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IL-2 signals during the primary response to infection are essential in shaping CD8+ T cell fate decisions. How CD8+ T cells integrate IL-2 signals in the development of functional memory is not well understood. Because IL-2 induces potent activation of the STAT5 transcription factor, we tested the role of STAT5 in CD8+ memory T cell differentiation and function using a model system in which STAT5 activity is inducibly abrogated upon CD8+ T cell activation. We report that STAT5 activity is broadly important for the expansion and effector function of all effector CTL subsets. After pathogen clearance, STAT5 was required for the survival of effector phenotype memory CTLs during the contraction phase. However, despite its role in supporting full primary CD8+ T cell expansion, and unlike IL-2, STAT5 activity is not required for the development of memory CD8+ T cells capable of robust secondary expansion upon rechallenge. Our findings highlight differential requirements for survival signals between primary and secondary effector CTL, and demonstrate that IL-2–dependent programming of memory CD8+ T cells capable of secondary expansion and secondary effector differentiation is largely STAT5 independent. The Journal of Immunology, 2013, 190: 3390–3398.

Environemntal signals, including those from cytokines, play an essential role in effector and memory CD8+ T cell differentiation (1). The common γ-chain (γc) family cytokine IL-2 has been shown to drive CD8+ T cell effector and memory differentiation (2–5). We and others have shown a role for IL-2 during the primary immune response in driving differentiation of effector CTL (2–4), as well as programming of memory CD8+ T cells capable of robust recall responses (3, 5).

How CD8+ T cells integrate signals from cytokines to determine fate decisions is not fully understood. Specifically, how do cytokines, such as IL-2, induce signaling pathways that result in long-lived memory CD8+ cells capable of protective recall responses? Several transcription factors, including T-box expressed in T cells (6–11), Eomesodermin (Eomes) (6, 8, 12, 13), B lymphocyte–induced maturation protein (PRMD1/Blimp-1) (14–18), and B cell lymphoma 6 protein (Bcl-6) (14, 19, 20), have been shown to have crucial roles in CD8+ T cell effector differentiation and effector versus memory fate decisions. In the case of T-bet (6, 8–11) and Blimp-1 (14, 16–18), increased activity promotes a more differentiated terminal effector state in CD8+ T cells. In contrast, Eomes (6, 8, 12, 13) and Bcl-6 (14, 19, 20) are associated with establishment of long-lived memory CD8+ T cell populations. In the case of effector CD4+ T cell differentiation, connections between cytokines and transcriptional programs are relatively well described and in several lineages involve STAT proteins (21). In contrast, the molecular connection between environmental signals such as cytokines received by CD8+ T cells during an immune response and the resulting transcriptional outcome driving differentiation toward terminal effector versus memory CD8+ T cell fate is less clear. The induction of transcription factors such as T-bet and Blimp-1 by cytokines appears to play a key role in this process (9, 22, 23).

We have shown a crucial role for the cytokine IL-2 in the differentiation of effector and memory CD8+ T cells. IL-2 signals during the primary response to infection are critical in promoting the differentiation of effector and effector memory CTL (3). Moreover, IL-2 signals received by CTL during the primary response program memory CD8+ T cells capable of robust secondary responses (3, 5) and secondary effector differentiation (3). We sought to determine the signaling pathways induced by IL-2 during a primary immune response that are involved in CD8+ T cell fate decisions and memory CD8+ T cell programming. The JAK3/STAT5, PI3K/Akt, and MAPK signaling pathways are potentially activated by IL-2 (24). Of these pathways, PI3K/Akt and MAPK are activated by a variety of other cytokine receptors, TCR ligation, and costimulatory signals (25–28) during T cell activation. Other γc cytokines in addition to IL-2, such as IL-7 and IL-15, also activate JAK3/STAT5, but the timing, magnitude, and context of this signaling differ because of cytokine availability and differential regulation of the specific receptor subunits (29). IL-2 potently activates STAT5 during the differentiation of effector CTL. Although IL-15 and IL-7 can also activate STAT5 during the effector CTL response, they play a dominant role in the maintenance of memory T cells after Ag clearance (29, 30). Moreover, IL-15 and IL-7 signals are not required for the programming of memory CD8+ T cells (3, 31, 32), as is the case with IL-2 (3, 5). IL-2 is an important catalyst for robust STAT5 activation during primary CTL responses, and we hypothesized that IL-2–mediated activation of STAT5 was a key step in the differentiation of highly functional CD8+ memory T cells.
Two isoforms of STAT5 exist, STAT5a and STAT5b, which have redundant functions in T cell homeostasis (33, 34). Mice with deletion of both stat5a/b fail to develop T cells (35, 36), demonstrating that STAT5 is essential during thymic selection. Recent reports examined the role of STAT5 in the survival of effector and memory CD8+ T cells after pathogen clearance (37, 38). In the absence of STAT5, effector CD8+ T cells show reduced accumulation at the peak of the primary response to infection, possibly because of their inability to induce Bcl-2 via STAT5 in response to IL-7 and IL-15 (38). Forced expression of a constitutively active form of STAT5 increased the numbers of effector CTL at the peak of the primary response to lymphomatis chroniogenitus virus (LCMV) and augmented survival of all CD8+ T cell subsets through contraction and memory (37). These studies support a role for STAT5 signaling in effector and memory CD8+ T cell survival, mainly in the context of IL-15 and IL-7 signaling. However, high IL-2 levels potently drive STAT5 activation during the primary CTL response, and to date no studies have examined the role of STAT5 in differentially promoting effector and memory CTL subsets or in the programming of secondary CTL responses, two functions that have been described for IL-2 (3, 5). In this study, we use a unique experimental system in which STAT5 deletion is selectively targeted to activated CTL in an otherwise normal immune environment.

