Increased IL-12 induced STAT-4 signaling in CD8 T cells from aged mice

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Abstract:

Aging is associated with poor immune function leading to increased susceptibility to infectious diseases and the development of immune associated disorders. Specific deficiencies in CD4 T cell function have been identified whereby CD4 T cells from old mice have defective T cell receptor (TCR) mediated antigen-specific responses compared to young T cells. Several of the defects described in T cells in old age can be attributed to dysfunctional cell signaling through the T cell receptor. Immune cell function is also dependent on cytokine production, and the capacity of cells to respond to cytokines. We have previously shown that a subset of CD8 T cells from the lungs of aged mice produce IFN-γ when exposed to IL-12 in an antigen-independent manner. Furthermore, IL-12 responsive CD8 T cells are present in apparently healthy old mice and represent a population that can mediate early non-specific control of *Mycobacterium tuberculosis*. For this reason understanding the mechanism underlying their ability to respond to IL-12 in the absence of a TCR signal may identify an alternate pathway to optimize protective immune responses in the elderly.

We investigated the ability of CD8 T cells from old mice to respond to IL-12 by determining the expression of the IL-12 receptor on the surface of young and old CD8 T cells, as well as by quantifying the activation of the downstream signaling molecule, STAT-4. We found IL-12Rβ2 expression on pulmonary CD8 T cells to be equivalent between young and old mice,

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and therefore hypothesized that IL-12 signaling in pulmonary CD8 T cells was increased in old age. To test this hypothesis, single cell suspensions from young and old mice were given IL-12 for 4 hours, and STAT-4 phosphorylation in CD8 T cells was determined using flow cytometric analysis. In response to IL-12, pulmonary CD8 T cells from old mice had increased levels of phosphorylated STAT-4 within individual CD8 T cells, and old mice possessed significantly more CD8 T cells that could phosphorylate STAT-4 in response to IL-12 compared to young, leading to an increased number of CD8 T cells that could secrete IFN-γ. To confirm STAT-4 activation was a direct consequence of IL-12 stimulation, STAT-4 phosphorylation was determined in purified $CD8^+$ cells from young and old mice. IL-12-induced STAT-4 phosphorylation was detected in purified $CD8^+$ cells from both ages of mice, illustrating that IL-12 could act directly on $CD8^+$ cells. Importantly, the proportion of $CD8^+$ cells that could phosphorylate STAT-4, and the amount of phosphorylated STAT-4 in each cell was significantly greater in $CD8^+$ cells from old mice. These data demonstrate that old mice possess a substantial population of CD8 T cells exhibiting increased STAT-4 phosphorylation in response to IL-12, which ultimately leads to enhanced IFN-γ production. We therefore define a mechanism for the increase in IL-12-induced IFN-γ secretion by CD8 T cells in old mice. A more comprehensive understanding of the unique contribution of CD8 T cells to innate immunity in old mice could lead to the development of more efficacious therapies in the elderly for combating pulmonary infections.

Introduction:

It is well established that immune function declines with age, rendering the elderly more susceptible to many infectious diseases [1-4]. Respiratory infections are particularly troublesome and are among the leading causes of death in the elderly, with nearly 80% of the deaths caused by influenza and pneumonia occurring in individuals over 65 years of age [5]. Tuberculosis is a devastating disease world-wide, accounting for 2 million deaths per year, and is also problematic in the elderly due to the decreased ability of this population to control infection [3]. In addition to being more susceptible to pulmonary infections, the elderly are also less responsive to the available prevention and treatment options for these infections, thus there is considerable need for new treatment strategies in this population [6]. A better understanding of basic immune function in old age could aid in the design of new vaccines and therapies for the elderly to combat respiratory infections.

Like humans, elderly mice are also more susceptible to respiratory infections, including tuberculosis [7]. Studies in mice have demonstrated that increased susceptibility to *Mycobacterium tuberculosis* (*M. tb*), the causative agent of tuberculosis, is due to defective T cell responses [7]. Several of the defects described in T cells in old age can be attributed to dysfunctional cell signaling through the T cell receptor (TCR), the molecule responsible for recognizing antigens presented by the major histocompatability complex (MHC) [8-12]. Engagement of the TCR with MHC results in activation of the T cell, leading to cytokine production and proliferation. Defects in any of these functions could lead to an inadequate immune response. T cell receptor signaling mechanisms have been well described in old age;

however the ability of these cells to respond to cytokines in the absence of TCR activation has not.

We have recently shown that old mice have a resident population of memory CD8 T cells within their lungs that are capable of producing the T_H1 cytokine, IFN-γ, in response to other cytokines, IL-12 and IL-18, in the absence of TCR activation, an event that is dependent on IL-12, however greatly enhanced by the addition of IL-18 [13]. Both IL-12 and IFN-γ are produced in abundance during an *M. tb* infection and have been shown to be essential for the control of this pathogen [14-16]. IL-12 responsive CD8 T cells are present in apparently healthy old mice and represent a population that can mediate early non-specific control of *Mycobacterium tuberculosis* [17]. For this reason understanding the mechanism underlying their ability to respond to IL-12 in the absence of a TCR signal may identify an alternate pathway to optimize protective immune responses in the elderly.

