an intrinsic ability to resist viral infection that was similar to *Stat2*^{+/-} MEFs (Table 1, experiment 3). In sum, these studies confirm that defects in the biological responses observed in *Stat2*^{-/-} cells can be attributed to a single gene, *Stat2*, and provide further support for the existence of a type I IFN autocrine loop.

Table 2. Splenic and Hepatic Viral Burdens from VSV-Infected Mice

Genotype	Spleen	Liver
wt	$1.60 \times 10^3 \pm 657$	59.5 ± 13.1
<i>Stat2</i> ^{-/-}	$5.48 \times 10^7 \pm 2.30 \times 10^7$	$1.23 \times 10^7 \pm 1.55 \times 10^7$
<i>Stat1</i> ^{-/-}	$6.97 \times 10^7 \pm 5.89 \times 10^7$	$1.59 \times 10^7 \pm 5.65 \times 10^6$
<i>Stat1/Stat2</i> dko	$4.18 \times 10^8 \pm 1.25 \times 10^8$	$6.51 \times 10^8 \pm 2.32 \times 10^8$

Thirty-six hours after intravenous infection with 4 pfu of VSV, spleens and livers were harvested. Viral burden (pfu/g) was determined by measuring the viral titer of organ homogenates on HeLa cells. Organs from four mice were included in each group.

Antiviral Response of *Stat2* Null Mice

Studies with IFN receptor knockout mice have determined that type I but not type II IFNs mediate a potent innate antiviral response to VSV infection (Müller et al., 1994). That is, *IFNAR1*^{-/-} mice were found to be exquisitely sensitive to infection with low doses of virus. Consistent with the requirement of *Stat1* for ISGF-3 formation, *Stat1* null mice exhibited a similar increase in their susceptibility to VSV infection (Meraz et al., 1996). In our studies, wild-type and *Stat2*^{+/-} mice were resistant to infection with doses of VSV of 2.5×10^6 pfu (HeLa cell units). However, *Stat1* and *Stat2* null mice succumbed to infection with doses that were ~6 logs lower. The majority of *Stat1*^{-/-} and *Stat2*^{-/-} mice died between 3 to 6 days after infection with doses of 1–3 pfu of VSV. Although there was a modest trend toward earlier death in the *Stat2*^{-/-} mice, this was more apparent when *Stat1/Stat2* double knockout mice were infected with VSV. In an effort to quantitate these differences more accurately, viral burdens in the spleen and liver were determined 36 hr postinfection in all three lines of mice (Table 2). As anticipated, these viral burdens were low in wild-type mice, especially in liver. Both the *Stat1*^{-/-} and *Stat2*^{-/-} mice exhibited substantially larger (i.e., up to 5 logs), yet similar, viral burdens. The viral burdens were modestly higher in the *Stat1/Stat2* double knockout mice. The demonstration of parity in the viral burdens in *Stat1*- and *Stat2*-deficient mice is consistent with the notion that a *Stat1*-*Stat2* heterodimer (i.e., ISGF-3) plays the critical role in mediating antiviral responses. However, the modestly elevated sensitivity of double knockout mice leaves open the possibility that these transcription factors may transduce some independent signals (e.g., *Stat1* homodimers).

Lymphocyte Function in *Stat2* Null Mice

Recent studies have suggested that type I IFNs regulate the maturation of lymphocytes. Resident bone marrow macrophages and stromal fibroblasts have been shown to secrete type I IFNs, which can impair lymphoid proliferation and maturation (Wang et al., 1995; Pilling et al., 1999; Oritani et al., 2000). Either virally induced or administered type I IFNs will block the development of B cells and T cells (Binder et al., 1997; Lin et al., 1998). Careful analysis of these T cells has determined the block to occur during the pro-T cell stage differentiation, leading to a profound loss in double-positive (i.e., CD4⁺/CD8⁺) thymocytes (Lin et al., 1998).

