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Anti-STAT6 CTL activity in *Stat6*$^{-/-}$ mice

A cautionary tale

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The generation of germline gene mutations in mice has been an invaluable tool for experimental biology. However, studying immune responses that develop in the absence of a specific protein that could alter thymic selection complicates experimental interpretations. We observed that CD8$^+$ T cells from *Stat6*$^{-/-}$ mice displayed "autoreactivity" to STAT6-expressing cells, associated with specific STAT6 peptides binding to MHC class I molecules. These results suggest caution in interpreting experiments where STAT6-expressing cells are transferred into *Stat6*$^{-/-}$ mice, or where adoptive transfer of *Stat6*$^{-/-}$ lymphocytes is performed. Our results further highlight additional considerations when studying immune responses involving cell transfer into gene-deficient mice.

Introduction

Signal transducer and activator of transcription (STAT) family proteins are requisite molecules in the development of T helper subsets. STAT1 and STAT4 are required for the development of Th1 cells that secrete IFNγ and mediate immunity to intracellular pathogens. STAT3 promotes Th17 development and the secretion of IL-17, a cytokine required for immunity to extracellular bacterial and fungal infections. STAT6 is required for the differentiation of Th2 and Th9 cells, which contribute to extracellular parasite immunity.[1]

The in vivo function of STAT6 has been defined largely following the generation of gene-deficient mice. *Stat6*$^{-/-}$ mice develop normally, breed as homozygotes, but have altered immune responses in vivo.[2-5] In the absence of STAT6, mice are resistant to the development of allergic inflammation, and have exaggerated responses to inflammatory insults resulting in greater immunity to intracellular pathogens, but greater susceptibility to autoimmune inflammation, with differences in phenotype attributed to different mouse lines with targeted *Stat6* alleles.[2,6] Some of the studies that defined these functions utilized experiments involving adoptive transfer of lymphocytes, or where cells or tumors were adoptively transferred into *Stat6*$^{-/-}$ hosts. Whether cells behave normally in these transfer experiments has not been extensively examined.

In experiments to test if deficiency in STAT4 or STAT6 affected development of CTL specific for non-classical MHC molecules, we observed autoreactivity of *Stat6*$^{-/-}$ CTL for STAT6-expressing cells. The development of STAT6-specific reactivity by *Stat6*$^{-/-}$ CD8$^+$ T cells might have important implications for the interpretation of experiments where *Stat6*$^{-/-}$ CD8$^+$ T cells are exposed to STAT6 expressing cells in vivo.

Results

To examine the responses of BALB/c, *Stat4*$^{-/-}$ and *Stat6*$^{-/-}$ mice (H2^d, Qa1^b and Mta^a) as responders against NZB/B1NJ (NZB) (H2^d, Qa1^a and Mta^b) stimulator cells, we generated CTL lines from each strain. We found no consistent differences in the ability of the *Stat4*$^{-/-}$ or *Stat6*$^{-/-}$ CD8$^+$ T cells to develop responder CTL against Qa1^a or Mta^b antigens, compared with BALB/c T cells (**Fig. 1A–C**). However, we did observe that *Stat6*$^{-/-}$ anti-NZB CTL could lyse target cells from *Stat4*$^{-/-}$ and BALB/c mice (**Fig. 1C**). In contrast, BALB/c and *Stat4*$^{-/-}$ responders did not lyse BALB/c, *Stat4*$^{-/-}$ or *Stat6*$^{-/-}$ cells (**Fig. 1A and B**). The ability of *Stat6*$^{-/-}$ CTL to lyse these target cells was unexpected because *Stat4*$^{-/-}$, *Stat6*$^{-/-}$ and BALB/c are syngeneic, differing only in the regions adjacent to the targeted *Stat4* or *Stat6* alleles.