To determine the contribution of STAT5 to CD8+ effector and memory T cell differentiation, we used a bone marrow (BM) chimera mouse model in which stat5a/stat5b are inducibly deleted upon CTL activation. We found that STAT5 was broadly important for all effector CD8+ T cell subsets during the primary response to acute infection, as well as the survival of effector phenotype CTL during contraction. After contraction, STAT5-independent memory CD8+ T cell populations were readily detectable, but whereas STAT5 was required for robust expansion and survival of primary effector CTL, STAT5-deficient memory CD8+ T cells mounted robust recall responses comparable with wild-type (WT) levels and underwent effective secondary effector CTL differentiation. Our findings highlight a differential requirement for survival signals mediated by STAT5 during primary and secondary CD8+ T cell responses. Moreover, our data suggest that IL-2–driven differentiation and programming of memory CD8+ T cells with robust recall potential is largely STAT5 independent.

Materials and Methods

Mice and infections

Four- to 6-wk-old B6.PL-Thy1.1(-/+) (B6.PL, Thy1.1+) and C57BL/6 (B6; Thy1.2+/-) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Granzyme B (Gzb)-Cre (39), Rosa26-/-stop/+-YFP (RosaYFP) (40) (The Jackson Laboratory), and stat5a/fl/fl (41) (supplied by L. Henighausen, The Jackson Laboratory), and stat5b/fl/fl (41) (supplied by L. Henighausen, The Jackson Laboratory), and stat5a/fl/fl (41) (supplied by L. Henighausen, The Jackson Laboratory) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), or Santa Cruz Biotechnology (Santa Cruz, CA). Abs were specific for the following Ags: CD8, Thy1.1, Thy1.2, killer cell lectin-like receptor subfamily G member 1 (KLRG1), IL-7Rα, CD62L, T-bet, Eomes, Bcl-6, Gzb, and STAT5-pY694. For cell-surface staining, single-cell suspensions were incubated with Abs in FACS buffer (PBS with 2% FBS and 0.02% sodium azide). For intracellular staining of T-bet and Gzb, cells were permeabilized and stained using the BD Cytotox/Cytoperm kit and manufacturer’s instructions (BD Biosciences). For intracellular staining of Eomes, cells were prefixed in 0.5% parformaldehyde to preserve the YFP signal, washed twice, then fixed using Foxp3 Fix/Perm Buffer kit and manufacturer’s instructions (eBioscience). To detect STAT5-pY694, we followed the BD Phosflow kit and manufacturer’s protocol (BD Biosciences). H-2D b/GP33–41 and H-2Db/NP396–404 tetramers were prepared as previously described (47, 48). Multiparameter analysis of stained cells was performed using a FACSCanto II flow cytometer (BD Biosciences) and results were analyzed using FlowJo software (Tree Star, Ashland, OR).

Intracellular cytokine staining

Single-cell suspensions in RPMI 1640 containing 10% FBS, t-glutamine, and penicillin/streptomycin were incubated with 0.1 μM GP33-41 or NP396-404 peptides in the presence of GolgiPlug (BD Biosciences) for 4–5 h. Cells were stained with cell-surface Abs, fixed, and permeabilized using a kit (BD Biosciences), and stained with fluorescently labeled anti-cytokine Abs specific to IFN-γ, TNF-α, and IL-2 (eBioscience).