Our preceding studies (Figure 4A) had already documented a significant defect in the ability of IFN- α to

induce Ly-6C expression in *Stat2*^{-/-} T cells. To extend this analysis to the antiproliferative activity of type I IFN on T cells, splenic lymphocytes were harvested. When wild-type and *Stat2*^{-/-} cells were stimulated with concanavalin A (Con A; a T cell mitogen), they exhibited the anticipated increase in proliferation, as measured by propidium iodide staining. However, the ability of IFN- α to block the proliferative response was lost in the *Stat2*^{-/-} lymphocytes (data not shown).

Next, the potential role of IFNs in T cell expansion was evaluated *in vivo*. Mice were challenged with an injection of dexamethasone, which is known to promote a dramatic loss of CD4/CD8 double-positive T cells (Wang et al., 1999). This is normally followed by a burst of lymphopoiesis that first entails a transient increase in a CD8⁺/CD3^{lo} precursor population. These cells rapidly mature into CD4⁺/CD8⁺ T cells, which then give rise to CD4/CD3^{hi} and CD8/CD3^{hi} single-positive cells that are found in the periphery. When wild-type and *Stat2* null mice were injected with dexamethasone, double-positive thymocytes were largely eliminated by 2 days (i.e., the nadir; Figure 5). There was, however, a slight suggestion that *Stat2*^{-/-} mice were beginning to repopulate the CD8⁺ and CD4⁺/CD8⁺ populations. Two days later (i.e., day 4 postdexamethasone), the difference between wild-type and *Stat2* null mice was striking. Wild-type mice had exhibited a dramatic increase in their CD8⁺ population (49% versus 23.9%), whereas the *Stat2* cells had already substantially repopulated the CD4/CD8 double-positive compartment (7.3% versus 46.8%). This trend continued on day 5, at which time the *Stat2* null mice had almost returned to baseline. Recovery in the wild-type mice continued to lag behind, although less dramatically. These observations support a model where endogenously secreted IFNs serve to antagonize stress-dependent expansions of T cells through a *Stat2*-dependent pathway.

Discussion

IFN receptor knockout studies have demonstrated that type I and type II IFNs promote distinct biological responses (Huang et al., 1993; Müller et al., 1994; Lu et al., 1998). Type I IFNs achieve this through the activation of two transcription factors, ISGF-3 (Stat1 + Stat2 + IRF-9) and Stat1 homodimers. IFN- γ (type II IFN) transduces signals exclusively through Stat1 homodimers. This led to the conclusion that Stat2 and ISGF-3 are responsible for the unique properties of type I IFNs (Schindler and Strehlow, 2000). Although gene targeting studies have confirmed the critical role Stat1 plays in the biological response to type I and II IFNs, they have provided little insight into the relative role of ISGF-3 and Stat1 homodimers in the response to type I IFNs (Durbin et al., 1996; Kimura et al., 1996; Meraz et al., 1996). The generation of *Stat2* knockout mice has, however, provided an opportunity to explore the unique role Stat2 and ISGF-3 play in the biological response to type I IFNs.

The initial studies were carried out in *Stat2*^{-/-} fibroblasts and indicated that these cells were defective in their ability to mediate a number of responses to type I IFN. This included defects in the activation of ISGF-3,

