Evidence of STAT5-dependent and -independent routes to CD8 memory formation and a preferential role for IL-7 over IL-15 in STAT5 activation

Roslyn A Kemp, Claire F Pearson, Georgina H Cornish and Benedict P Seddon

Interleukin (IL)-7 and IL-15 have non-redundant roles in promoting development of memory CD8+ T cells. STAT5 is activated by receptors of both cytokines and has also been implicated as a requirement for generation of memory. To determine whether STAT5 activity was required for IL-7 and IL-15-mediated generation of memory, we expressed either wild type (WT) or constitutively active (CA) forms of STAT5a in normal effector cells and then observed their ability to form memory in cytokine replete or deficient hosts. Receptor-independent CA-STAT5a significantly enhanced memory formation in the absence of either cytokine but did not mediate complete rescue. Interestingly, WT-STAT5a expression enhanced memory formation in a strictly IL-7-dependent manner, suggesting that IL-7 is a more potent activator of STAT5 than IL-15 but did not mediate complete rescue. These data suggest that the non-redundant requirement for IL-7 and IL-15 is mediated through differential activation of both STAT5-dependent and STAT5-independent pathways.

Keywords: memory; T cells; transcription factors

Establishment of a functional memory CD8+ T-cell population is necessary for an effective secondary response to antigen. The specific requirements for the development of memory from effector cells and for the maintenance of such a population have not been fully elucidated, although it is clear that survival and proliferation are the key functions. Two γ chain receptor family cytokines, interleukin (IL)-7 and IL-15, promote survival and proliferation, and both receptors are upregulated on memory CD8+ T cells, indicating an important role for these molecules in maintaining memory populations. In support, Il7−/− and Il15−/− or Il15ra−/− mice have fewer memory CD8+ T cells after infection or stimulation than mice with both cytokines intact, whereas IL-7 or IL-15 transgenic over-expressing mice have more memory cells. Although mice deficient in either cytokine can generate memory cells, those without IL-7 do not maintain this population and those without IL-15 maintain only a very small, non-dividing population of cells. Loss of both cytokines results in rapid ablation of memory cells. At a molecular level, both induce expression of the anti-apoptotic molecule, Bcl2. Thus, each cytokine can mediate memory cell establishment and can also independently control survival and proliferation.

A major signaling pathway downstream of both IL-7 and IL-15 involves recruitment of JAK proteins to tyrosine kinase residues on IL-7Rα or IL-15Rα. This event leads to recruitment and phosphorylation of STAT5 and its subsequent dimerization and translocation to the nucleus. There is also evidence that other signaling pathways are also activated by these cytokine receptors: PI-3 kinase and Ras downstream of IL-15, and PI-3 kinase and JNK downstream of IL-7. STAT3 has already been implicated in memory cell development—STAT5a−/− or STAT3b−/− mice have fewer, and STAT3b transgenic mice more, memory phenotype T cells than wild type (WT) mice. Thus, we hypothesized that the non-redundant roles of IL-7 and IL-15 in establishing memory cells were due to differences...
in their downstream signaling pathways controlling survival and proliferation. To this end, we designed a study to examine the role of STAT5a signaling in memory development downstream of either IL-7 or IL-15.

RESULTS

Transduction of naïve TCR transgenic cells with mutant STAT5a expressing retrovirus
To investigate the role of STAT5a activity in the formation of CD8+ memory, we took advantage of previously described retroviral constructs expressing either WT-STAT5a or a H299R and S711F mutant of STAT5a that spontaneously dimerizes and provides constitutive STAT5a activity in expressing cells (constitutively active (CA-STAT5a)).20 Retroviral constructs also included an EGFP reporter expressed by virtue of upstream IRES sequence so as to monitor relative expression levels. We first established that both WT-STAT5a and CA-STAT5a expressing constructs transduced and expressed in comparable manner. Expression level of EGFP driven by IRES sequence is a faithful reporter upstream construct expression (data not shown). Antigen-stimulated CD8+ F5 TCR transgenic T cells were used as targets of STAT5a expressing retroviral transduction. F5 T cells express a Class I restricted TCR specific for an epitope of influenza A nucleoprotein (NP68 hereon). Transduction of in vitro activated F5 T cells with WT-STAT5a and CA-STAT5a expressing retroviruses resulted in similar frequencies of transduced T cells (Figures 1a and b). Importantly, the relative levels of construct expression, determined by measuring the MFI of EGFP expression, was virtually identical between the two constructs (Figure 1c). Finally, examination of STAT5 Y694 phosphorylation (pSTAT5) in EGFP+ve cells revealed a high level of phosphorylation in CA-STAT5a transduced F5 T cells compared with EGFP−ve T cells or F5 T cells transduced with an empty EGFP expressing vector (Figure 1d). Interestingly, WT-STAT5a expression resulted in low but detectable level of pSTAT5, suggesting that WT-STAT5a over-expression was resulting in a low level of STAT5 activation.

