Published in final edited form as:

Cell Immunol. 2008 February ; 251(2): 93–101. doi:10.1016/j.cellimm.2008.04.007.

Low dose IL-15 induces snap arming of CD44^{low} T lymphocytes in the absence of antigen

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Abstract

It is widely accepted that naïve T cells require two signals, antigen recognition and co-stimulation, to become cytotoxic over the course of 3–5 days. However, we observed that freshly isolated murine splenocytes without exposure to antigen become cytotoxic within 24 hours after culture with IL-15. IL-15 is a cytokine that promotes homeostatic proliferation, maintenance and activation of memory T cells. The induced cytotoxicity, measured by anti-CD3 redirected ⁵¹Cr release, represented the combined activity of T cells regardless of their antigen specificity, and proceeded even when CD44^{hi} (memory-associated phenotype) CD8⁺ T cells were depleted. Cytotoxic capacity was perforin-dependent and occurred without detectable up regulation of granzyme B or cell division. After induction, the phenotypic markers for the memory subset and for activation remained unchanged from the expression of resting T cells. Our work suggests that T cells may gain cytotoxic potential earlier than currently thought and even without TCR stimulation.

Keywords

Granzyme B; cytotoxic T lymphocyte; IL-15; IL-2

1. Introduction

T cells freshly isolated from pathogen-free mice lack the capacity to engage and kill target cells. Induction of cytotoxic potential or ‘arming’ occurs after infection, and many specific details of the process are unresolved at this time. The current paradigm maintains that two signals are required for T cell activation. One signal is initiated by the T cell recognizing its cognate antigen [1;2]. The second signal is CD28 costimulation. After both activation signals are received, T cells then undergo proliferation and upregulation of their cytotoxic arsenal. It is worth noting is that both events, proliferation and arming, may be dissociated events. Indeed, Dr. Ann Kelso and colleagues demonstrated that IL-2 can up-regulate granzyme message without concurrently increasing T cell growth [3]. In these studies, day 3 was the earliest time point examined for culture with cytokines, and the authors focused on expression of genes for

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cytotoxic proteins rather than cytotoxic function. Further, the T cells were T cell receptor for antigen (TCR)-stimulated and the experiments provide support for a paradigm for lymphocyte activation where proliferation and cytotoxic potential are independent factors. Here we have addressed activation in the absence of TCR stimulation, supported by a T cell growth factor, IL-15.

IL-15 as a T cell growth factor supports adaptive immune responses, induces homeostatic proliferation and maintenance of memory T cells [4;5]. IL-15 and the predominant T cell growth factor IL-2 share a pair of receptor subunits, the gamma chain common to several cytokine receptors (γ_c , CD132) combined with the IL-2/15 beta receptor chain (IL-2R β , CD122) [6]. Binding of IL-15 to the β - γ_c , with a $K_d \sim 10^{-9}$ M [7–9], will activate this dimeric receptor to transmit intracellular signals via the JAK1/3-STAT3/5 pathways [10–12]. Thus, at elevated doses of cytokine (10^{-8} M), these β - γ_c receptors will be saturated. Responses to lower doses of IL-15 require a unique signaling mechanism. IL-15 has a specific high affinity receptor ($K_d \sim 10^{-11}$ M) that is formed when separate specific alpha receptor chains combine into trimeric receptors with the CD122/CD132 receptor pair [6]. There are few of these IL-15 α -trimers per T cell [7;13] and the intracellular signaling is less well characterized than for the β - γ_c receptor. It is known that IL-2 or IL-15 will activate NK cells without α -receptor stimulation [14;15]. It is also known that IL-15 without antigen(s) can activate cytotoxic capacity of human T cells with a memory-associated phenotype and may also activate naïve human CD8⁺ T cells [16;17] after days 4 or 6 of culture, respectively. Here we demonstrate that cytotoxicity can be induced by low IL-15 in T cells more rapidly than previously described, that activation of cytotoxicity is antigen independent and that cytotoxicity proceeds in the absence of memory-phenotype CD8⁺ effector lymphocytes.