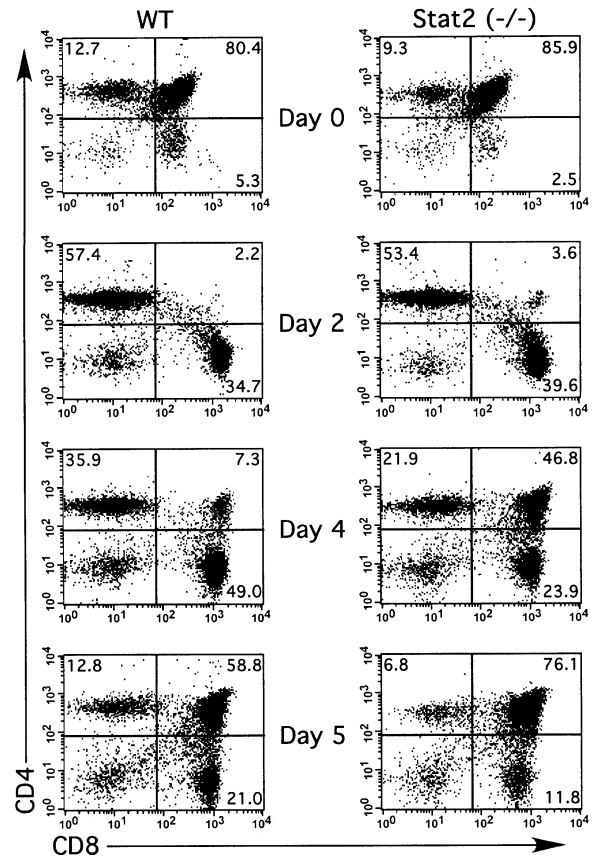


Figure 5. T Cell Proliferation after a Dexamethasone Challenge

Wild-type and *Stat2*^{-/-} mice were intraperitoneally injected with 1.0 mg dexamethasone or PBS vehicle. Two, four, and five days later, thymic cells were harvested, stained for CD4 and CD8, and evaluated by flow cytometry. The number of cells present in each quadrant are indicated as a percent of total cells plotted. Similar results were obtained in three independent experiments.

Thirty-six hours after intravenous infection with 4 pfu of VSV, spleens and livers were harvested. Viral burden (pfu/g) was determined by measuring the viral titer of organ homogenates on HeLa cells. Organs from four mice were included in each group.

induction of ISGF-3 target genes, and stimulation of an antiviral response. However, defects were also noted in the activation of GAS-driven genes. Previous studies had suggested that Stat2 serves as the docking site necessary for Stat1 activation by type I IFNs (Leung et al., 1995; Farrar et al., 2000). Although our studies in *Stat2*^{-/-} fibroblasts were consistent with this model, our studies with *Stat2*^{-/-} macrophages were not (see below). In addition, immunoblotting studies determined that the level of Stat1 protein was reduced in *Stat2*^{-/-} fibroblasts, suggesting a second reason for the defect in IFN- α -stimulated Stat1 homodimer signaling.

The reduced levels of Stat1 in the *Stat2*^{-/-} fibroblasts highlighted a previously unrecognized role for Stat2 in the regulation of basal levels of this transcription factor and raised the possibility of a *Stat2*-dependent auto-crine loop. Consistent with this idea, previous studies have determined that type I IFNs function in an autocrine manner, both in tumor lines (Resnitzky et al., 1986; Mattei et al., 1994; Kawabata et al., 1997; Oritani et al.,

2000) and primary cells (Erlandsson et al., 1998; Sato et al., 1998; Takaoka et al., 2000). This loop mediates the secretion of basal levels of type I IFN as well as the robust IRF-7-dependent production of late type I IFNs during viral infection (Marie et al., 1998; Sato et al., 1998; Takaoka et al., 2000). Additional roles have been suggested for the autocrine loop as well (Lin et al., 1998; Lee et al., 1999; Marrack et al., 1999; Oritani et al., 2000). The current study both supports the existence of this loop in fibroblasts and splenocytes (see below) and demonstrates that it is Stat2 dependent. The data in fibroblasts include (1) the observation that both *IFNAR1*^{-/-} and *Stat2*^{-/-} cells are inherently more susceptible to viral infection than wild-type cells, (2) the demonstration that reexpression of Stat2 in *Stat2*^{-/-} cells restores basal Stat1 expression to its normal level, and (3) sensitive assays demonstrating a basal level of ISGF-3 and target gene expression in unstimulated MEFs (data not shown; Lee et al., 1999; Takaoka et al., 2000). Of note, the formation of basal ISGF-3 activity correlated directly with cell density, was absent in *Stat2*^{-/-} cells, and blocked by the addition of IFN- α/β -blocking antibodies (data not shown). Finally, we would like to argue that the inability of *Stat2*^{-/-} fibroblasts to mediate an effective antiviral response to IFN- γ stimulation (in vitro) is, at least in part, a manifestation of this autocrine loop, as other studies on *IFNAR1*^{-/-} tissues have indicated (Müller et al., 1994; Takaoka et al., 2000).