To confirm these data and to rule out problems due to the use of one specific mouse strain (NZB), we examined responses of BALB/c, *Stat4*$^{-/-}$ and *Stat6*$^{-/-}$ as stimulators and responders of CTL in similar assays. *Stat6*$^{-/-}$ anti-BALB/c and *Stat6*$^{-/-}$ anti-*Stat4*$^{-/-}$ responder CTL also lyse *Stat4*$^{-/-}$ and BALB/c target cells (**Fig. 1D and E**), whereas *Stat4*$^{-/-}$ anti-*Stat6*$^{-/-}$ CTL do not lyse *Stat4*$^{-/-}$, *Stat6*$^{-/-}$ or BALB/c target cells (**Fig. 1F**).

To determine the restricting MHC molecule involved in these responses, we examined mice expressing different MHC haplotypes and found the responses were restricted to *H2*^{d}. *Stat6*$^{-/-}$ anti-*Stat4*$^{-/-}$ or anti-BALB/c CTL lysed target cells from H2^d (BALB/c, *Stat4*$^{-/-}$ and NZB) mouse strains (**Figs. 1D and E**

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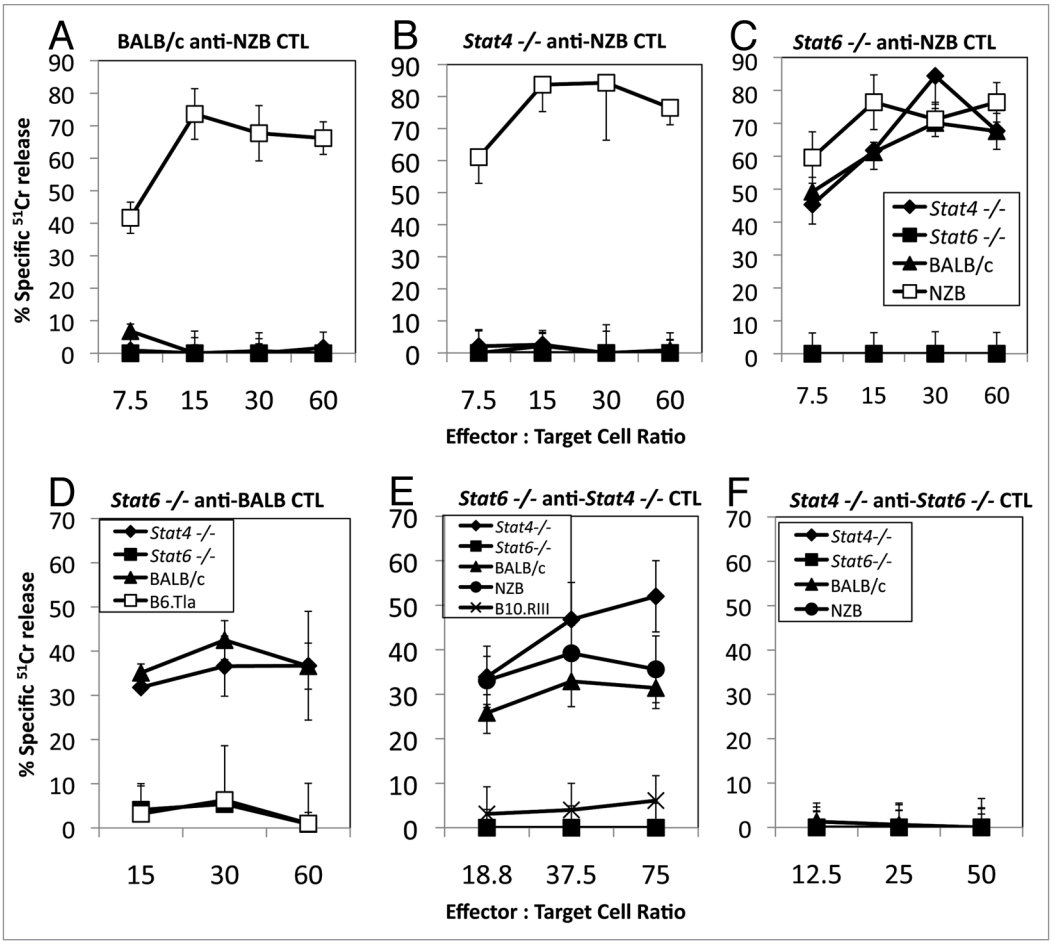