For induction of cytotoxicity, which in our case was *without antigen*, we evaluated low interleukin concentrations (10 ng/ml IL-15; 7×10^{-10} M) at a very early time point (16–24 hours). IL-15 induced cytotoxic capacity in the resting T cell population. We use the word ‘capacity’ advisedly because, physiologically, in the absence of antigen, the capacity would likely be unutilized. We measured induced cytotoxic capacity with anti-CD3 antibody redirected lysis of P815 cells and then extended our investigation to characterize intracellular levels of granzyme B (Gzm B) and the phenotype of the multi-clonally activated cells. At 24 hours, the granule protein Gzm B was undetectable by flow cytometry. The general phenotype of the effector T cell population resembled a resting state, and the cells were also non-dividing. Furthermore, CD44^{low} T cells acquired cytotoxic capacity and the cytotoxicity was perforin-dependent. This rapid or snap arming is a newly discovered cellular property. This discovery extends our knowledge of the antigen-independent T cell arming by IL-15 that has been previously characterized by several investigators [17–23], including ourselves [24].

2. Methods and Materials

2.1 Animals

The animal protocols for this study were approved by the University of Nevada Animal Use Review Committee. We used wild type (WT) C57BL/6 mice (Jackson Labs or the NCI) and perforin-gene ablated mice (Pfn1^{-/-} order number 002407 from Jackson [25] or order number PFPN12-M-F from Taconic Farms [26]). The animals were between 6–16 weeks of age.

2.2 Cell Culture

Spleens were harvested and the cells cultured at 5×10^5 cells/ml in T-75 tissue culture flasks (CoStar) with RPMI-1640 media (Sigma Chemical Co.), 10% FBS (HyClone), and 1% Pen-Strep (Sigma) with or without mouse recombinant (r-) IL-15 (eBiosciences mainly and also PeProtech). The specific activity of the eBiosciences r-IL-15 was approximately 10^6 CTLL-2

growth units per mg protein and $\sim 1.4 \times 10^{13}$ units/mole. In some experiments, r-IL-2 from PeProtech, specific activity 10^7 u/mg, was used for comparison of ILs 2 and 15. Incubation was at 37°C with 5% CO₂. Cytokine stimulated cultures received 10 ng/ml IL-15 (7×10^{-10} M) unless otherwise stated.

2.3 Flow Cytometric Analysis

Cells were harvested and phenotyped with mAbs to the following mouse antigens: CD3e (hamster IgG, clone 145-2C11), CD4 (rat IgG2b, clone GK1.5), CD8a (rat IgG2a, clone 53-6.7), NK1.1 (mouse IgG2a, clone PK136), Gr-1/Ly-6G (rat IgG2b, clone RB6-8C5), CD62L (rat IgG2a, clone MEL-14), CD25 (rat IgG1, clone PC61.5) and anti-mouse/human CD44 (rat IgG2b, clone IM7). The fluorochromes included fluorescein (FITC), R-fluorophycoerythrin (PE), PE-cyanine 5 (PC5) and PEcyanine 7 (PC7) as indicated in the figures. After surface labeling, the cells were washed, fixed, and permeabilized (IntraPrep Kit, Beckman Coulter). Granzyme (Gzm) B was detected with PE anti-human Gzm B mAb (mouse IgG1, clone GB-12, Caltag) that cross-reacts with mouse Gzm B and failed to react with mouse Gzm B^{-/-} T cells (data not shown). Samples were measured with a Beckman Coulter XL/MCL flow cytometer with optical filters to detect 525, 575, 670, and >740nm. The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR), including proliferation kinetics. For histograms, the “relative cell number” ordinate value represents a scale normalized to 95% as equal to the number of cells in the histogram channel with the most cells. Cells were labeled with carboxyfluorescein succinimidylester (CFSE, Molecular Probes/Invitrogen, 2.5 μM for 5 min) and cellular fluorescence monitored for reduction of label after each cell division.

2.5 Cytotoxicity Assays

T cell lysis (activated without antigens or anti-TCR or CD3 antibodies) was redirected to the FcIgGR-bearing P815 cells with anti-mouse CD3e mAb (hamster IgG, clone 145-2C11). Anti-CD3e antibody was added during the assay at a concentration of 1 ug/ml. P815 target cells were labeled with Na⁵¹CrO₄ (Amersham CJS4) and assays run in quadruplicate with 10,000 cells per well for 4 hours. Specific release was calculated by the formula: % Specific Release = [(Experimental CPM) – (Spontaneous Release)] / [(SDS Total Release) – (Spontaneous Release)]. Splenocyte: target ratios were determined by hemocytometer counts. CD8⁺ effector:target ratios were calculated by using the splenocyte population and multiplying it by the fraction of CD3⁺CD8⁺ as determined by flow cytometry. Comparison of the activities of different cytotoxic T cells was made by comparing lytic units of activity per 10⁶ effector cells, where 1 lytic unit is defined as the number of cells that will kill half the targets in the 4 hour assay [27].