IL-7 and IL-15 are required to establish memory CD8+ T cell-populations
Transduction of T cells with STAT5A expressing retrovirus requires the T cells to be activated and cycling. Therefore, to investigate the role of STAT5 mutants in memory formation and maintenance, we took advantage of the fact that transfer to naive hosts of in vitro generated F5 effector T cells results in formation of memory-like cells.21 Although it is not known whether memory generated from cells primed in vitro bears all the hallmarks of in vivo primed memory generated after infection or antigen-adjuvant challenge, we and others have shown earlier in vitro primed memory cells do have identical homeostatic, phenotypic and cytotoxic functional properties to their in vivo generated counterparts22-24 and are therefore a reasonable model of memory cell behavior, at least for these characterized features.

As STAT5 activation occurs downstream of both IL-7R and IL-15R, we first confirmed that memory generation from in vitro primed F5 T cells was dependent on these cytokines. CD8+ effector F5 T cells were generated in vitro as described in 'Methods' and transferred into Rag1−/−, Rag1−/−Il15ra−/− or Rag1−/−Il7−/− mice. Mice deficient in IL-15Rα are also functionally IL-15 deficient because the receptor is absolutely required for trans-presentation of the cytokine.25 The number of donor CD8+ TCR+ cells recovered from the spleen of Rag1−/− hosts after 21 days is shown in Figure 2. Similar data were obtained from peripheral lymph nodes, bone marrow and peritoneal

![Image](319x414 to 545x724)

![Image](320x149 to 544x252)

![Image](325x100)

Figure 1. Expression of WT-STAT5a and CA-STAT5a in F5 T cells increases basal pSTAT5 levels. F5 T cells were activated in vitro with NP68 peptide and transduced with either empty, WT-STAT5a or CA-STAT5a expressing retrovirus. Viable effector T cells were isolated and re-cultured for a further 4 days in exogenous IL-2. (a) Scatter plot shows EGFP versus SSC by cultured F5 T cells transduced with WT-STAT5a or CA-STAT5a expressing retrovirus as compared with uninfected controls T cells. (b) Bar chart shows mean frequency of F5 T cells successfully transduced by WT-STAT5a or CA-STAT5a expressing retrovirus. (c) Bar chart shows mean fluorescence intensity of EGFP by EGFP+ and EGFP− T cells transduced with the indicated retroviral construct. (d) Histograms show pSTAT5 expression by EGFP+ (solid line) and EGFP− (gray fill) blasts at the end of the culture period in T-cell cultures transduced with the indicated retrovirus. Data are representative of (a, d) or pooled from (b, c) three independent experiments.

![Image](325x100)

Figure 2. Non-redundant roles of IL-7 and IL-15 in formation of CD8+ T-cell memory from F5 effector T cells. F5 T cells were activated in vitro with NP68 peptide for 3 days followed by 4 days further culture in IL-2. Cells were injected (5×106 per recipient) into groups of Rag1−/−, Il15ra−/−, Rag1−/−Il15ra−/− and Il7−/−Rag1−/− hosts. After 21 days, spleens were recovered and numbers of CD8+ TCR+ F5 T cells were determined by flow cytometry and cell counting. Histogram shows the mean cell recovery ± s.d. from different hosts. Data are pooled from four independent experiments with minimum 18 mice per group. Statistics: *P<0.0001.
cavity, and also in experiments in which cells were left 60 days after transfer (data not shown). Recovered cells had a memory phenotype—CD44hi CD122hi and IL-7Rαhi as published earlier. The number of cells recovered from mice lacking endogenous IL-7 or IL-15 was far lower than that recovered from control hosts in which both cytokines function normally (Figure 2) and we have shown earlier that populations of cells lacking both IL-7 and IL-15 signals do not endure in vivo. These data confirm in our system a non-redundant role for each cytokine in establishing a memory CD8+ T-cell population.

