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

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Abstract

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 *in vivo*. 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

Introduction

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 gamma chain receptor family cytokines, IL-7 and IL-15, promote survival and proliferation, and both receptors are upregulated on memory $CD8^+$ T cells 1, 2, 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 following infection or stimulation than mice with both cytokines intact 2-4, while IL-7 or IL-15 transgenic over-expressing mice have more memory cells 5, 6. While mice deficient in either cytokine can generate memory cells, those without IL-7 do not maintain this population 2, 7 and those without IL-15 maintain only a very small, non-dividing population of cells 8. Loss of both cytokines results in rapid ablation of memory cells 7.

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The non-redundant roles of IL-7 and IL-15 in the generation and maintenance of memory imply differing roles for each cytokine. Such a view is supported by the distinct patterns of their receptor expression during $CD8^+$ T cell responses. CD122, required for functional IL-15R signaling, is rapidly upregulated following activation in effector cells before memory formation and plays a role in the induction of the response 8; indeed, IL-15-dependent memory cells are $CD122^{hi}$ while IL-15-independent CD8 memory cells are $CD122^{lo}$ 9. In contrast, IL-7Rα is lost soon after activation and its re-expression much later in the response correlates with development of memory 1. Functionally, however, differences between IL-7 and IL-15 activity are less clear. Both cytokines are found to promote survival 7, 10, 11 and proliferation 2, 7, 12 during formation and maintenance of memory. At a molecular level, both induce expression of the anti-apoptotic molecule, Bcl2 10, 13. 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 14. This event leads to recruitment and phosphorylation of STAT5 and its subsequent dimerisation and translocation to the nucleus 15. There is also evidence that other signaling pathways are also activated by these cytokine receptors: PI-3 Kinase and Ras downstream of IL-15 16, and PI-3 Kinase and JNK downstream of IL-7 17, 18. STAT5 has already been implicated in memory cell development - $STAT5a^{-/-}$ or $STAT5b^{-/-}$ mice have fewer, and STAT5b transgenic mice more, memory phenotype T cells than wild type mice 19. 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.

Materials and Methods

Mice

$Rag1^{-/-}$, $Il7^{-/-}Rag1^{-/-}$, $Il15ra^{-/-}Rag1^{-/-}$ and F5 $Rag1^{-/-}$ mice were bred in a conventional colony free of pathogens at the National Institute for Medical Research, London, U.K. All lines were of $H\text{-}2^b$ haplotype. Animal experiments were performed according to local and national regulations.

Constructs and retrovirus production

pMX retroviral constructs expressing either wild type STAT5a (pMX-STAT5a-IRES-GFP) or constitutively active STAT5a and downstream IRES-EGFP reporter (pMX-STAT5a1*6-IRES-GFP) were kindly provided by Prof Toshio Kitamura (Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo). CA-STAT5a in the pMX-STAT5a1*6-EGFP construct bears specific H299R and S711F mutations 20. Infective retroviral particles were generated following 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), CD8-PETR (Caltag Laboratories, Burlingame, CA), and TCRβ-PECy5 (eBioscience), AlexaFluor 647 phospho-STAT5 (pY694; BD Biosciences, San Jose, CA). Acquisition was carried out on Cyan (Dako, Glostrup,□Denmark) or FACSCalibur (BD Biosciences) and analysed with FlowJo (Treestar, Ashland, OR). 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′ at RT, washed in FACS buffer and permeabilised in 90 % methanol 10 % water solution for 30′ on ice. Cells were then washed in FACS buffer and stained with AlexaFluor 647 phospho-STAT5 at RT for 30′.

In vitro culture and retrovirus infection

Single cell suspensions from axial, brachial, inguinal and cervical lymph nodes and spleens of transgenic TCR F5 *Rag1*$^{-/-}$ mice were obtained and incubated with 1 nM NP68 peptide (synthesised 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). 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 IL-2 (Peprotech, Rocky Hill, NJ) for 96 h. At the end of culture, effector T cells were CD44^{hi}, CD62L^{lo} and CD25^{hi}, 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; 10ng/ml) or IL-15 (Peprotech; 10ng/ml) for various times in PBS or with PBS alone as control. Expression of CD8, CD44 and pSTAT5 was detected as described earlier.