2.6 Depletion of memory-phenotype T cells

Spleens were harvested from C57BL/6 mice and splenocyte preparations were filtered through 03–35/16 Nitex (Sefar American Inc.) to remove large debris. CD16/32 mAb was added to block non-specific Fc binding sites. Anti-Gr1 mAb was added at a ratio of 8 ug/10⁷ splenocytes. The cells were incubated at 4°C for 30 minutes and washed. After washing, the cells were brought up in 200 ul of streptavidin magnetic particles (BD Biosciences iMag, Cat# 557812) and incubated for 45 minutes at room temperature. After incubation, the tube was placed in the iMag (BD biosciences) magnetic apparatus and the cells separated after 10 minutes. The separation procedure was repeated 3–5 times to ensure depletion. Separated cells were placed into culture or labeled for flow cytometry to monitor depletion of the CD44^{hi} cells (which were also Gr1⁺).

2.7 Reproducibility and Statistics

Representative experiments are illustrated from at least two replicate experiments. Standard errors of the mean were calculated for cytotoxic assays and are indicated where appropriate. The flow cytometric data were subjected to Overton subtraction statistical analyses [28] to assess frequency of antibody-positive cells in the populations.

3. Results

3.1. IL-15 induces snap arming of cytotoxic potential in splenocytes within 24 hours

We harvested splenocytes from C57BL/6 mice and placed them in culture for one day with or without 10 ng/ml IL-15. In an anti-CD3e redirected lytic assay against P815 targets (H-2^b C57BL/6 mice against H-2^d P815 mastocytoma), we found that there was little killing directly *ex vivo* (Fig. 1A). The initial splenocyte population consisted of ~1% cells that were positive for the NK cell marker NK1.1 and ~6% of all the cells were CD8⁺ with few detectable NK1.1⁺ CD8⁺ NK-T cells in these starting splenocytes. After 24 hours, splenocytes cultured without cytokine continued to lack substantial cytotoxic capability, just as the *ex vivo* splenocytes did (Fig. 1B). This lack of activity was the case despite an increase in the proportion of CD8⁺ cells during culture (from 5.6% to ~15% of all cells, see Fig. 1B, flow data). Splenocytes cultured with 10 ng/ml of IL-15 exhibited markedly increased cytotoxicity from ~5% to over 30% at the CD3⁺ T cell:target E:T ratio of 1:1 (Fig. 1C). The frequency of CD8⁺ T cells in the IL-15 treated culture (15%) remained similar to the untreated cultured cells. NK1.1⁺CD8⁺ T cells remained undetectable (Fig. 1C, flow data). Thus increased T cell-mediated killing was independent of the frequency or survival of the CD8⁺ T cells.

There is evidence for differentiation of the T cells during the single day of culture without specific antigens. High side scatter often represents granular features of T cells and changes may be interpreted to be indicative of functional alterations. With IL-15, the T cells with high side scatter were 12% of all cells (circumscribed by the triangle above Fig. 1C). In comparison, the proportion of high side scatter T cells for the control cultured cells was only 8.3%, up from 3.4% *ex vivo*. Thus the frequency of high side scatter cells after culture with IL-15 indicates that some differentiation may have started.

Splenocytes were treated with a dose titration of IL-15 and the cytotoxicity was measured after one day in culture (Fig. 1D). Cytotoxic potential was IL-15 dose dependent over the range of cytokine concentrations tested. Concentrations as low as 1 ng/ml (7×10^{-11} M) induced killing potential. The splenocytes treated with the highest concentration of cytokine tested, 50 ng/ml (approximately 3.5×10^{-9} M, approaching the K_d of 10^{-8} M of IL-15 for the $\beta\gamma$ receptor complex), demonstrated the highest killing. Additionally, the IgG control (Fig. 1D) indicates that the cytotoxic capacity is CD3-dependent for efficacy, evidence that the observation is independent of NK cells.

Taken together, these observations suggest that resting T cells can be rapidly potentiated and are capable of killing target cells.