Over-expression of STAT5a enhances memory CD8+ T-cell formation

To investigate the specific effect of STAT5a in effector T cells and their transition to memory cells, effector F5 T cells transduced with either empty, WT- or CA-STAT5a expressing retrovirus were transferred into Rag1−/− mice, recovered after 21 days and the frequency and number of retrovirally transduced F5 T cells evaluated. Total cultures of F5 T cells were used for transfer to naive hosts and therefore included a mixture of blasts either successfully transduced with retroviral constructs or remaining untransduced, the two cases distinguishable by their expression of EGFP. EGFP− blasts co-transferred with transduced EGFP+ cells provided a convenient internal control as they shared identical culture conditions but lacked retroviral construct expression. Comparing frequencies of EGFP+ F5 cells before and 21 days after adoptive transfer revealed that empty virus had no advantageous effect on memory formation (Figure 3a). In contrast, F5 blasts expressing WT-STAT5a or CA-STAT5a were enriched at day 21 (Figure 3a) and EGFP expressing cells were represented at a significantly higher frequency at this time compared with their representation (see Methods) among effector cells injected at d0 (Figure 3b). The increase in representation by CA-STAT5a expressing cells over EGFP− non-transduced cells was much greater than observed in WT-STAT5a expressing cells (Figure 3b) and was particularly evident when determining absolute numbers of EGFP+ cells recovered (Figure 3c). As expected, recoveries of non-transduced EGFP− memory cells from the same hosts were similar regardless of which retrovirus had been used to transduce the cultured T cells (Figure 3c).

Previous studies have shown that IL-7 signaling can itself regulate IL-7Rα expression. Therefore, to ensure that differences in memory development, we observed in STAT5a expressing T cells, were not a consequence of altered cytokine receptor expression, we examined their expression of CD122 and IL-7Rα. CD122 expression by F5 T cells was identical in both EGFP+ and EGFP− F5 cells in the same host, as well as compared with control F5 memory generated from cultures not treated with retrovirus at all (Figure 4a). Similarly, IL-7Rα expression by EGFP+ F5 T cells expressing WT-STAT5a was identical to that of EGFP− F5 cells in the same host and control F5 memory cells (Figure 4b). Interestingly, EGFP+ F5 cells expressing CA-STAT5a had reduced IL-7Rα as compared with EGFP− F5 cells in the same host and control F5 memory cells (Figure 4b). It is possible that this reduced expression could have had a detrimental effect on memory formation by CA-STAT5a expressing cells. However, CA-STAT5a expression still resulted in greatly enhanced memory formation, and rather supports the view that CA-STAT5a is in fact functioning in a receptor-independent manner. Furthermore, these data show for the first time that IL-7 signal induced downregulation of IL-7Rα may be mediated by STAT5 activation, which would be consistent with the potent ability of IL-2 to inhibit IL-7Rα expression, which also signals through STAT3.

CA-STAT5a can partially rescue memory cell development in the absence of IL-7 or IL-15

The data in Figure 2 highlight the non-redundant roles of IL-7 and IL-15 in the establishment of memory T-cell populations, as published earlier. The data in Figure 3 show a potent role for STAT5 activation, a downstream target of both IL-7 and IL-15 receptors, for establishment of memory from effector cells, consistent with the observation that STAT5-deficient mice have a reduced CD8 memory pool. To examine whether activation of STAT5a was the key molecular event downstream of IL-7 and/or IL-15 during memory generation, we asked whether receptor-independent CA-STAT5a could substitute for either cytokine for the full development of memory. Effector F5

![Figure 3](https://example.com/figure3.png)