Adoptive Transfer

5×10^6 effector T cells in PBS were adoptively transferred into host mice by tail vein injection and left for at least 21 d, 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:

$$\text{Freq}\quad \text{EGFPexpressing cells} = (\%\text{EGFP}^+ / (1 - \%\text{EGFP}^+)).$$

Changes in the representation of EGFP$^+$ transduced F5 cells (EGFP - % input) recovered from recipients following adoptive transfer of cultured cells was determined by normalising EGFP frequencies amongst 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 was assessed using Student t test assuming unpaired data with unequal variance, using Kaleidagraph V4.1 software.

Results

Transduction of naïve TCR transgenic T cells with mutant STAT5a expressing retrovirus

In order 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 dimerises and provides constitutive STAT5a activity in expressing cells (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). Tranduction of *in vitro*

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activated F5 T cells with WT-STAT5a and CA-STAT5a expressing retroviruses resulted in similar frequencies of transduced T cells (Fig. 1A and 1B). Importantly, the relative levels of construct expression, determined by measuring the MFI of EGFP expression, was virtually identical between the two constructs (Fig. 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 (Fig. 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 be activated and cycling. Therefore, in order to investigate the role of STAT5 mutants in memory formation and maintenance, we took advantage of the fact that transfer to naïve 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* bear all the hallmarks of *in vivo* primed memory generated following infection or antigen-adjuvant challenge, we and others have previously shown *in vitro* primed memory cells do have identical homeostatic, phenotypic and cytotoxic functional properties to their *in vivo* generated counterparts 7, 22-24 and are therefore a reasonable model of memory cell behaviour, at least for these characterised features.

Since 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 Materials and Methods and transferred into *Rag1*$^{-/-}$, *Rag1*$^{-/-}$*Il7*$^{-/-}$ or *Rag1*$^{-/-}$*Il15ra*$^{-/-}$ mice. Mice deficient in IL-15Rα are also functionally IL-15 deficient since 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 cavity, and also in experiments in which cells were left 60 days after transfer (data not shown). Recovered cells had a memory phenotype - CD44^{hi}, CD122^{hi} and IL-7Rα^{hi} as previously published 7. 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 (Fig. 2) and we have previously shown that populations of cells lacking both IL-7 and IL-15 signals do not endure *in vivo* 7. 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 naïve 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 since they shared identical culture conditions but lacked retroviral construct expression. Comparing frequencies of EGFP$^+$ F5 cells before and 21 d after adoptive transfer revealed that empty virus had no advantageous effect on memory formation (Fig. 3A). In contrast, F5 blasts expressing WT-STAT5a or CA-STAT5a were enriched at d21 (Fig. 3A) and EGFP expressing cells were represented at a significantly higher frequency at this time compared with their representation (See

Material and Methods) amongst effector cells injected at d0 (Fig. 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 (Fig. 3B) and was particularly evident when determining absolute numbers of EGFP$^+$ cells recovered (Fig. 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 (Fig. 3C).

Previous studies have shown that IL-7 signalling can itself regulate IL-7Rα expression26. 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 (Fig. 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 (Fig. 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 (Fig. 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 down-regulation of IL-7Rα may be mediated by STAT5 activation, which would be consistent with the potent ability of IL-2 to inhibit IL-7Rα expression27, which also signals via STAT5.

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 previously published. The data in Figure 3 demonstrate 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 19. 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 T cells transduced with WT or CA-STAT5a expressing RV were generated and transferred to *Rag1*$^{-/-}$, *Rag1*$^{-/-}$*Il7*$^{-/-}$ or *Rag1*$^{-/-}$ *Il15ra*$^{-/-}$ mice and memory formation examined. As before (Fig. 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 (Fig. 5A). Significantly, a near identical increase in frequency of CA-STAT5a expressing cells was also observed in hosts lacking IL-7 or IL-15 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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. Also, 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, since reporter EGFP expression by cells was identical in all three hosts (Fig. 5D). This suggests an additional requirement for STAT5 independent signalling by both IL-7 and IL-15 during memory formation.