Figure 1. *Stat6*$^{-/-}$ CTL lyse *Stat6*$^{+/+}$ targets cells. CD8^{+} CTL lines generated from secondary MLCs were assayed against *Stat6*$^{+/+}$ and *Stat6*$^{-/-}$ Con A blast (CAB) target cells in a standard ^{51}Cr release assay. (**A**) BALB/c, (**B**) *Stat4*$^{-/-}$ and (**C**) *Stat6*$^{-/-}$ anti-NZB responder CTL were assayed against NZB, BALB/c, *Stat4*$^{-/-}$ or *Stat6*$^{-/-}$ CAB target cells. Lysis of NZB cells in (**A**) and (**B**) represents Mta^{b} and/or Qa1^{a} restricted responses. *Stat6*$^{-/-}$ anti-NZB CTL (**C**) lyse NZB, *Stat4*$^{-/-}$ and BALB/c, but not *Stat6*$^{-/-}$ target cells, indicating lysis of *Stat6*$^{+/+}$ cells. (**D**) *Stat6*$^{-/-}$ anti-BALB/c, (**E**) *Stat6*$^{-/-}$ anti-*Stat4*$^{-/-}$ CTL and (**F**) *Stat4*$^{-/-}$ anti-*Stat6*$^{-/-}$ MLC were assayed against BALB/c, *Stat4*$^{-/-}$, *Stat6*$^{-/-}$, *H2*^{b}, B6.*Tla*^{a} or H2^{r} (B10.RIII) CAB target cells. All data are the mean ± SD of triplicate assays and are representative of three or more experiments.

and 2), but not cells expressing H2^{b} (B6.Tla^{a}), H2^{r} (B10.RIII), or H2^{k} (C3H/HeJ and B10.BR) (**Figs. 1D and E and 2C**), suggesting that the CTL were not alloreactive. To determine whether the restricting element(s) are H2-K^{d}, -D^{d} and/or -L^{d} molecules, we further compared *Stat6*$^{-/-}$ CTL responses against A/J (H2-K^{k}, -D^{d} and -L^{d}), BALB/c (H2-K^{d}, -D^{d} and -L^{d}) and BALB.dm2 (H2-K^{d}, -D^{d} and L null) target cells using two different *Stat6*$^{-/-}$ CTL lines. **Figure 2** illustrates that either H2-K^{d}, -D^{d} or -L^{d} (**Fig. 2A and B**) are recognized by the polyclonal responder CTL. Since H2^{k} cells (C3H.HeJ or B10.BR) were not lysed by *Stat6*$^{-/-}$ CTL (**Fig. 2C**), recognition of cells from A/J (H2-K^{k}, -D^{d} and -L^{d}) by the 241.44 line shows restriction to H2-D^{d} and/or -L^{d} (**Fig. 2A**). In contrast, the lack of response of the 240.43 line demonstrates the failure of these cells to recognize H2-D^{d}, suggesting the reactivity against another H2^{d} class I molecule, most likely H2-K^{d} (**Fig. 2B**). H2-K^{d} restricted CTL were also able to lyse BALB.dm2 cells, which express H2-K^{d} and H2-D^{d}, but not H2-L^{d}, further suggesting that H2-L^{d} restriction is not a prominent reactivity in these CTL lines (**Fig. 2D**). Thus, *Stat6*$^{-/-}$ CTL can develop with specificities restricted to H2-K^{d}, H2-D^{d} or H2-L^{d}.