3.2 Early activation by IL-15 occurs in the absence of memory-phenotype T cells

IL-15 is a cytokine known to maintain homeostatic proliferation of memory T cells [29;30] and can activate resting memory T cells to become cytotoxic [31;32]. As such, memory T cells are prime candidates as mediators of the cytotoxic action observed after IL-15 treatment. To determine if memory-phenotype T cells were the cytotoxic population mediating target cell death, we used biotinylated anti-Gr-1 antibody conjugated to streptavidin-microspheres to selectively deplete the CD44^{hi} memory-phenotype T cell population (Fig. 2A). Anti-Gr-1 cross-reacts with Ly-6c, which is co-expressed with CD44 on memory-phenotype effector T

cells [33]. The population of CD3⁺CD8⁺CD44^{hi} cells was approximately 26% of all CD8⁺ splenic cells obtained directly *ex vivo*. After Gr-1 depletion, the proportion of CD3⁺CD8⁺CD44^{hi} cells was reduced to 7% of the CD3⁺CD8⁺ T cells, a more than 3-fold reduction. The depleted and control cells were cultured overnight with or without IL-15.

When the IL-15 treated and untreated populations were assayed for cytotoxicity, we found that allogeneic killing (undirected lysis) of the H2^d P815 targets by the H2^b C57BL/6 effector cells was elevated in both of the IL-15 treated groups (Fig. 2B). Indeed, lysis was more elevated in the Gr-1 depleted group than in the unseparated population, contrary to what one might expect if the induced allogeneic cytotoxicity were attributable to memory T cells. Redirected lysis was also significantly elevated in the cytokine-treated series (Fig. 2C). There was little difference in killing between the Gr-1 depleted and intact groups, an observation that would be surprising if one expected only the memory T cells to be lytic. Lytic potential of the cells after treatment with IL-15 was largely independent of the memory-phenotype effector T cells, as a three-fold shift in cytotoxic activity would have been readily detectable. Because the redirected killing is CD3 dependent, these data suggest that cells within the naïve-phenotype T cell population are mediating the cytotoxic effect.

3.3. Cytotoxicity is induced by IL-15 without measurable increases in granzyme B, proliferation and is perforin-mediated

Resting T cells are considered to be in an “unarmed” state. To become effective killers, T cells must be activated by receptor and/or cytokine stimulation and initiate production of cytotoxic proteins. Given the rapid CD3-dependent cytotoxic response observed in the naïve-phenotype lymphocytes, we investigated if cytotoxic granule maturation occurred in such a limited time frame. We chose Gzm B as our granule marker because it is a major granule protein and is intimately involved in the cytotoxic process.

At the onset of culture (not illustrated) and after 1 day, Gzm B levels were below the limits of detection by flow cytometry (Fig. 3A–B). The IL-15 treated CD8⁺ T cells had a Gzm B median fluorescent index (MFI) of ~1 while for the isotype control group the Gzm B MFI was ~0.7. The lack of Gzm B was a marked contrast to the Gzm B of splenocytes cultured for 3–4 days with 1000 u/ml of IL-2 (which had a Gzm B MFI of 86, not illustrated). These data suggest that the cytotoxic potential induced by IL-15 may occur in the absence of a significant increase in many granule proteins. It is important to note that Gzm B may be present at levels below the detection threshold for flow cytometry, but the observed cytotoxic potential was present before Gzm B levels indicated lymphocyte activation.

The Kelso group has shown independence of proliferation and granzyme expression [3] and others have demonstrated that naïve T cells can be induced to proliferate over time after IL-15 exposure [17]. To determine if the splenic T cells were dividing by day 1, CFSE-labeled splenocytes were placed in culture with or without IL-15 (Fig. 3C). CFSE staining was similar between the IL-15 treated and untreated groups and the CFSE signal itself remained unchanged, for both CD44^{hi} and CD44^{low} T cells. These data suggest that the cytotoxic potential induced by IL-15 (Fig. 1) occurs before either detectable Gzm B up-regulation or proliferation.

We are confident that killing is *via* perforin through the granule-mediated pathway because Prf1 WT lymphocytes treated with 50ng/ml of IL-15 for 1 day demonstrate 3–4 fold greater killing than prf1^{−/−} lymphocytes after cytokine treatment (Fig. 3D). In totality, these data suggest that the T cells are killing targets rapidly by granule-dependent mechanisms without detectable increases of major granule proteins or proliferation.