In contrast to CA-STAT5a transduced cells, F5 effectors expressing WT-STAT5a behaved differently in the absence of IL-7 versus IL-15. Transduction of F5 effectors with WT-STAT5a promoted subsequent memory formation in cytokine sufficient *Rag1*$^{-/-}$ hosts, albeit to a lesser extent than CA-STAT5a (Fig. 5A). Similarly, following transfer to IL-15 deficient (*Il15ra*$^{-/-}$ *Rag1*$^{-/-}$) hosts, an enhancement of F5 memory formation was observed amongst EGFP^{+} WT-STAT5a expressing cells compared with EGFP^{-} F5 cells derived from the same culture and present in the same host (Fig. 5A). In contrast, WT-STAT5a 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 (Fig. 5A) and reflected also in cell recoveries from these mice (Fig. 5B), in which numbers of EGFP^{+} and EGFP^{-} cells recovered reflected their representation in the original *in vitro* culture used for cell transfer. Therefore, while CA-STAT5a appeared able to enhance F5 memory formation independently of cytokine signaling, WT-STAT5a 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 behaviour of WT-STAT5a transduced cells in IL-15 and IL-7 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 following *in vitro* stimulation with IL-7 or IL-15. While 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 (Fig. 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 low but functionally significant level 4. However, to confirm this observation, we also measured the kinetics of pSTAT5 induction in CD8^{+} CD44^{lo} naïve and CD8^{+} CD44^{hi} memory phenotype cells from WT mice. In CD8^{+} CD44^{lo} naïve T cells, pSTAT5 induction in response to IL-15 was both slower and reduced compared to the response to IL-7. CD8^{+} CD44^{hi} memory phenotype cells, however, exhibited similar kinetics and magnitude of pSTAT5 in response to IL-7 and IL-15 (Fig. 6B).

Discussion

In this study, we examined the role of the Jak-Stat signalling downstream of IL-7 and IL-15 for generation and maintenance of memory CD8 T cells. Introduction of CA-STAT5a expression into normal T cells greatly enhanced memory formation from *in vitro* generated effector cells, consistent with findings from others 19. However, CA-STAT5a only partially rescued memory formation in the absence of IL-7 or IL-15, suggesting that other signalling 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-STAT5a 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*, since IL-15R is rapidly up-regulated and maintained after activation 4, 7, 8 while IL-7Rα is lost on effectors and re-expression associated with development of memory 1, 7. 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-STAT5a 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-STAT5a 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-7Rα and IL-15R as we and others observed similar pSTAT5 induction by IL-7 and IL-15 *in vitro* 14. The requirement for IL-7 to enhance memory formation by WT-STAT5a 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 28. 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-STAT5a expressing F5 cells is even more significant as lymphopenic *Rag1*$^{-/-}$ hosts were used in the present study and, in the absence of host T cells, cytokine is not limiting in such a host.

The precise cellular mechanism by which CA-STAT5a and WT-STAT5a expression enhances memory formation remains to be fully elucidated. We have previously published 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 signalling. Transfer of F5 blasts to *Rag1*$^{-/-}$ hosts is accompanied by both their survival and homeostatic proliferation 7. In the present 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 *Il7*$^{-/-}$ and *Il15ra*$^{-/-}$ hosts where expression of 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 signalling, 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 remain 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 PI3Kinase as a target downstream of both IL-7 18, 29 and IL-15 16 signaling and, via downstream activation of Akt, is thought to regulate multiple pathways involved in controlling both survival and proliferation, such as p27^{kip}, FoxO3a 30 and Bad 31. However, a recent study suggests that PI3K 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 PI3K activation in this context are not known 29. In contrast, IL-15R, like IL2R can directly activate PI3K 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-7Rα expression via activation of PI3K/Akt 33. However, the role of PI3K activation in lymphocyte survival remains unclear with studies both supporting 29 and

refuting 10 its role in survival, and using a CA myristilated PI3K isoform, we could find no role for PI3K 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 PI3kinase downstream of both receptors. Shc couples Ras-MAPK pathway to IL-15 signaling 32, 34 but a role in memory formation has yet to be fully addressed. In conclusion, while 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*.