Confirming these data, in **Figure 3** we show that pre-incubating A/J or *Stat4*$^{-/-}$ target cells with monoclonal antibodies reactive with H2-K^{d}, -D^{d} and H2-L^{d} molecules blocks target cell recognition and lysis by these CTL. Lysis of Stat4$^{-/-}$ or A/J target cells is blocked by monoclonal antibody 30-5-7S that reacts with H2-D^{d} or -L^{d} but not H2-K^{d} or H2-K^{k}, confirming restriction of this line to H2-L^{d} (**Fig. 3A and B**). In **Figure 3C**, lysis of *Stat4*$^{-/-}$ targets by the *Stat6*$^{-/-}$ anti-BALB 240.43 line is not blocked by 30-5-7S, but rather is blocked by the two antibodies which bind H2-K^{d} molecules, 34-1-2S and 34-7-23S, demonstrating restriction to H2-K^{d}. Taken together, the data clearly demonstrate that *Stat6*$^{-/-}$ anti-BALB/c or anti-*Stat4*$^{-/-}$ CTL are restricted to either H2-K^{d}, -D^{d} or -L^{d}.

Since the primary difference between BALB/c or *Stat4*$^{-/-}$ and *Stat6*$^{-/-}$ mice is the *Stat6* gene product, we tested whether

Stat6$^{-/-}$ CTL recognized peptides derived from the STAT6 protein. We tested a STAT6 peptide previously shown to be presented by tumor cells[7] and observed the H2-K^d restricted CTL recognize *Stat6*$^{-/-}$ target cells incubated with the SYWSDRLIL peptide (**Fig. 4**), which fits the H2-K^d consensus motif of XYX$_6$(I/L/V)L. We additionally tried several STAT6 peptides that fit the H2-D^d or H2-L^d consensus motifs, XGPX(K/R)X$_3$(L/I/F) and XPX$_6$(L/M/F), respectively, but none conferred reactivity.

Discussion

These data demonstrate responses by *Stat6*$^{-/-}$ CTL against H2^d cells from *Stat6*-expressing mouse strains including BALB/c, NZB and *Stat4*$^{-/-}$. The polyclonal CTL populations generated from the *Stat6*$^{-/-}$ responder spleen cells initially recognized K^d, -D^d and -L^d restricted antigens, although with weekly passage in vitro some populations eventually self-selected for a predominantly H2-K^d, -D^d or -L^d restricted phenotype. Use of target cells from recombinant inbred mouse strains and blocking by antisera specific for H2^d class I molecules confirms their MHC restriction. The H2-K^d restricted CTL recognized the STAT6-derived peptide SYWSDRLIL.

These data clearly illustrate the importance of running negative control experiments, particularly when working with genetically altered strains of mice. In some situations the responses described here may not obviously affect experimental results. For example, in evaluations of immune responses within *Stat6*$^{-/-}$ mice against exogenous antigens or allergens, or in studies of inflammation or hypersensitivity, the results may show no effects easily attributable to altered peptide presentation by MHC class I molecules on *Stat6*$^{-/-}$ cells.[2,8] However, it is possible that results could be influenced by subtle changes in reactivity of cells maturing in the *Stat6*$^{-/-}$ environment and/or by the unusual peptide repertoire transferred on H2^d molecules with *Stat6*$^{-/-}$ cells. Such changes in immune responses due to these altered peptide expression might be negligible or difficult to identify and quantitate, but should nonetheless be considered.