CTL assays

We used a CTL assay as previously described (6). EL-4 target cells were incubated with 0.1 μM GP33-41 peptide for 2 h at 37°C. Cells were washed and then incubated with sorted STAT5 WT or STAT5 conditional knockout (CKO) P14 CTLs for 2 h at CTL to target ratios ranging from 3:1 to 0.1:1. The percentage of Annexin V+ target cells was determined by FACS using Annexin V–allophycocyanin apoptosis detection kit (eBioscience). Specific lysis was determined by comparison of killing of target cells without peptide loading.

Real-time PCR

RNA was extracted from sorted YFP+ CD8+ T cells using TRIzol (Invitrogen, Carlsbad, CA). cDNA was prepared from the RNA, and real-time RT-PCR was performed using Superscript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA) following the manufacturer’s guidelines. Primer sets used are as follows: EOMES: forward (f), 5′-CCGCCCACTAAGTGTTC-3′, reverse (r), 5′-GAAATCTCTCGTCCCTCCA-3′; Tbet: f, 5′-TGGCCCAACTATAGAGATGCG-3′, r, 5′-CCTCT- GTTGGTGGTGAAGCTT-3′; Blimp-1: f, 5′-GGGGATGGAACATCTACTT-3′, r, 5′-TTTCTTTCACGGCTGACTTCTCTC-3′; Bcl-6: f, 5′-CCGGCCCAATATCATCGCTGAA-3′, r, 5′-GGGTGCTGATGAACTGTTG-3′; FasL: f, 5′-TGACCCATGTCAGCTCTGTTTGA-3′, r, 5′-CCTCTGTCTCAGGAGACCGATGGA-3′; Bim: f, 5′-CTTCATCACAAGACAGCTTGG-3′, r, 5′-GGAGCTCTGCGAGATGTTG-3′, r, 5′-TTCCAGCGCTCGGAATATC-3′; myeloid leukemia cell differentiation protein 1 (mcl-1): f, 5′-AGAGCGTCGAGACCCCTG-3′, r, 5′-CTACTCTTGCGGAAATATC-3′.
Results

STAT5 is required for accumulation of primary CTL during acute infection

To study the role of STAT5 signals in CD8+ T cell effector and memory differentiation, we created a mouse BM chimera model system in which stat5a and stat5b are deleted in activated CD8+ T cells, and the resulting STAT5-deficient CD8+ T cells are traceable by permanent expression of YFP (Fig. 1A). BM was harvested from mice with the following genetic components: Cre recombinate driven by the GzB promoter (39) (GzB-Cre), RosaYFP reporter construct (40), and the stat5a/b genes flanked by loxp sites (41) (stat5fl/fl), which we will subsequently refer to as “STAT5 CKO” BM. The donor BM was mixed 1:1 with WT B6.PL BM (“WT B6”) and transplanted into irradiated B6.PL hosts. This set of chimeras will subsequently be referred to as STAT5 CKO chimeras. A second set of BM chimeras was made in an identical fashion, except the donor BM contained the stat5a/b genes without loxp sites (stat5fl/wt) and will be referred to as STAT5 WT chimeras (Fig. 1A). We distinguished between STAT5 (Thy1.2+) and B6.PL (Thy1.1+) donor-derived T cells over the course of infection based on differential expression of congenic Thy1 alleles. In this model system, activation of Cre activity upon CTL activation, driven by GzB induction, results in the deletion of loxp-flanked stat5a/b as well as a loxp-flanked stop codon preceding YFP under the control of the Rosa26 promoter. Because GzB expression is an early event after CTL activation (39), permanent YFP expression serves as an effective surrogate marker for Cre-mediated gene deletion during CTL differentiation and expansion. Importantly, the mixed BM chimera model system allows for the study of CTL-specific STAT5 activity without complications arising from differences in pathogen clearance.

Before infection, small numbers of YFP+ cells could be detected among the STAT5 WT (~5%) and at much lower levels among the STAT5 KO (~0.5%) CD8+ T cells (Fig. 1B). These cells were largely CD44hiCD122lo and CD62Llo, indicating that they did not arise through homeostatic proliferation and do not represent a pre-existing memory phenotype population (Supplemental Fig. 1). Postinfection with LCMV-Armstrong, CD8+ T cell expansion was evident in both STAT5 WT and STAT5 KO chimeras, and both groups of chimeras cleared the infection as expected (data not shown). A clearly distinguishable CD8+ YFP+ population was seen in both STAT5 WT and STAT5 KO chimeras on day 8 postinfection (Fig. 1B). When gated on STAT5 WT or STAT5 KO donor CD8+ T cells (Thy1.2+), >90% of KLRG1+ effector CTL also expressed YFP, confirming that activation of YFP and expression of the YFP reporter was highly efficient (Fig. 1C). In addition, YFP+ CD8+ T cells from either WT or KO donors populated the effector (KLRG1hiIL-7Rαlo) and memory precursor (KLRG1hiIL-7Rαhi) pools with similar frequency (Fig. 1C).