**Figure 3** Expression of WT-STAT5a and CA-STAT5a in F5 effector T cells enhances CD8+ T-cell memory formation in vivo. F5 T cells were transduced with either empty, WT-STAT5a or CA-STAT5a expressing retrovirus as described in Figure 1. (a) After culture and retroviral transduction, viable effector T cells were injected into Rag1−/− hosts (5×10⁶ per recipient, n=5). After 21 days, splenocyte number and phenotype were determined by flow cytometry and cell counting. Dot plots show frequency of EGFP expressing CD8+ TCR+ F5 T cells from the indicated cultures at the end of the culture period, before injection into Rag1−/− hosts (d0) and the frequency of EGFP+ cells from the same cultures 21 days after transfer in vivo (d21). (b) Histogram shows percent change in representation of EGFP expressing cells at d21, as normalized to the starting frequency in each culture before injection at d0. (c) Histogram shows cell recovery of EGFP+ (filled) and EGFP− (empty) F5 T cells at 21 day after transfer from the indicated cultures. Data are representative of five independent experiments. Statistics: *P<0.0001.
T cells transduced with WT- or CA-STAT5a expressing RV were generated and transferred to Rag1−/− or Rag1−/−/Il7−/− or Rag1−/−/Il15ra−/− mice and memory formation examined. As before (Figure 3b), CA-STAT5a profoundly increased the frequency of memory cells generated in control Rag1−/− hosts with intact IL-7 and IL-15 compared to WT-STAT5a (Figure 5a). Significantly, a near identical increase in frequency of CA-STAT5a expressing cells was also observed in hosts lacking IL-7 or IL-15 (Figure 5a). This increase in CA-STAT5a expressing cells was particularly evident when cell recoveries were determined. More EGFP+ than EGFP− cells were recovered in all three host strains (Figure 5c), despite the initial low frequency (23%) in the cell cultures used for transfer into the hosts. This suggests that CA-STAT5a could rescue memory formation independently of IL-7 or IL-15 signaling. However, absolute numbers of CA-STAT5a expressing F5 T cells were significantly reduced in both IL-7 and IL-15-deficient hosts compared with control hosts (Figure 5c). Therefore, although CA-STAT5a expression did confer a near identical competitive advantage over non-transduced cells in cytokine-deficient hosts, reflected in their relative increase in frequency (Figure 5a), transduced cells were still subject to a similar survival defect as EGFP− cells in the absence of IL-7 or IL-15 that could not be overcome by CA-STAT5a expression. Moreover, reduced cell recoveries in IL-7 and IL-15-deficient hosts could not be accounted for by preferential selection/exclusion of F5 cells expressing CA-STAT5a at different levels, as reporter EGFP expression by cells was identical in all three hosts (Figure 5d). This suggests an additional requirement for STAT5-independent signaling by both IL-7 and IL-15 during memory formation.

Figure 4 CA-STAT5a expression suppresses IL-7Rα expression. F5 T cells, transduced with either WT-STAT5a or CA-STAT5a expressing retrovirus, or cultured free of retrovirus as control, were transferred to Rag1−/− hosts. At day 21, recipient splenocytes were recovered and phenotype determined by FACS. Histograms show CD122 (a) or IL-7Rα (b) expression by EGFP+ (solid lines) and EGFP− (broken lines) F5 T cells from the same recipients of cultures transduced with either WT-STAT5a or CA-STAT5a expressing retrovirus as indicated. As control, CD122 (a) or IL-7Rα (b) expression by F5 cells not exposed to retrovirus during culture are shown (gray fills). Data are representative of three independent experiments.