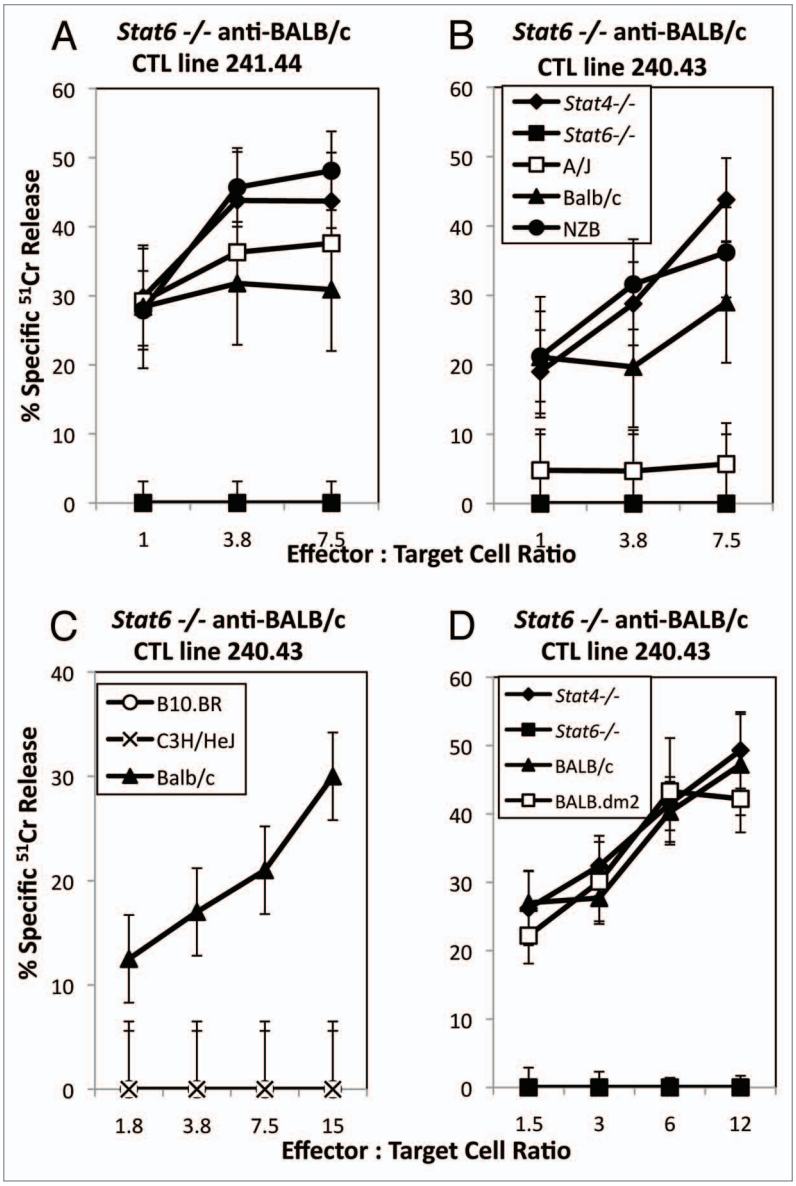

Figure 2. *Stat6*$^{-/-}$ anti-*Stat6*$^{+/+}$ responses are H2-K^d and H2-D^d or -L^d restricted. *Stat6* $^{-/-}$ anti-BALB/c CTL lines (**A**) 241.44, (**B–D**) 240.43 were incubated with target cells from *H2*^{d}, or *H2*^{k} expressing mouse strains in a standard ^{51}Cr release assay. CTL line 241.44 (**A**) (H2-D^d and -L^d-restricted) lyses A/J (H2-K^k, -D^d and -L^d) target cells, whereas CTL line 240.43 (**B**) (H2K^d-restricted) did not lyse A/J or (**D**) BALB.dm2 (H2K^{d}D^d). Neither line lysed H2^k target cells (**C**, data not shown). Data are the mean ± SD of triplicate assays and are representative of two or more experiments.