We confirmed that STAT5 activity was absent from the CD8+ YFP+ population within the STAT5 KO chimeras at effector and memory time points. Using an anti–phospho-STAT5 Ab that efficiently stains phospho-STAT5 in mouse T cells (37, 38), we observed that STAT5 KO YFP+ CTLs failed to phosphorylate STAT5 upon ex vivo exposure to IL-2, as compared with STAT5 WT CTLs, as well as unstimulated (Fig. 1C) and isotype (data not shown) controls. We also measured the deletion of stat5a/b by

FIGURE 1. STAT5 is required for full primary expansion of CD8+ T cells. (A) Schematic showing creation of STAT5 WT and STAT5 KO BM chimeras as a model system to study CD8+ T cell responses in the absence of STAT5, absent complications arising from differences in viral clearance. (B) Representative flow plots showing Thy1.2+CD8+YFP+ populations in both STAT5 WT and STAT5 KO chimeras before infection and at day 8 post LCMV-Arm infection. (C) Representative flow plots show YFP expression by KLRG1hiThy1.2+CD8+ T cells, as well as IL-7Rα and KLRG1 expression by CD8+ Thy1.2+YFP+ T cells, in the spleen at day 8 postinfection. (D) Splenocytes from STAT5 WT or STAT5 KO chimeras harvested on days 8 or 40 post LCMV infection were cultured with 500 U/ml murine IL-2 for 20 min, then stained for phospho-STAT5. Representative histogram shows phospho-STAT5 levels in unstimulated controls (light gray shaded), CD8+YFP+ T cells from STAT5 WT chimeras (dark gray shaded), and CD8+YFP+ T cells from STAT5 KO chimeras (black line). (E) The ratio of “WT” (Thy1.1+) to “STAT5” (Thy1.2+) within the CD8+ T cell compartment is shown for both sets of chimeras before (day 0) and day 8 post LCMV infection. (F) Total numbers of CD8+ T cells from “WT” and “STAT5” components on day 8 post LCMV infection in STAT5 WT and CKO chimeras. Error bars indicate the SEM (n = 3–4 mice per time point). Results are representative of four to five independent experiments.
detect tetramer-binding CTL in the spleen. Although we cannot pinpoint in vivo STAT5 inactivation precisely, in vitro activation of CD8+ STAT5 KO T cells resulted in undetectable STAT5 activity within 48 h of activation (data not shown). Therefore, we conclude that our model system results in rapid inhibition of STAT5 activity, and that STAT5 is an important survival signal for CD8+ T cells undergoing expansion during primary immune challenge. We cannot rule out the possibility that STAT5 provides key survival and/or differentiation signals in the first hours after activation.

**STAT5 influences CTL function but does not drive CTL differentiation**

To explore whether STAT5 influenced effector CTL differentiation, we examined the localization, expression of effector molecules, and function of effector CTLs in the absence of STAT5. Ag-specific WT and STAT5 KO CD8+ T cells were present at similar frequencies in the liver of LCMV-infected mice on day 8 postinfection (Fig. 3A), demonstrating that CTL form and traffic to peripheral sites of infection even in the absence of STAT5. In these cases, the overall loss in CD8+ T cells, the total number of STAT5 KO CD8+ T cells was almost entirely derived from the WT donor (Fig. 1F), indicating a decreased accumulation of STAT5 KO CD8+ T cells at day 8 postinfection compared with WT. We examined WT and STAT5 KO CD8+ T cells for differences in antiapoptotic, prosurvival, or proapoptotic gene expression on day 8 postinfection with LCMV and found no significant differences in expression of the prosurvival genes Bcl-2 and Mcl-1, and no difference in expression of the proapoptotic gene Bim (Table I). STAT5 WT CD8+ T cells showed a modest but significant increase in expression of the prosurvival molecule B cell lymphoma extra large (Bcl-xl) over STAT5 KO CD8+ T cells (Table I).