Indirect studies have suggested that the type I IFN autocrine/paracrine loop serves to regulate lymphocyte function. This includes studies identifying stromal and dendritic cells that secrete type I IFNs (Siegal et al., 1999; Oritani et al., 2000) and studies demonstrating that type I IFNs both downregulate lymphopoiesis (Binder et al., 1997; Lin et al., 1998) and promote the survival of memory T cells, especially from the CD8⁺ lineage (Tough et al., 1996; Marrack et al., 1999). Studies on lymphocytes from *Stat2*^{-/-} mice support these observations. They indicate that a Stat2-dependent autocrine/paracrine loop regulates both the rapid expansion of lymphocytes in response to stress and the basal expression of cell surface markers. Preliminary observations suggest that Stat2 may also participate in the IFN- α -dependent preservation of memory T cells. This raises the question as to how type I IFNs can mediate both an antiapoptotic process in mature cells and an antiproliferative response in immature cells. We hypothesize that both are dependent on the antiproliferative activity of these IFNs. In immature cells, the effect is direct, but, in mature cells, a block in proliferation protects cells from programmed cell death. Perhaps some of these activities account for the beneficial effect of IFN- β in the treatment of multiple sclerosis.

Another potentially important immunoregulatory response of type I IFNs appears to be on the expression of MHC class I (Biron, 1998). The regulation of this locus is complicated and involves at least three promoter elements (Martin et al., 1997). One element fits an ISRE consensus site, binds IRF-1, and has been shown to regulate response to type I IFNs (Johnson and Pober, 1994). Consistent with the activity of an autocrine loop, recent studies have shown that the basal level of MHC class I expression is decreased in Stat1 knockout lymphocytes (Lee et al., 1999). Intriguingly, our studies indi-

cate that the IFN- α -dependent regulation of MHC class I may vary in a tissue-specific manner. We find in fibroblasts that the IFN- α -stimulated expression of MHC class I (as well as all target genes we examined) is dependent on Stat2. For Stat1-dependent genes (i.e., GAS-driven), this appears to be achieved both through the regulation of Stat1 levels and the IFN- α -dependent activation of Stat1. In contrast, in macrophages, IFN- α -stimulated expression of GAS-driven (i.e., Stat1 homodimer sites) genes is independent of Stat2. Of note, the IFN- α -dependent expression of MHC class I is a secondary response. However, our studies indicate that expression of this locus is dependent on Stat1 homodimers (e.g., via IRF-1; Johnson and Pober, 1994). Thus, in striking contrast to what is found in fibroblasts, the IFN- α -induced expression and activation of Stat1 in macrophages is independent of Stat2. This surprising dichotomy in type I IFN response may reflect the unique role macrophages play in regulating immune response. It also challenges the notion that Stat2 is physically involved in the IFN- α -stimulated activation of Stat1 (Leung et al., 1995). In sum, these observations define the critical role Stat2 and ISGF-3 play in type I IFN biology and the pervasive role this family of IFNs plays in the regulation of immune response.