However, the situation could be more complicated in adoptive transfer experiments. Although most adoptive transfer experiments involving *Stat6*$^{-/-}$ mice use transferred purified CD4 T cells, in experiments where *Stat6*$^{-/-}$ splenocytes or CD8 T cells are transferred to wild type recipients,[9,10] it is possible that STAT6-specific CD8 T cells could mediate a graft vs. host response, resulting in background inflammation, apart from the specific responses being studied. Conversely, when STAT6-expressing cells are transferred into Stat6$^{-/-}$ recipients, it is possible that *Stat6*$^{-/-}$ CD8 T cells reactive against STAT6 peptides might eliminate transferred cells, masking some functional activity of the transferred cells.[10-13] The results from most of these experiments are consistent with a phenotype arising from STAT6-deficiency, suggesting that the contribution of anti-STAT6 immunity may be modest.

The contribution of anti-STAT6 CTL responses may be more important in studies of graft rejection. Two reports[14,15] demonstrated that *Stat6*$^{-/-}$ mice demonstrated shorter graft survival times in models of cardiac transplant with minor histocompatibility differences, and where CTLA4-Ig was used to block graft rejection. Although it is possible that some of this increase is due to the increased propensity of *Stat6*$^{-/-}$ T cells to develop into inflammatory T cells, it is likely that anti-STAT6 immunity may also be contributing to the observed phenotype.

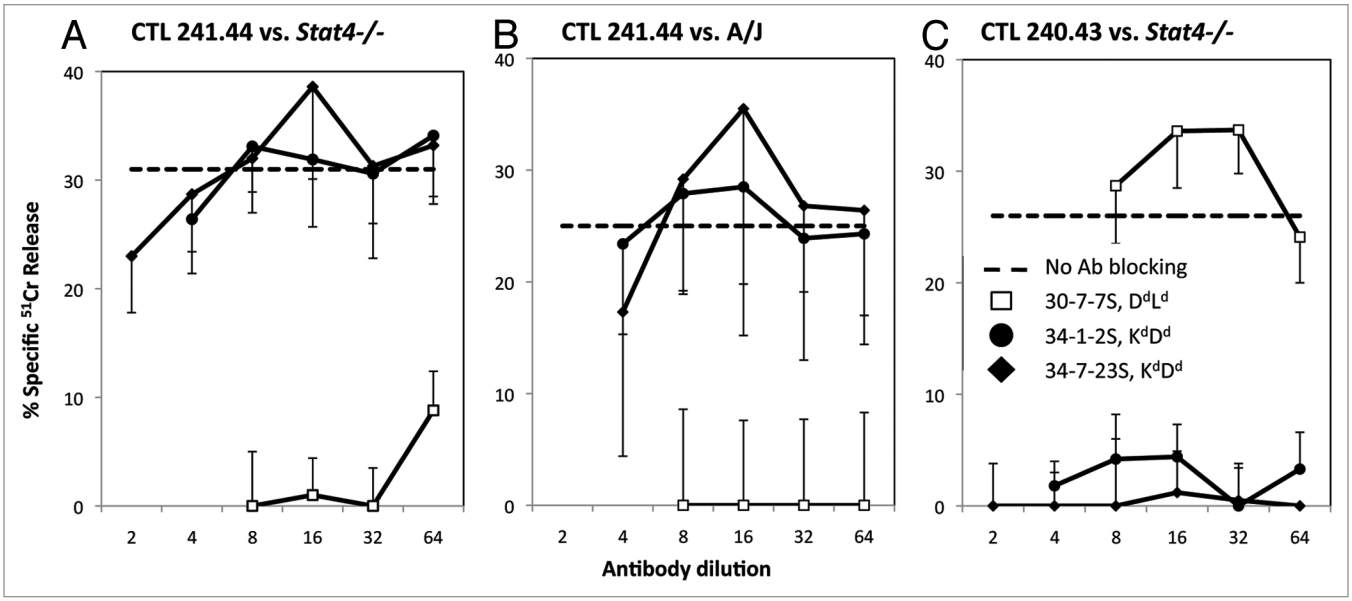

Figure 3. *Stat6*$^{-/-}$ anti-BALB/c or *Stat4*$^{-/-}$ CTL are H2-K^d, -D^d or -L^d restricted. (**A–C**) *Stat6*$^{-/-}$ anti-BALB/c CTL lines 241.44 (**A and B**), and 240.43 (**C**) were incubated with CAB target cells from *Stat4*$^{-/-}$ (**A and C**) or A/J (**B**) mice and cytotoxicity was measured in a standard ^{51}Cr release assay. The ability of anti-MHC I antibodies to block lysis was tested. Lysis in the absence of blocking antibodies is indicated as a dashed line. Data are the mean ± SD of triplicate assays and are representative of two or more experiments.