Table I. Gene expression in WT STAT5 CD8+ compared with KO STAT5 CD8+ T cells on day 8 postinfection with LCMV

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Expression in WT Compared with KO</th>
</tr>
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<tbody>
<tr>
<td>T-bet</td>
<td>1.57 (p = 0.008)</td>
</tr>
<tr>
<td>Blimp-1</td>
<td>1.82 (p = 0.021)</td>
</tr>
<tr>
<td>Prf1</td>
<td>1.55 (p = 0.010)</td>
</tr>
<tr>
<td>Bcl-6</td>
<td>0.23 (p = 0.041)</td>
</tr>
<tr>
<td>EOMES</td>
<td>1.42 (p = 0.150)</td>
</tr>
<tr>
<td>FasL</td>
<td>0.95 (p = 0.859)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.71 (p = 0.365)</td>
</tr>
<tr>
<td>Bcl-X1</td>
<td>1.77 (p = 0.046)</td>
</tr>
<tr>
<td>Bim</td>
<td>1.17 (p = 0.642)</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>0.88 (p = 0.654)</td>
</tr>
</tbody>
</table>

Significant fold differences (p < 0.05, n = 4/group) are in boldface.
To test effector function more directly, we compared target cell lysis of STAT5 WT and STAT5 CKO CD8+ T cells on day 8 postinfection. We found approximately a 2-fold decrease in killing on a per-cell basis in the absence of STAT5 (Fig. 3D). This could be potentially explained by the lower levels of Prf1 mRNA expressed by STAT5 CKO CTL compared with WT CTL (Table I) and is consistent with a report that STAT5 binds the Prf1 promoter and drives Prf1 expression in CTL (4).

Overall, our data suggest that on a per-cell basis, STAT5 deficiency has a modestly adverse impact on CTL effector function. This is similar to the modest decrease in effector function seen in CTLs that do not receive IL-2 signals (3), and it is likely that IL-2–driven acquisition of effector function is mediated, at least to some degree, by STAT5. However, taking into account that STAT5 does not affect peripheral localization of effector CTLs and that STAT5 CKO CTLs express GzB, cytokines, transcription factors, and FasL equal to WT (Fig. 3, Table I), we conclude that STAT5 plays only a modest role in the development of primary effector CTL function.

CTL differentiation is heterogeneous, including effector and memory precursor CD8+ T cell subsets that form part of the overall effector CTL pool. These two populations can be distinguished based on cell-surface expression of KLRG1 and IL-7Rα (9). We and others have shown that IL-2 signals drive differentiation of terminal effector phenotype CTLs during the primary response (2, 3). To determine whether IL-2–driven effector differentiation could be mediated by STAT5, we examined the differentiation of STAT5 CKO Ag-specific CD8+ T cells based on IL-7Rα and KLRG1 expression post LCMV infection. We found that the total numbers of STAT5 CKO IL-7RαKLRG1hi CD8+ T cells were decreased at the peak of the effector response and declined at nearly a 10-fold faster pace than STAT5 WT IL-7RαKLRG1hi CD8+ T cells between days 8 and 40 postinfection (Fig. 4A, 4C). Whereas CD8+ T cells activated in the absence of IL-2 signals generate normal numbers of memory precursor CTLs (3), STAT5 CKO memory precursor IL-7RαKLRG1hi CD8+ T cells were also reduced at effector time points and declined rapidly between days 8 and 40 postinfection (Fig. 4B, 4C). We conclude that in contrast with IL-2, STAT5 does not selectively drive differentiation of effector phenotype CTLs and is instead important for the emergence of all CTL subsets through the peak of expansion. After clearance of pathogen, STAT5 signals are important for maintaining IL-7RαKLRG1hi phenotype CTLs through contraction and into the memory phase.

**STAT5 signals are important for maintenance of tissue-residing memory CTLs after pathogen clearance**

Memory CD8+ T cell populations were detectable in the spleen in the absence of STAT5 post LCMV infection, although at lower numbers than WT (Fig. 5A). Importantly, surviving memory cells did not express detectable STAT5 (Fig. 6A), indicating that memory CTLs can emerge in the absence of STAT5, albeit at lower levels. We did not examine memory CD8+ T cell survival and homeostasis beyond 6 wk postinfection because of the known role of IL-15 and IL-7 in long-term memory T cell maintenance (30). At early memory time points, the absence of STAT5 resulted in rapid loss of CD62Llo effector memory CTLs in the spleen (Fig. 5B), and the preferential and specific decline of tissue-homing effector memory CTLs in the liver (Fig. 5C). These findings are consistent with the role of IL-2 in effector memory CTL differentiation and suggest that IL-2–driven STAT5 activation is a key driver of effector memory CTL establishment (3, 5). After pathogen clearance, STAT5 signaling is particularly important for ef-
fector memory and tissue-residing memory CD8+ T cell survival (Fig. 5B, 5C).