Figure 5 Differential rescue of CD8+ T-cell memory formation by CA-STAT5a and WT-STAT5a in the absence of IL-7 or IL-15. F5 effector T cells were transduced with either WT-STAT5a or CA-STAT5a and transferred (5x10^6 per recipient) into groups of Rag1−/−, Il15ra−/−Rag1−/−, or Il7−/−Rag1−/− mice (n=4). After 21 days, spleens were taken and number and phenotype of F5 memory cells were determined by flow cytometry and cell counting. (a) Histogram shows the change in EGFP reporter expression, normalized to frequency at day 0, by F5 blasts transduced with either WT-STAT5a (empty bars) or CA-STAT5a (filled bars). Initial absolute frequencies of retroviral transduction at day 0 for the experiment depicted were 12% for WT-STAT5a and 23% for CA-STAT5a. (b, c) Histograms show total cell recoveries of non-transduced EGFP− (empty bars) or transduced EGFP+ (filled bars) F5 T cells from spleens of indicated recipients of either WT-STAT5a transduced blasts (b) or CA-STAT5a transduced blasts (c). (d) Histograms show EGFP expression by F5 cells transduced with CA-STAT5a and recovered from Rag1−/−, Il15ra−/−Rag1−/− or Il7−/−Rag1−/− mice in (c). Numbers are EGFP MFI of positively gated EGFP expressing F5 T cells and is the average ± s.d. observed among individual recipients in the different hosts. Data are representative of two independent experiments. Statistics: *P<0.05, **P<0.005.
In contrast to CA-STAT5α transduced cells, F5 effectors expressing WT-STAT5α behaved differently in the absence of IL-7 versus IL-15. Transduction of F5 effectors with WT-STAT5α promoted subsequent memory formation in cytokine sufficient Rag1−/− hosts, albeit to a lesser extent than CA-STAT5α (Figure 5a). Similarly, after transfer to IL-15-deficient (Il15ra−/−Rag1−/−) hosts, an enhancement of F5 memory formation was observed among EGFP+ WT-STAT5α expressing cells compared with EGFP− F5 cells derived from the same culture and present in the same host (Figure 5a). In contrast, WT-STAT5α expression failed to enhance memory formation in the same blasts transferred to IL-7-deficient Rag1−/− hosts, both in terms of increased representation of EGFP+ transduced cells (Figure 5a) and reflected also in cell recoveries from these mice (Figure 5b), in which numbers of EGFP+ and EGFP− cells recovered reflected their representation in the original in vitro culture used for cell transfer. Therefore, though CA-STAT5α appeared able to enhance F5 memory formation independently of cytokine signaling, WT-STAT5α was only capable of enhancing memory formation in the presence of IL-7 in Rag1−/− or Il15ra−/−Rag1−/− hosts. These data therefore suggest that, although IL-15 may be capable of activating STAT5 activity, it is IL-7 that is the principle means of activating STAT5 during memory formation in vivo.

**IL-7 and IL-15 activate STAT5 in a comparable manner in vitro**

To ask whether the differences in behavior of WT-STAT5α transduced cells in IL-15 and IL-15-deficient hosts reflected fundamental biochemical differences in the ability of these cytokines to activate STAT5, we examined IL-7 and IL-15-induced STAT5 phosphorylation in T cells after in vitro stimulation with IL-7 or IL-15. Although IL-7 induced a higher level of pSTAT5 than IL-15 in naïve F5 T cells, pSTAT5 levels in memory cells were similar in response to IL-7 and IL-15 (Figure 6a). These data suggest that IL-7 and IL-15 induce comparable pSTAT5 in memory F5 T cells. The difference between naïve and memory cell responses to IL-15 is likely explained by the difference in CD122 expression in these cells, and naïve cells express IL15R at a lower but functionally significant level. However, to confirm this observation, we also measured the kinetics of pSTAT5 induction in CD8+ CD44hi memory phenotype cells from WT mice. In CD8+ CD44hi naïve T cells, pSTAT5 induction in response to IL-7 was both slower and reduced compared to the response to IL-7. CD8+ CD44hi memory phenotype cells, however, exhibited similar kinetics and magnitude of pSTAT5 in response to IL-7 and IL-15 (Figure 6b).

**DISCUSSION**

In this study, we examined the role of the Jak-Stat signaling downstream of IL-7 and IL-15 for generation and maintenance of memory CD8 T cells. Introduction of CA-STAT5α expression into normal T cells greatly enhanced memory formation from in vitro generated effector cells, consistent with findings from others. However, CA-STAT5α only partially rescued memory formation in the absence of IL-7 or IL-15, suggesting that other signaling pathways activated by these receptors are required for optimal memory formation.