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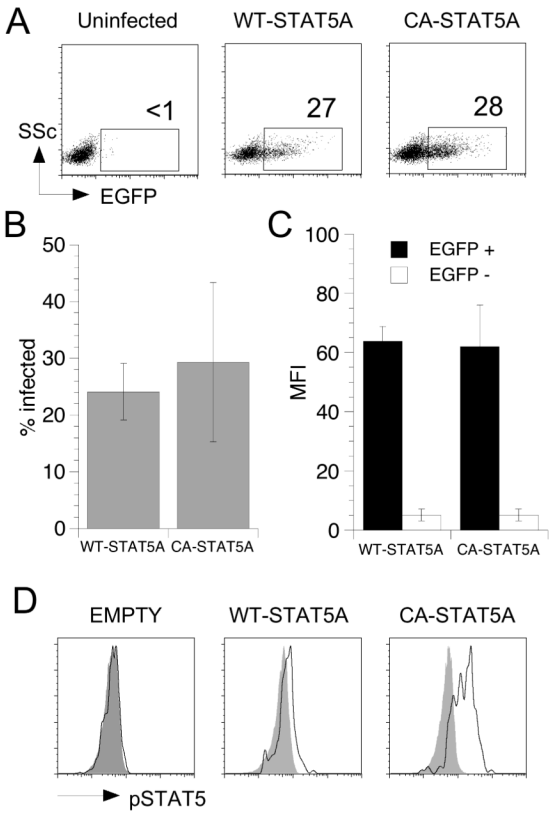

Figure 1.

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 d in exogenous IL-2. (A) Scatter plot shows EGFP vs 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 $EGFP^{-}$ (grey fill) blasts at the end of the culture period in T cell cultures transduced with the indicated retrovirus. Data are representative of (A and D) or pooled from (B and C) three independent experiments.

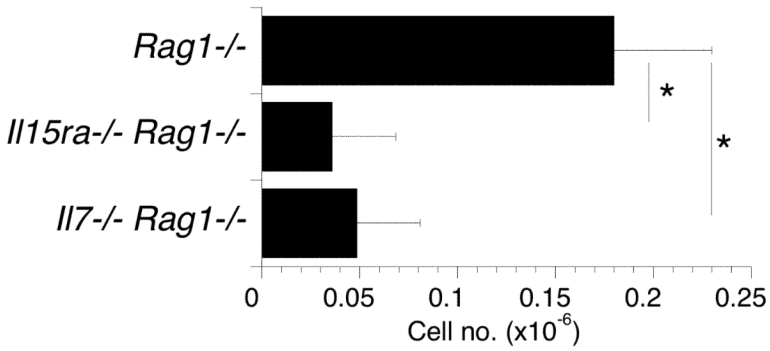

Figure 2.

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 d followed by 4 d further culture in IL-2. Cells were injected (5×10^6 / recipient) into groups of $Rag1^{-/-}$, $Il15ra^{-/-}$ $Rag1^{-/-}$ and $Il7^{-/-}$ $Rag1^{-/-}$ hosts. After 21 d, spleens were recovered and numbers of $CD8^+$ TCR^+ F5 T cells 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: * $p<0.0001$.