STAT5 CKO memory CTLs have robust recall capacity

Because IL-2 signals during primary CTL activation are required for the formation of memory CTLs capable of robust secondary responses (3, 5), we assessed the ability of STAT5 WT and STAT5 CKO memory CTLs to respond to rechallenge. Before rechallenge, we verified that Ag-specific memory CD8+YFP+ T cells from STAT5 CKO chimeras did not express detectable STAT5 (Fig. 6A). Direct rechallenge of memory-phase BM chimeras is difficult to interpret due to the inability to distinguish between recall responses by YFP+ memory CD8+ T cells and recruitment of YFP− memory or naive CD8+ T cells that subsequently express YFP. Therefore, we used an adoptive transfer strategy to determine recall capacity in the absence of STAT5. STAT5 WT or STAT5 CKO CD8+ YFP+ memory CTLs were FACS purified from the spleens of mixed chimeras 6 wk after LCMV infection, analyzed for frequency of CD8+tetramer-binding cells, and transferred into naive B6 hosts. Twenty-four hours after adoptive transfer, the naive B6 hosts were infected with LCMV clone 13 to induce recall responses by YFP+ memory CD8+ T cells (Fig. 6B). Similar

FIGURE 4. STAT5 signals during primary expansion are broadly important for all CD8+ T cell subsets responding to acute infection. Graphs display the total number of KLRG1hiIL-7Rαlo (terminal effector phenotype) (A) and KLRG1loIL-7Rαhi (memory precursor/memory phenotype) (B) at the indicated time points postinfection with LCMV-Arm for STAT5 WT and STAT5 CKO tetramer-binding CD8+ T cells. Numbers within the plot indicate fold decline between days 8 and 40. (C) Representative flow plots display the frequency of CD8+ T cell subsets as determined by KLRG1 and IL-7Ra expression at the indicated time points for STAT5 WT and STAT5 CKO tetramer-binding YFP+CD8+ T cells. Results are representative of three to four separate experiments. Error bars represent the SEM (n = 3–4/group).

FIGURE 5. STAT5 signals are important for establishment and maintenance of effector memory and tissue-residing CD8+ T cells. (A) Graphs display the total numbers of Ag-specific STAT5 WT and STAT5 CKO YFP+CD8+ T cells in the spleen at the indicated time points post LCMV infection. (B) Histograms show representative CD62L staining in STAT5 WT and CKO YFP+CD8+ T cells in the spleen at indicated time points. (C) Ratio of H2Db-GP33–41 tetramer-binding CD8+ T cells derived from WT CD8+YFP+ memory T cells were FACS sorted from pooled spleens derived from either STAT5 WT or STAT5 CKO chimeras ~6 wk post LCMV-Arm infection. Sorted CD8+YFP+ T cells were analyzed for the frequency of H2Db-GP33–41 and NP396–404 tetramer-binding memory CD8+ T cells and then adoptively transferred into naive B6 hosts. The next day, the naive B6 hosts were infected with LCMV clone 13. (B) Representative flow plots show the frequencies of tetramer-binding YFP+CD8+ STAT5 WT and STAT5 CKO secondary responders in the spleens of B6 hosts on day 5 postinfection. (C) Bar graphs display the fold expansion of Ag-specific STAT5 WT and STAT5 CKO CD8+YFP+ cells on day 5 postinfection compared with numbers adoptively transferred before infection. Results are representative of three to four independent experiments; error bars represent the SEM (n = 3–4/group).

FIGURE 6. STAT5-deficient memory CD8+ T cells are capable of robust recall responses. (A) Schematic showing adoptive transfer strategy for determining recall capacity of STAT5 CKO CD8+ T cells compared with WT. CD8+YFP+ memory T cells were FACS sorted from pooled spleens derived from either STAT5 WT or STAT5 CKO chimeras ~6 wk post LCMV-Arm infection. Sorted CD8+YFP+ T cells were analyzed for the frequency of H2Db-GP33–41 and NP396–404 tetramer-binding memory CD8+ T cells and then adoptively transferred into naive B6 hosts. The next day, the naive B6 hosts were infected with LCMV clone 13. (B) Representative flow plots show the frequencies of tetramer-binding YFP+CD8+ STAT5 WT and STAT5 CKO secondary responders in the spleens of B6 hosts on day 5 postinfection. (C) Bar graphs display the fold expansion of Ag-specific STAT5 WT and STAT5 CKO CD8+YFP+ cells on day 5 postinfection compared with numbers adoptively transferred before infection. Results are representative of three to four independent experiments; error bars represent the SEM (n = 3–4/group).
rechallenges were done using LCMV Armstrong or recombinant Lm-gp33 as a secondary stimulus, with similar results (data not shown).