3.4. CD8⁺ T cells snap armed by IL-15 retained a resting phenotype

Phenotypic changes in the CD8⁺ T cells in response to IL-15 induced cytotoxicity were investigated. Table 1 lists the different markers examined. NK1.1 and DX5 are NK cell markers that are also associated with NK-T cells. Changes were negligible in NK-T cells with regard to frequency relative to the control groups of either day 0 cells (shaded) or cells cultured without cytokine (Fig. 4A–B). CD122 is the β -chain component of the IL-2 and IL-15 signaling receptor complex. Prior results show that this marker is up regulated on memory and activated T cells. We found that after one day in culture there was a slight elevation in the proportion of CD122⁺ CD8⁺ T cells compared to directly *ex vivo*, but the intensity of the CD122 signal was slightly diminished (Fig. 4C).

Multiple markers on CD8⁺ T cells were examined for IL-15 induced changes. The marker status was largely unchanged after 24 hours of culture. CD44 is a classical memory phenotype marker, and anti-Gr-1 reacts with Ly-6c [33], a marker co-expressed on memory cells with CD44. The frequency of CD44^{hi} T cells increased after 1 day in culture regardless of cytokine culture (Fig. 4D). Because CFSE staining indicated cell proliferation was static (Fig. 3), it seems possible that either some of the naïve cells perished and/or that a change in phenotype occurred spontaneously among the CD44^{low} T cells. Gr-1 staining was slightly reduced on the IL-15 treated CD8⁺ T cells, but both treated and control groups were similar to *ex vivo* expression levels (Fig. 4E). CD62L is a lymphoid homing marker up regulated on central memory T cells. The T cells demonstrated elevated CD62L after 1 day in culture, with the cytokine-free cultured control group having a greater proportion of CD62L dim cells (Fig. 4F). It appears that culture conditions support CD62L expression independently of IL-15 influences. Because CD62L is an adhesion marker for lymph homing, and given our starting cells are of splenic origin, it is possible microenvironment changes drove the changes in CD62L. CD25 is the IL-2 receptor alpha chain and surface expression increases during T cell activation. Levels of CD25 expression after IL-15 exposure were insignificant from the *ex vivo* population (Fig. 4G) and similar to the control population cultured without cytokine (~13% of the IL-15 cells were higher for CD25 by Overton subtraction statistical analysis). In totality, these data indicate that the gross phenotype of the responding cells is similar to the resting phenotype. While changes in phenotype may be beginning, the data indicate that cytotoxic potential can be induced by IL-15 independent of the classical activated phenotype (*e.g.* Gzm B⁺ and CD25⁺).

4. Discussion

It is possible that the naïve T cell population is capable of responding to IL-15 and initiating cytotoxic activity against cellular targets without antigen and within 24 hours. A biological implication of this potential is snap arming of effector lymphocytes in the periphery (*e.g.* gut, skin, or mucosal membranes) during early stages of infection. Macrophages or dendritic cells, the primary IL-15 producing immune cells, at the site of a nascent infection may activate the cytotoxic capacity passing naïve-phenotype T cells. Keratinocytes during inflammation [34], activated dermal fibroblasts [35] and smooth muscle cells after injury [36] may also provide IL-15 at sites of infection. The regional T cells can gain the capability to destroy targets, in a mode that appears distinct from classical antigen priming and clonal expansion. It should be noted that the efficacy of the snap armed cells still depends on interaction of the T cell receptors for antigen (TCRs) with cognate *de novo* antigens, foreign peptides that would also start to appear in MHC I molecules within 24 hours of viral infections. Even though the frequency of T cells to new antigens is low without cellular expansion, the location of these T cells at the site of infection may eliminate subclinical viral infections by snap-armed naïve T cells. Such snap arming may also involve T cells entering antigen-dependent activation pathways in the

periphery and would represent an instance of a specific cytotoxic cell finding itself in the right place at the right time.

Another implication of T cell snap arming is the clearance of antigen-presenting cells (APCs). Dendritic cells (DCs) that provide antigenic stimulation in the traditional sense may be cross-presenting antigen from a limited store of antigen that was phagocytosed. Antigen would also be presented if the DCs themselves were actively infected with viruses. If dendritic cells are chronically infected, they will present relevant antigen indefinitely. Interacting naïve or immune T cells may be induced to attack and clear the infected DCs. Further, this effect suggests that infected cells draining into the lymphoid organs may be quickly cleared, protecting the site from the spread of the virus to other resident DCs.