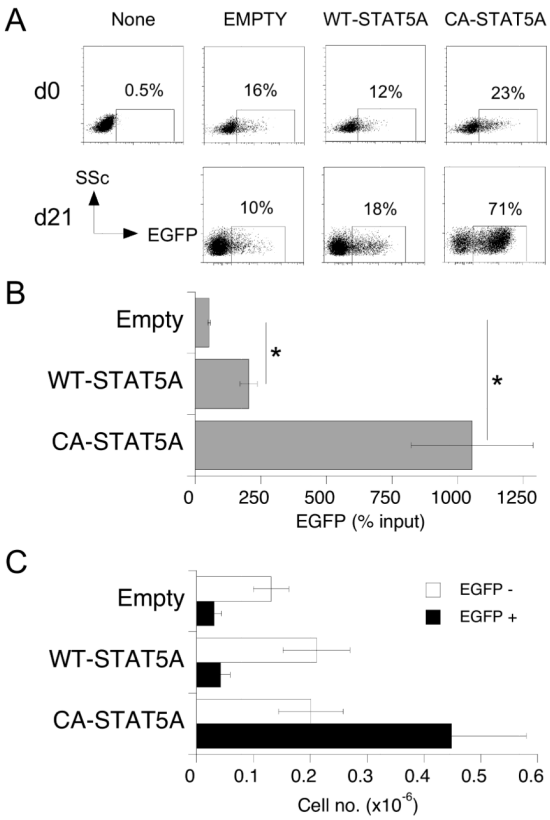

Figure 3.

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 Fig. 1. (A) After culture and retroviral transduction, viable effector T cells were injected into *Rag1*$^{-/-}$ hosts (5×10^6 / recipient, n=5). After 21 d, splenocyte number and phenotype was 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, prior to injection into *Rag1*$^{-/-}$ hosts (d0) and the frequency of $EGFP^+$ cells from the same cultures 21 d after transfer *in vivo* (d21). (B) Histogram shows % change in representation of EGFP expressing cells at d21, as normalized to the starting frequency in each culture prior to injection at d0. (C) Histogram shows cell recovery of $EGFP^+$ (filled) and $EGFP^-$ (empty) F5 T cells at 21 d following transfer from the indicated cultures. Data are representative of five independent experiments. Statistics: * $p<0.0001$.

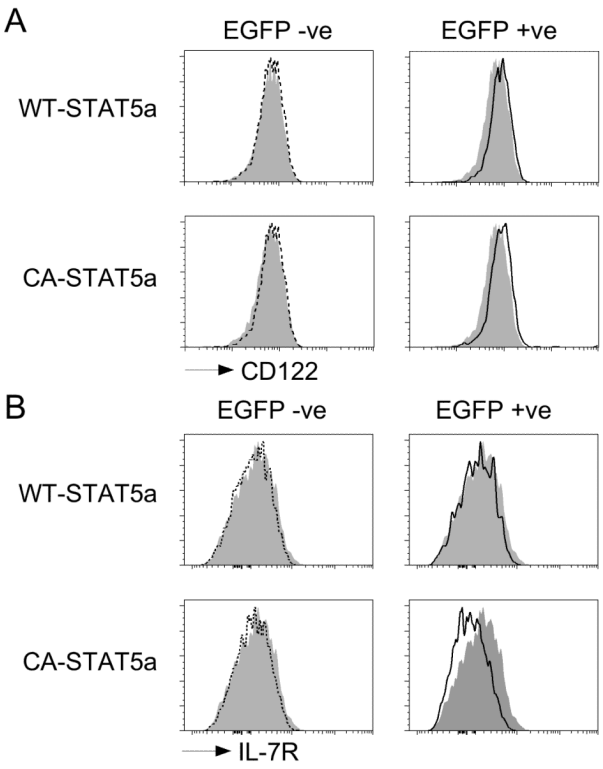

Figure 4.

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 d 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 (Grey fills). Data are representative of three independent experiments.