Experimental Procedures

Generation of Stat2-Deficient Mice

Screening a 129/Sv genomic phage library (Stratagene) with a murine Stat2 cDNA probe led to the isolation of two overlapping genomic clones (14.5 and 15.5 kb). After mapping the genomic locus, a targeting vector consisting of a 7 kb 5' arm (a NotI/EcoRV fragment) and a 1.1 kb 3' arm (an XbaI/Sall fragment) was assembled in pBlue-script (Figure 1A). The linearized targeting vector was electroporated into W9.5 embryonic stem (ES) cells (gift of C. Stewart) and recombinants selected with G418 and gancyclovir (Wurst and Joyner, 1993). Homozygous null mutant ES lines were generated by raising the level of G418 for *Stat2*^{+/-} ES to 5 mg/ml (Mortenson et al., 1992). To create Stat2 null mice, *Stat2*^{-/-} ES cells were introduced into blastocysts harvested from *RAG1*^{-/-} mice (Jackson Labs). Chimeric male offspring were mated with 129Sv/J females to yield heterozygous Stat2 knockout mice. These were then intercrossed to homozygosity. Recombination events were initially identified by Southern blotting with a Sall-BamHI (750 bp) genomic Stat2 DNA fragment that lies 3' to the targeted locus (probe SB; see Figure 1). However, subsequent genotyping was carried out either with a more robust murine Stat2 cDNA probe (a PstI fragment spanning bp 1338-2118; probe P; see Figure 1; Park et al., 1999) or by PCR (common-3'-St2, GAGGTAAGAGGTTCCGAGTGTGTT; NeoP, CAGCGCATCCGCTTCTATCGCCTTCTTG; and wt-5'-St2, GGATTCTGAATCAGGCTCAAAGAG). Stat1 null and IFNAR1 null mice were generous gifts from R. Schreiber and M. Aguet (Müller et al., 1994; Meraz et al., 1996). Stat1/Stat2 double knockout mice were obtained by crossing the appropriate strains. Genotyping was done by established PCR assays (see above and Meraz et al., 1996) and confirmed by immunoblotting.

RNA

Primary murine embryonic fibroblast (PMEF) RNA was prepared by guanidinium isothiocyanate lysis (Chomczynski and Sacchi, 1987). Immortalized murine embryonic fibroblast (MEF) RNA was prepared with RNazol (Life Technologies), as directed by the supplier. RNA was fractionated on 1.0% agarose/formaldehyde gels, transferred to nylon membranes, and hybridized with [³²P]cDNA probes labeled to a specific activity of $\sim 5 \times 10^8$ cpm/ μ g (Random Prime Labeling; Boehringer Mannheim and NEN). Probes included MxA cDNA (gift of D. Levy), full-length OAS cDNA (gift of D. Levy), full-length PKR cDNA (gift of A. Koromilas), full-length murine Stat1 cDNA (C. Schind-

ler, unpublished data), IRF-1 cDNA (Pine et al., 1994), IGTP cDNA (1.0 kb EcoRI fragment) (Taylor et al., 2000), and rat GAPDH cDNA (Azam et al., 1997). Macrophage RNA was prepared with an RNAeasy kit, as directed (Qiagen), and then evaluated by RT-PCR. In brief, 375 ng of total RNA was reverse transcribed with Superscript, in a volume of 20 μ l (Life Technologies, Inc). Two microliters of reverse transcription (RT) template was then incubated with specific primers and amplified with Taq polymerase (Life Technologies, Inc.) for 25 to 30 cycles (95°C for 1 min, 55°C for 30 s, and 72°C for 1 min). PCR primers included IRF-1 (CTGAGGTGTAAGGCAGAGGC, TCTAGGGCCAGTCTATGCT), IP-10 (CAGTGGATGGCTAGTCC TAATTG, AGTATCTTGATAACCCCTGGGA), MIG (TACAAATCCCT CAAAGACCTCAA, ACGGAAGGCTTTTCAGTACAATC), and GAPDH (ACCACAGTCCATGCCATCAC, TCCACCACCCTGTTGCTGTA).