The anti-STAT6 immunity that develops in mice lacking endogenous STAT6 clearly contributes to the increased anti-tumor immunity in *Stat6*$^{-/-}$ mice. For example, Ostrand-Rosenberg et al.[16] have shown delayed and reduced primary mammary carcinoma growth in *Stat6*$^{-/-}$ mice using 4T1, a BALB/c mammary carcinoma that is usually malignant, non-immunogenic and metastatic. The authors hypothesize that the deletion of the *Stat6* gene facilitates development of potent anti-tumor immunity via a CD4$^+$-independent pathway. Our data suggest that immunization with the H2^d STAT6-expressing tumor might simply stimulate CD8$^+$ CTL specific for H2^d restricted antigens from STAT6-expressing cells, which lyse the cells regardless of expression of tumor antigens. Indeed, Jensen et al. demonstrate that enhanced immunity to 4T1 cells in *Stat6*$^{-/-}$ mice is dependent upon the STAT6 peptide used in our studies.[7] Similarly, Kacha et al.[17] show that effector cells from *Stat6*$^{-/-}$ mice have increased lytic activity and produce increased levels of IFNγ in response to the H2^d tumor P1.HTR. Thus, it is not clear that these studies actually demonstrate an effect of STAT6 function, but rather may reflect immune responses that are not tolerant to STAT6 peptides presented in the context of MHC I.

It is important to note that "autoreactivity" to a missing self-protein is not a ubiquitous phenomenon. Although there is clearly an anti-STAT6 CTL response in *Stat6*$^{-/-}$ mice, there is no corresponding anti-STAT4 CTL response in *Stat4*$^{-/-}$ mice. There could be multiple reasons for the restricted nature of these observations. First, not all proteins have MHC class I binding peptides. It is possible that STAT4 peptides do not compete effectively for binding to MHC class I such that no reactive cells are positively selected in the thymus. Alternatively, MHC class I binding peptides from STAT4 might be homologous enough to other endogenous peptides that reactive T cells are negatively selected in the thymus. The differences between the phenotypes of *Stat4*$^{-/-}$ and *Stat6*$^{-/-}$ CTL illustrate that the autoreactive phenomenon described here must be tested empirically for each mutant strain being studied.

In summary, our data illustrate the importance of demonstrating maintenance of self-tolerance in mice made defective in a particular gene by homologous recombination. Particularly in genes that have immune function, where common techniques involve transplantation and adoptive transfer, it is critical to define the ability of T cells to recognize peptides from the targeted proteins as foreign. Failure to do so could result in misinterpretation of how a gene of interest contributes to immune responses.

Material and Methods

Mice. *Stat4*$^{-/-}$ and *Stat6*$^{-/-}$ mice were generated and backcrossed for at least 10 generations to BALB/c mice as described previously.[3,18] B6.*Tla*^{a}, B10.BR, C3H/HeJ breeder mice and BALB/c.dm2 spleen cells were the kind gift of Dr James Forman. BALB/c, C57BL/6J, NZB/B1NJ and A/J breeder mice were purchased from The Jackson Laboratory. All animals were bred and housed in the IUSM-Evansville animal facility and procedures were approved by the IUSM IACUC. Where indicated, mice were immunized intraperitoneally with 25 × 10^6 spleen cells in 0.5 mL balanced salt solution, rested for at least 7 d, euthanized, and their spleens removed for the in vitro generation of CTL.