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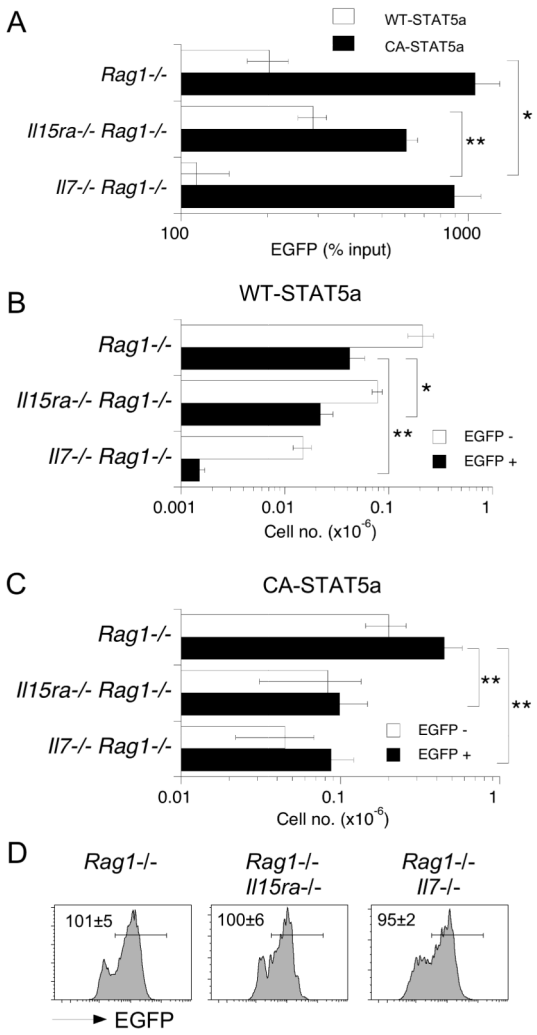

Figure 5.

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 (5×10^6 / recipient) into groups of *Rag1*$^{-/-}$, *Il15ra*$^{-/-}$ *Rag1*$^{-/-}$, or *Il7*$^{-/-}$ *Rag1*$^{-/-}$ mice (n=4). After 21 d, spleens were taken and number and phenotype of F5 memory cells 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 ± sd observed amongst individual recipients in the different hosts. Data are representative of two independent experiments. Statistics: * $p < 0.05$, ** $p < 0.005$.

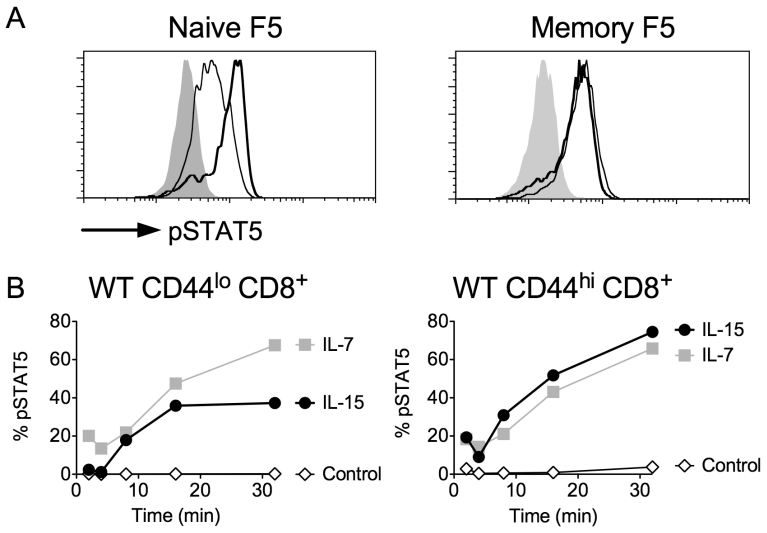

Figure 6.

Similar induction of pSTAT5 in memory CD8$^+$ T cells by IL-7 and IL-15 *in vitro*.

(A) F5 memory CD8$^+$ T cells were generated by transfer of effector T cells into *Rag1*$^{-/-}$ recipients. At d21, memory F5 T cells were recovered and stimulated with IL-7, IL-15 or PBS alone for 30′. Naïve F5 T cells were similar treated as control. Histograms show pSTAT5 induction in response to IL-7 (thick line), IL-15 (thin line) or PBS (grey fill) by naïve and memory F5 T cells. (B) CD8$^+$ splenocytes were isolated from WT mice and stimulated with IL-7, IL-15 or PBS for different times. Cells were stained with antibodies for expression of CD8, CD44 and pSTAT5. Line graphs show % pSTAT5 +ve staining with time by CD8$^+$ CD44^{lo} or CD8$^+$ CD44^{hi} WT cells stimulated with IL-7 (grey squares), IL-15 (black circles) or PBS alone (empty diamonds). Data are representative of 3 independent experiments.