Our data suggest that the non-redundant roles of IL-7 and IL-15 in promoting memory formation are mediated by distinct signaling activities of their receptors and that optimal activation of STAT5 is at least one key difference. Although the receptor-independent activity of CA-STAT5α could partially rescue memory formation in the absence of either IL-7 or IL-15, rescue was not complete in either case and therefore STAT5 activity alone cannot account for the function of these cytokine receptors. The fact that a single factor cannot account for the activity of both cytokines is consistent with their non-redundant requirement in memory formation and suggests roles for receptor-specific activation of other pathways. We did, however, find evidence that IL-7 was a more potent activator of STAT5 activity than IL-15 in vivo, providing evidence of a key functional difference between IL-7 and IL-15. Regulation of receptor expression is one level at which the biological affects of these cytokines are controlled in vivo, because IL-15R is rapidly upregulated and maintained after activation whereas IL-7Rα is lost on effectors and re-expression is associated with development of memory. Here, we show evidence that differential activation of a key downstream effector, STAT5, is another important contribution to the distinct and non-redundant roles for these two cytokines. Expression of the WT-STAT5α retroviral construct in F5 T cells resulted in a small increase in basal pSTAT5 that revealed a significant difference in the ability of IL-7 and IL-15 to activate STAT5 to facilitate memory formation in vivo. Expression of the WT-STAT5α construct enhanced memory formation from F5 effector T cells only in IL-7 replete hosts. This difference could not be explained by biochemical differences in IL-7Ra and IL-15R as well as others observed similar pSTAT5 induction by IL-7 and IL-15 in vitro. The requirement for IL-7 to enhance memory formation by WT-STAT5α expressing cells may rather represent differences in abundance and/or location of expression of IL-7 and IL-15 in different lymphoid sites. The bone marrow has been identified as a key site of IL-15 expression for CD8 memory. However, IL-7 is expressed at high levels in both primary and secondary lymphoid tissues. The failure of IL-15 to enhance memory formation from WT-STAT5α expressing F5 cells is even more significant as lymphopenic Rag1−/− hosts were used in this study and, in the absence of host T cells, cytokine is not limiting in such a host.

The precise cellular mechanism by which CA-STAT5α and WT-STAT5α expression enhances memory formation remains to be
fully elucidated. We have published earlier that F5 blasts transferred to naïve hosts rapidly acquire a small resting phenotype and that cells exhibit both functional and phenotypic hallmarks of memory cells. However, their establishment as long-lived functional memory population is dependent on intact IL-7 and IL-15 signaling. Transfer of F5 blasts to Rag1−/− hosts is accompanied by both their survival and homeostatic proliferation. In this study, the substantial augmentation of memory formation by CA-STAT5a, and to a lesser extent WT-STAT5a, is most likely explained by an enhanced proliferative activity conferred by STAT5a expression. This was particularly evident in the presence of memory formation by CA-STAT5a conferred identical competitive advantages over non-transduced EGFP+ F5 T cells as observed in control Rag1−/− hosts. Despite this, in the absence of IL-7 and IL-15 signaling, recoveries of EGFP+ cells were still reduced. However, it is also possible that enhanced survival could be a contributing factor in the effects of STAT5a expression. The relative contribution of survival and proliferation to the affects of STAT5 activity remains to be fully determined.

The question, however, remains as to which other signaling pathways are activated by IL-15 and IL-7. Studies have implicated PI-3 kinase as a target downstream of both IL-728,29 and IL-15,16 signaling and, through downstream activation of Akt, is thought to regulate multiple pathways involved in controlling both survival and proliferation, such as p27kip1, FoxO3α30 and Bad.31 However, a recent study suggests that PI-3 K activation is not in fact a direct target of IL-7R but rather depends on STAT5 activation and transcriptional activity, although the gene targets necessary for PI-3 K activation in this context are not known.29 In contrast, IL-15R, like IL-2R can directly activate PI-3 K through association with Shc,32 and this may represent an important difference to IL-7R signaling. In support of this interpretation, administration of IL-15 increased proliferation human memory CD8+ T cells with low IL-7Rz expression through activation of PI-3K/Akt.33 However, the role of PI-3 K activation in lymphocyte survival remains unclear with studies both supporting30 and refuting10 its role in survival, and using a CA myristilated PI-3 K isoform, we could find no role for PI-3 K in F5 memory formation in any context (data not shown). Additionally, the failure of CA-STAT5a to completely substitute for IL-7 argues for the requirement of signaling pathways other than STAT5 or PI-3 kinase downstream of both receptors. Shc couples Ras-MAPK pathway to IL-15 signaling32,34 but a role in memory formation has yet to be fully addressed. In conclusion, though the role of STAT5-independent pathways remains to be elucidated, differential STAT5 activation by IL-7 and IL-15 represents a key strategic difference with a functional impact on memory formation in vivo.