In experiments not presented, we found that IL-2 is also capable of inducing snap arming. Murine interleukin-2 at 10 ng/ml was less effective than 10 ng/ml IL-15. The IL-2 activity in the presence of low CD25, the high affinity IL-2 receptor, suggests that the IL-2 induced observed effects are the result of beta/gamma chain (CD122/CD132) signaling. It is worth noting that other gamma chain cytokines, such as IL-7 [37], may have similar effects.

IL-15 signaling is unique in that small amounts can be extremely effective because the cytokine is “presented” in membrane-bound form by APCs and other cells to responding T effector cells [13;38]. In this mode, IL-15 is produced either endogenously by the APCs or secreted by exogenous sources, and is sequestered by IL-15Ra on the membrane of the presenting cell. The APC then interacts with the T cells, trans-presenting the IL-15 molecules to T cells, with T cell signal transduction occurring through trimeric IL-15 receptors or through the common IL-2/15 beta/gamma chains. Under the conditions of our experiments, it is highly probable that the dendritic cells were loaded with low levels of cytokine that are then presented to CD8⁺ effector cells. It is curious that the snap-arming effect occurs in the absence of memory-phenotype T cells. Previous reports suggest that T cells of both mice and men have responsiveness to IL-15 and the ability to expand without antigen [16;17] at time points after day 1. These findings on day 1 build upon the observations and it is now documented that IL-15 induces rapid activation of T cells for cytotoxicity. Lack of phenotypic changes suggests that synthesis of a minimal number of proteins is required.

Effector NK-T cells are an unlikely candidate for the IL-15 activated cytotoxic effector cells even though they are CD3⁺ and would be triggered in redirected lysis. This cytotoxic cell group has properties of innate immunity, and its cytotoxic action is generally thought to occur through recognition of CD1d antigens [39;40]. First, the percentage of CD3⁺NK1.1⁺ cells was less than a fraction of 1% of the total effector lymphocyte population before or after depletion (Fig. 1) and it is unlikely that 1% NK-T cells could kill 30% of the targets at a 1:1 CD3⁺ T: P815 ratio. Second, depletion of NK1.1⁺DX5⁺ cells produced little change in cytotoxicity in the IL-15 treated groups (not illustrated). Thus, we consider NK-T cells to be unlikely effectors for the cytotoxic action we observed.

The results presented in this paper leave open the issue of snap arming of memory cells but describe the more unexpected IL-15 induced antigen-independent snap arming of naïve T cells. Given the effects of IL-15 on the memory T cell population, the major body of published work describes IL-15 and antigen-specific effector/memory cell interactions. Less attention has been given to effects on naïve T cells by IL-15. There are some reports demonstrating that IL-15 induces naïve T cells to proliferate *in vitro* without antigen [24;41] and *in vivo* when IL-15 is administered [42]. Further, naïve T cell survival is maintained for longer in the presence of IL-15 [43]. The cytotoxic potentiation observed here has potential to occur anywhere cells may be producing IL-15 and naïve T cells are present, raising interesting perspectives for therapeutic application where a large bolus of soluble IL-15 is administered [44;45].

Acknowledgements

We gratefully acknowledge that this work was supported by the grants NIH RO1CA38942 (DH), NIH T32CA9090563 (DT and BNA), NIH INBRE P20RR016463 (for flow cytometry), the Fraternal Order of the Eagles Research Award (DT), and the University of Nevada, Reno Graduate Student Association Student Research Grant Award (DT).

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Fig. 1. Low IL-15 induces rapid cytotoxic potential

Splenocytes were harvested and placed into culture without antigen and with or without exogenous 10 $\mu\text{g}/\text{ml}$ (7×10^{-10} M) IL-15. T cell lysis was redirected against P815 tumor targets with anti-CD3 mAb. The cells were labeled with anti-CD3 and anti-CD8 and analyzed by flow cytometry to assess NK T cells and potential changes in the CD8 T cell population. **(A)** Splenocytes from naïve mice have little cytotoxic potential *ex vivo*. T cells immediately harvested from naïve mouse spleens demonstrate little (less than 10%) killing. The splenic population directly *ex vivo* contained approximately 1% NK effectors and NK1.1⁺ CD8⁺ NK-T cells were undetectable. **(B)** Splenic T cells retain unprimed functional properties after 24 hours in culture without cytokine. After 24 hours in culture with only endogenous cytokines, splenic T cells were harvested and assayed for lytic potential, which was unchanged from *ex vivo* T cells. **(C)** Low concentrations of IL-15 without specific antigens induced rapid CD3 dependent lytic potential in splenocytes. Splenic T cells cultured with cytokine demonstrated elevated levels of cytotoxicity, even though the percentage of CD8⁺ splenocytes was similar to the cells cultured without cytokine. It should be noted in the flow data for **B & C** that the frequency of CD8⁺ T cells was unaltered on day 1 when the cytokine was present. **(D)** An IL-15 titration was performed to evaluate the range of cytokine effect. Cytotoxic potential was dependent on the dose of cytokine administered. The effect was anti-CD3 antibody dependent as indicated by the lack of activity of cells cultured with 50 ng/ml IL-15 and assayed without antibody to CD3 (filled triangles).