Cell lines and assays. Using specific strain combinations as described, CTL were generated in secondary MLC as described.[19] Briefly, 5 × 10^6 responder and 5 × 10^6 irradiated (3000Rad) stimulator spleen cells were incubated for 6–7 d in RPMI 1640 supplemented with 7–10% fetal bovine serum, 1% L-glutamine and 50 ng/mL 2-mercaptoethanol at 37°C in 7% CO_2. Some CTL

lines were re-stimulated weekly with irradiated stimulator cells in supplemented Mishell-Dutton medium that also contained T-cell growth factors from $(NH_4)_2SO_4$-precipitated supernatants of phorbol myristic acetate-stimulated EL4.IL2 cells.[20] Generation of concanavalin A (ConA) blast cells (CAB), ^{51}Cr labeling of target cells, the standard 4–6 h ^{51}Cr release assay, controls, and the method for determination of the % specific lysis of target cells have been described.[21] Standard error was computed by propagation of errors as previously described.[21] ^{51}Cr release assays for analysis of peptide binding, peptide blocking and antibody blocking were performed using pre-incubations as previously described.[22] RMA-S-D^d, T2-D^d, E3 and LTKD cell lines were provided by Dr D. Marguiles. RMA-S-L^d cell lines were provided by Dr Ted Hansen. Hybridomas producing anti-MHC antibodies were purchased from ATCC. Peptides were purchased from Genemed Synthesis. Flow cytometry analyses of CTL lines was performed as previously described.[19] using anti-CD4 antibody GK1.4 (PharMingen) and anti-CD8α antibody YTS169.4 (kindly provided by Dr. James Forman,) The CTL were $CD8^+$ and $CD4^-$, as expected.

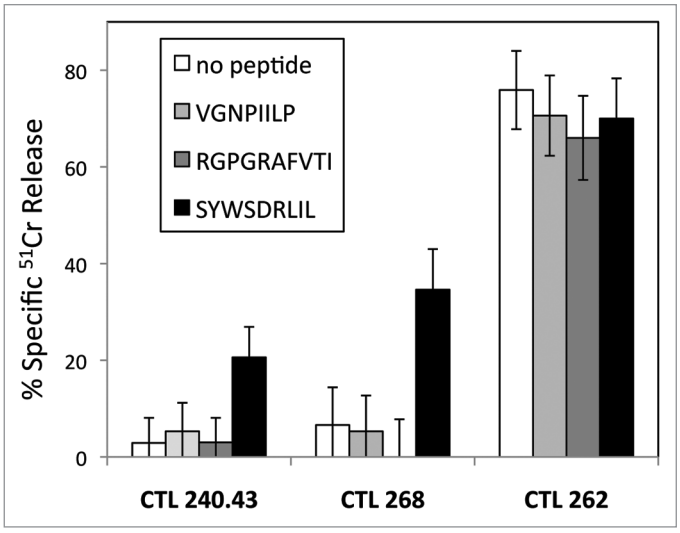

Figure 4. K^d-restricted *Stat6*$^{-/-}$ CTL recognize a peptide derived from the STAT6 protein. *Stat6*$^{-/-}$ anti-BALB/c CTL 240.43 (left) or *Stat6*$^{-/-}$ anti-*Stat4*$^{-/-}$ CTL 268 (middle) were incubated with *Stat6*$^{-/-}$ CAB target cells at an effector:target cell ratio of 12:1 in a standard ^{51}Cr release assay. Cytotoxicity was assessed in the absence or presence of 9-mer peptides from $H2K^d$ binding peptides from HIV gp160 (RGPGRAFVTI, VGNPIILP), or STAT6 (SYWSDRLIL). The alloreactive B10 ($H2^b$) anti-BALB/c ($H2^d$) CTL 262 (right) did not require peptide for lysis and was not affected by target cell incubation with peptides. Data are the mean ± SD of triplicate assays and are representative of three or more experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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