Cell Culture and Protein Extracts

PMEFs were prepared from E12.5 day embryos (Todaro and Green, 1963) and their genotype confirmed by PCR analysis. Immortalized MEFs were generated by continuous passage of subconfluent PMEFs. Stat1 immortalized MEFs were a generous gift from R. Schreiber. All PMEFs and MEFs were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (Life Technologies). HeLa S3 cells (ATCC) were grown in DMEM, supplemented with 10% newborn calf serum and penicillin/streptomycin (Life Technologies). Splenocytes were harvested through a nylon mesh, counted, and then cultured in RPMI/10% FCS. Resident peritoneal macrophages were harvested by lavage and then cultured in DMEM/10% FCS (Meraz et al., 1996).

Whole cell and nuclear extracts were prepared from cells before or after treatment with IFN ($\text{IFN-}\alpha_{\text{AD}}$ at 1000 U/ml; murine $\text{IFN-}\gamma$ at 66 U/ml; Pestka Biomedical Laboratories) for 20 min at 37°C, as previously described (Park and Schindler, 1998). Extracts were evaluated by electrophoretic mobility shift assay (EMSA) or immunoblotting, as previously described (Pine et al., 1994; Park and Schindler, 1998). The probes employed for EMSA were the OAS ISRE site (5'-agct TCTGAGGAAACGAAACCAACAG-3' [Matsumoto et al., 1999]) and the IRF-1 GAS element (5'-gatc GATTTCCCGAAAT-3' [Pine et al., 1994; Park and Schindler, 1998]). Antibodies employed for supershifts and immunoblotting studies included α -Stat1⁰⁰⁰ (Schindler et al., 1992), α -MuStat2 (Park et al., 1999), α -Stat3 (H-190; Santa Cruz), and α - β -actin (Sigma).

Stat2 cDNA (Park et al., 1999) was introduced into Stat2 null MEFs by calcium phosphate transfection (Strehlow and Schindler, 1998). Positive cells were isolated by sorting for cells with $\text{IFN-}\alpha_{\text{AD}}$ -dependent (1000 U/ml; 72 hr) expression of MHC I (H-2K^b; AF6-88.5; Pharmingen). The most intensely staining cells (top 10%) were collected.

VSV Infections

Vesicular stomatitis virus (VSV, Indiana strain; gift from R. Pine) was cultured and titered on HeLa cells, as previously described (Pine, 1992). For viral yield assays, 100 μ l of virus was absorbed to cells (2.0×10^4) in a microtiter well and then washed. Twenty-four hours later, cell supernatants were collected and titered by plaque assay. For animal studies, mice were injected in their tail vein with 100 μ l of virus. Thirty-six hours later, organ homogenates were prepared by dounce homogenization and titered.

Flow Cytometry

Single-cell suspensions were prepared from thymus, spleen, resident peritoneal macrophages, or typsinized fibroblasts and then stained with the appropriate antibodies (PharMingen). Antibodies employed in these studies included (1) FITC-conjugated antibodies (α -CD8a [53-6.7], α -H-2K^b [AF6-88.5], IgG_{2a}K [the isotype-matched control; G155-178], and α -Ly-6C [AL-21]), (2) PE-conjugated antibodies (α -CD4 [GK1.5]), and (3) APC-conjugated antibodies (α -CD3e [145-2C11], α -CD4 [RM4-5], α -CD8a [53-6.7], α -CD11b [Mac-1 α ; M1/70]). Biotin stains were revealed with PerCP-conjugated streptavidin. All analyses were performed using a FACSCalibur flow cytometer and analyzed with CellQuest software (Becton Dickinson). Ly-6C (splenocyte) and MHC class I (H-2K^b; macrophage and fibroblast) expression was evaluated after 72 hr of stimulation with $\text{IFN-}\alpha_{\text{AD}}$ (1000 U/ml) or $\text{IFN-}\gamma$ (66 U/ml).

For the dexamethasone challenge, mice were injected intraperitoneally, with 1.0 mg dexamethasone (Sigma) or PBS in 200 μ l bolus. After 2, 4, and 5 days, single-cell suspensions were prepared from thymi, stained for CD4 and CD8, and analyzed by flow cytometry.

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