Fig. 2. IL-15 induces snap arming and occurs in the naïve CD8⁺ T cells lacking phenotypic markers of memory T cells

Ex vivo splenocytes were depleted of GR-1⁺ memory-phenotype CD8⁺ T cells and cultured for 24 hours with or without 10 ng/ml IL-15. The cultures were harvested and cytotoxic potential evaluated as described in Figure 1. (A) CD44⁺CD8⁺ T cells were depleted from *ex vivo* splenocytes. Magnetic beads coupled with anti-Gr1 mAb were used to deplete the memory phenotype CD8⁺ T cells. The frequency of memory phenotype effector cells (CD3⁺CD8⁺CD44^{hi}) was reduced from 27% to 7%. (B) IL-15 induced snap arming and facilitated allogeneic killing. A reaction between C57BL/6 splenocytes (H2^d) against DBA P815 tumor targets (H2^b) demonstrated moderate allogeneic killing capacity. Depletion of T

cells with memory effector phenotype has limited effect. **(C)** IL-15 induced cytotoxic potential was maximized by CD3 redirection and occurred in the absence of CD8⁺CD44^{hi} T cells. Anti-CD3 redirected lysis of splenocytes after IL-15 treatment was markedly greater than allogeneic killing alone. Furthermore, the cytotoxic potential observed was independent of the action of memory phenotype T killers.

Fig. 3. IL-15 snap arming occurs before T cell maturation to a Gzm B⁺ effector phenotype, happens prior to proliferation, and is perforin-independent

(A–B) The cytotoxic protein granzyme B was monitored by flow cytometry in CD3⁺ T cells after stimulation with IL-15. IL-15 treated and untreated T cells were stained with either anti-Gzm B (A) or an isotype control antibody (B). The interleukin 15 and cytokine-free controls had negligible Gzm B staining profiles that were similar to the isotype control label. (C) Cells were labeled with CFSE and placed in culture with or without IL-15 stimulation. After 1 day, both groups of T cells demonstrated similar CFSE label with few, if any, proliferating cells that would have had lower CFSE staining. (D) Perforin WT and perforin^{-/-} splenocytes were

cultured for 1 day with 50 ng/ml IL-15 and then cytotoxicity was redirected against P815 target cells. The WT cells had 3–4 fold more snap-armed cytotoxicity than the perforin^{-/-} cells.

Fig. 4. CD8⁺ T cells retained a resting phenotype after IL-15 snap arming
 Splenocytes were cultured with or without 10ug/ml IL-15. Cells were stained for flow cytometry, and the indicated markers were assessed *ex vivo* and for change at 24 hours.

Table 1
Cell surface proteins monitored in figure 4 and their functions.

Marker	Function
<i>NK-T cell markers:</i>	
NK1.1	Marker expressed by NK and NK-T cells [36;37]. Present on C57BL/6 mice, but not on Balb/c. Expression is correlative to DX5 on C57BL/6 mice.
DX5	Pan-NK marker that recognizes CD49b (alpha 2 integrin, very late antigen-2) [38]. Co-expressed with NK1.1 in C57BL/6 mice.
<i>Molecules involved in memory responses:</i>	
CD25	IL-2 receptor alpha chain. Binds with high affinity to soluble IL-2 and acts in coordination with the beta-gamma receptor chains to initiate intracellular signals [44;45].
CD44	Adhesion molecule found on lymphocytes that is associated with memory T cells, up regulated during homeostatic proliferation and activation [40;41].
CD62L	Adhesion molecule that is a member of the selectin family and is associated with memory T cell homing to the lymphoid organs [42;43].
CD122	IL-2/15 receptor beta chain, up regulated on memory phenotype T cells and during activation [26;39].