Ag-specific STAT5 WT and STAT5 CKO YFP^+CD8^+ T cells were clearly visible in the spleens of the infected hosts at day 5 after rechallenge (Fig. 6C). In contrast with the primary response, STAT5 CKO memory CD8^+ T cells expanded at levels similar to WT when rechallenged (Fig. 6C, 6D). This is in striking contrast with the defective accumulation upon rechallenge of memory CD8^+ T cells generated in the absence of IL-2 (3, 5). As in the primary response, STAT5 CKO secondary CTLs expressed normal levels of GzB (Fig. 7A), effector cytokines (Fig. 7B), and differentiation-associated transcription factors T-bet and Eomes (Fig. 7A). When rechallenged with Lm-gp33, STAT5 CKO secondary CTLs trafficked to the liver normally (Fig. 7C). There was a modest decrease in formation of secondary KLRG1^{hi}IL-7Rα^{lo} phenotype CD8^+ T cells in the absence of STAT5 (Fig. 7D), but STAT5 CKO secondary CTLs expressed normal levels of Prf1 mRNA (Fig. 7E), in contrast with the primary CTL response. In addition, STAT5 CKO secondary effector CTLs degranulated normally after restimulation, as measured by surface CD107a expression (data not shown). Our results indicate that STAT5 has little effect on secondary effector CTL differentiation. We conclude that IL-2-driven programming of memory CD8^+ T cells capable of robust secondary expansion is largely STAT5 independent.

**Discussion**

Our results demonstrate a broad role for STAT5 signaling in the primary response to infection in promoting the accumulation of effector CTLs, and a more selective role for STAT5 in the survival of effector phenotype and establishment and survival of tissue-residing memory CD8^+ T cells after pathogen clearance. Despite its role in promoting robust primary effector CTL responses, we make two novel observations. First, STAT5 is not required for IL-2-driven memory differentiation, reflecting the potential participation of other IL-2-dependent signaling pathways in this process. Second, the requirement for STAT5 activity in the primary and secondary CTL response is different, with secondary CTLs differentiating and expanding normally in the absence of STAT5. A study using direct infection of mice with conditional stat5 deletion driven by a type I IFN-responsive promoter showed that cell division of WT and STAT5-deficient CD8^+ T cells on day 8 post-infection was the same (38). Our observations of poor primary expansion in the absence of STAT5 also likely reflect poor survival, and we propose that the survival of secondary effector CTLs is controlled by distinct mechanisms. In addition, the absence of IL-2 signaling during the in vivo response to acute infection impacts survival whereas having little effect on cell division (5).

Consistent with reduced survival in the absence of STAT5, we observed reduced expression of the prosurvival molecule Bcl-xL in STAT5 CKO CD8^+ T cells at day 8, similar to its expression in the absence of IL-2 signals (3). It is known that STAT5 proteins, including STAT5, can induce Bcl-xL in several cell types (51). Although it is possible that IL-2–induced STAT5 could promote survival of CD8^+ T cells during the primary response through induction of Bcl-xL, T cells lacking Bcl-xL (all isoforms) were able to mount normal responses to Listeria monocytogenes infection (52). Thus, the functional impact of differences in Bcl-xL mRNA levels during primary CTL expansion in the absence of STAT5 is not clear.

We find that STAT5 signals are broadly important for all subsets of effector CTL cell during primary expansion, suggesting that STAT5 does not drive CD8^+ T cell fate decisions during the primary response. This is in contrast with the specific role of IL-2 in preferentially promoting the differentiation of effector phenotype CTL (3). Another γC family member, IL-21, is able to activate STAT5, although to a lesser degree than IL-2 (53), and has recently been identified as being important in CD8^+ T cell differentiation in the context of chronic (54–56) and acute infection (57). Additional STAT5 activation during the primary response is likely stimulated by IL-7 and IL-15, and we have found that the combined absence of IL-2 and IL-15 enhances the defect in terminal effector CTL differentiation during the primary response (3). Our results suggest that STAT5 may have graded effects on CTL differentiation. Although some loss of STAT5 activation induced by IL-2 could influence effector phenotype CTL formation, STAT5 activation induced by other cytokines may be sufficient for the differentiation of memory precursor CTL. Complete loss of STAT5, however, results in a defect in both terminal effector phenotype and memory precursor CTL.