METHODS

Mice

Rag1−/−, IGF1R−/−, IGF1R−/− II15ra−/− Rag1−/− and F5 Rag1−/− mice were bred in a conventional colony free of pathogens at the National Institute for Medical Research, London, UK. All lines were of H-2b haplotype. Animal experiments were performed according to local and national regulations.

Constructs and retrovirus production

pMX retroviral constructs expressing either WT STAT5a (pMX-STAT5a-ires-EGFP) or CA-STAT5a and downstream IRES-EGFP reporter (pMX-STAT5aI6-IRES-EGFP) were kindly provided by Prof. Toshiro Kitamura (Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan). CA-STAT5a in the pMX-STAT5aI6-EGFP construct bears specific H299R and S711F mutations.35 Infective retroviral particles were generated after transfection of Plat E packaging cells with the plasmid constructs and viral supernatants used fresh or frozen at −70 °C.

Flow cytometry

The following monoclonal antibodies were used: CD8-PerCpCy5.5, CD44-FITC (eBioscience, San Diego, CA, USA), CD8-PETR (Caltag Laboratories, Burlingame, CA, USA) TCRβ-PECy5 (eBioscience), and AlexaFluo 647 phospho-STAT5 (pY694; BD Biosciences, San Jose, CA, USA). Acquisition was carried out on Cyan (Dako, Glostrup, Denmark) or FACSColor (BD Biosciences) and analyzed with FlowJo (Treestar, Ashland, OR, USA). Staining was performed using standard protocols and standard PBS/BSA/Azide FACS staining buffer. For detection of pSTAT5, cells were first surface stained for CD8 and TCR expression, fixed in 4% paraformaldehyde for 15 min at RT, washed in FACS buffer and permeabilized in 90% methanol 10% water solution for 30 min on ice. Cells were then washed in FACS buffer and stained with AlexaFluor 647 phospho-STAT5 at RT for 30 min.

In vitro culture and retrovirus infection

Single cell suspensions from axillary, brachial, inguinal and cervical lymph nodes and spleens of transgenic TCR F5 Rag1−/− mice were obtained and incubated with 1 μg NP68 peptide (synthesized at National Institute for Medical Research) in RPMI supplemented with 10% FCS, 2 mM glutamine, 1% penicillin-streptomycin, 5 × 10−5 M 2- ME (all from Sigma, St Louis, MO, USA). At 24 h, half of the media was replaced with virus-containing media and incubated for a further 48 h. Cells were washed and incubated in media supplemented with 5 ng ml−1 IL-2 (Peprotech, Rocky Hill, NJ, USA) for 96 h. At the end of culture, effector T cells were CD44hi, CD62Lhi and CD25hi and only cultures with >5% viral infections were used. For in vitro stimulation with cytokines, cells were stimulated with saturating levels of IL-7 (Peprotech; 10 ng ml−1) or IL-15 (Peprotech; 10 ng ml−1) for various times in PBS or with PBS alone as control. Expression of CD8, CD44 and pSTAT5 was detected as described earlier.

Adoptive transfer

Effector T cells of 5 × 106 in PBS were adoptively transferred into host mice by tail vein injection and left for at least 21 days, then recovered from spleen, peripheral lymph nodes, bone marrow and peritoneal cavity. Single cell suspensions were obtained and cells enumerated. Frequency of EGFP expressing cells was calculated as a fraction of non-transduced cells:

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\text{Freq} \text{EGFP expressing cells} = (\% \text{EGFP}^+/(1 - \% \text{EGFP}^+)).
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Changes in the representation of EGFP+ transduced F5 cells (EGFP−—% input) recovered from recipients after adoptive transfer of cultured cells was determined by normalizing EGFP frequencies among F5 cells recovered from host mice to those measured in the original pre-injection cultures. Thus, 100% indicates no change in representation of EGFP+ transduced F5 cells recovered from hosts as compared with the inoculating T-cell culture. This controlled for differences in transduction rates between different experiments and comparison of cells transduced with different retroviral constructs that could also result in different transduction rates. Statistically significant differences between groups of data were assessed using Student’s t-test assuming unpained data with unequal variance, using Kaleidagraph V4.1 software.

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