IL-15 selectively promotes survival of effector phenotype CTL after viral clearance. Although IL-15 induces STAT5 signaling, we observed a switch from a broad requirement for STAT5 during expansion to a more selective requirement for its activity in the maintenance of effector phenotype and long-lived effector memory CD8^+ T cells during contraction and into the memory phase. This
could be because of different levels of STAT5 activation induced by IL-15 and IL-7 (58). IL-7 also efficiently activates STAT5, and it has roles in both the generation and survival of memory CTLs (32). Alternatively, another report has shown that effector phenotype CD8+ T cells are more dependent on STAT5 signals for survival during contraction (37, 38). Collectively, these reports and ours emphasize that for different CD8+ T cells subsets, there appears to be differential usage of and access to common signaling cascades induced by γc, cytokines at distinct stages of differentiation.

Importantly, we observed the emergence of a STAT5-deficient memory CD8+ T cell population 6 wk postinfection, albeit at much lower levels than what was seen for WT. This brings to question the survival signals that are used in the absence of STAT5 and to what degree these signals are used under normal conditions. A recent report suggests that STAT5-independent survival signals could be used in the formation of memory CD8+ T cell populations (58), but the nature of those signals remains unknown. Another recent study showed that memory precursor CTLs were able to activate PI3K/Akt in an IL-15–dependent manner more efficiently than effector phenotype CTLs (37). However, hyper-activation of this pathway was detrimental to long-term survival of memory CD8+ T cells.

Despite being a key mediator of IL-2 signals, we found that STAT5 was dispensable for the ability of memory CD8+ T cells to mount robust secondary responses and undergo secondary effector CTL differentiation. This highlights a different requirement for STAT5 during primary and secondary CD8+ T cell responses, because STAT5-deficient primary responses were significantly reduced compared with WT. Moreover, this is in striking contrast with what was seen for memory CD8+ T cells generated in the absence of IL-2 signals, which failed to mount successful recall responses and failed to differentiate into secondary effector CTLs (3, 5). Thus, the mechanism by which IL-2 signals during the primary response program memory CD8+ T cells is independent of STAT5 and remains to be defined.

Alternative candidate pathways induced by IL-2 that could play a role in memory CTL differentiation include STAT3 and PI3K/Akt. STAT3 signals are also induced by IL-2 (53), and a role for STAT3 in persistence of memory precursor CTLs in mice (57) and CD8+ central memory T cell formation in humans (59) has been suggested. Like STAT5, STAT3 is activated by a variety of other cytokines, such as IL-21 and IL-6 (53, 60), and it is unclear what proportion of STAT3 activity during the primary CTL response is IL-2 dependent. The phenotype of STAT3-deficient and IL-2Rα–deficient CD8+ T cells do not align, because IL-2 is important for terminal effector CTL (3), whereas STAT3 appears to be important for memory precursor phenotype CD8+ T cells (57).

A more likely candidate is IL-2–dependent PI3K/Akt activation. Although this signaling pathway is induced in CD8+ T cells by a variety of sources, the additive effects of multiple signals, including IL-2, in the induction of PI3K/Akt activation for memory CD8+ T cell differentiation could be important. Moreover, several recent reports have suggested that the kinase mammalian target of rapamycin, which is downstream of PI3K/Akt activation, is a key regulator of effector and memory CTL fate decisions and survival (61–63). Therefore, a likely future hypothesis is that IL-2 mediates its effects on the programming of secondary effector CTL responses, at least in part, through PI3K/Akt activation.

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Disclosures

The authors have no financial conflicts of interest.

References


Supp. Fig. 1: Splenocytes from naïve STAT5 WT and STAT5 CKO chimeras were stained with the indicated activation markers and gated on CD8^+YFP^+ and CD8^+YFP^- populations. The top row of representative flow plots (n=3/group) indicates CD62L expression, while the bottom row indicates the frequency of CD44^hiCD122^hi memory phenotype cells.
Supp. Fig. 2: At day 8 post-infection CD8⁺YFP⁺ cells were FACS-sorted from the spleens of either STAT5 WT or STAT5 CKO chimeras. Genomic DNA (left panel) was amplified with primers for stat5a and relative expression normalized to the cd3z gene. Real-time RT-PCR was also done with RNA isolated from each population (right panel) and relative expression normalized to GAPDH.