The biological activity of IL-12 is mediated by the IL-12 receptor (IL-12R), which is composed of a β1 and β2 subunit [18]. Binding of IL-12 to the receptor induces a signaling cascade, resulting in the phosphorylation, and thus activation, of the transcription factor STAT-4 which then dimerizes and translocates to the nucleus where it ultimately induces the production of IFN-γ [19]. Phosphorylation of STAT-4 therefore indicates IL-12-induced activation of the IL-12 receptor and initiation of the signaling cascade that leads to IFN-γ production [20]. To determine whether IL-12 signaling was intact in CD8 T cells from old mice, we examined IL-12 receptor expression and IL-12 signaling in pulmonary and splenic CD8 T cells from young and old mice. IL-12Rβ2 expression on CD8 T cells was equivalent between young and old mice, however we found that STAT-4 phosphorylation was enhanced in old mice. These data provide evidence that IL-12 signaling in CD8 T cells not only remains intact in old age, but appears to be

increased, thus providing a potential therapeutic target for vaccines or therapies designed to help the elderly combat respiratory infections.

Results:

IL-12Rβ2 expression on CD8 T cells is unaltered with age

CD8 T cells from old mice can secrete IFN-γ in response to a combination of IL-12, IL-18, and IL-2 more effectively than similar cells from young mice [13]. CD8 T cells from old mice have been shown to constitutively express more receptors for IL-18 [13] on their surface, we therefore sought to determine whether the responsiveness of CD8 T cells from old mice to IL-12 was a consequence of increased IL-12 receptor expression. Lung cells from old and young mice were stained for the IL-12Rβ2 subunit and analyzed by flow cytometry. Flow cytometric analysis allows us to compare the proportion of cells expressing the IL-12 receptor (depicted as percentages) as well as the amount of receptor expression on each individual cell (depicted as the mean fluorescence intensity (MFI)). Expression of the IL-12Rβ2 subunit was analyzed because this chain is required for signaling and, unlike the β1 subunit, is not shared by any other receptor [18].

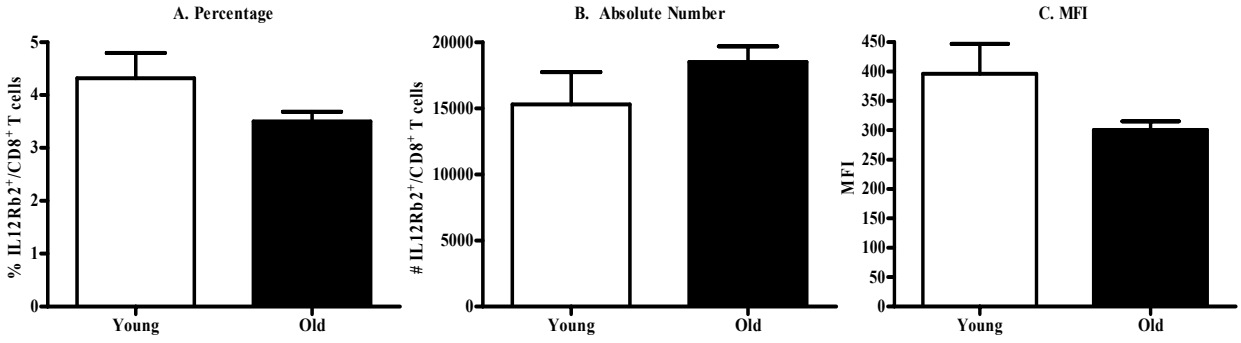

Figure 1: IL-12Rβ2 expression on lung-derived CD8 T cells from young and old mice
Single cell suspensions were isolated from the lungs of young (open bars) and old (closed bars) BALB/c mice. Cells were fixed in media containing sodium azide and flow cytometry was performed using antibodies against CD3, CD8, and IL-12Rβ2. Lymphocytes were gated according to their characteristic forward and side scatter and CD8 T cells were identified by CD8+/CD3+ staining. An isotype control antibody for IL-12Rβ2 was used to set the positive gates. A.) Percentage of CD8 T cells expressing IL-12Rβ2. B.) Percentages were used to calculate the absolute number of CD8 T cells expressing IL-12Rβ2. C.) Mean fluorescence intensity (MFI) of IL-12Rβ2 on CD8 T cells.

A very low frequency of CD8 T cells from the lungs of young and old mice expressed IL-12Rβ2 however, there were no significant differences between young and old in the proportion (Fig. 1a) or absolute number (Fig. 1b) of pulmonary CD8 T cells that expressed IL-12Rβ2. To determine if pulmonary CD8 T cells from old mice had increased levels of IL-12R on the surface of individual cells, we analyzed the MFI of IL-12Rβ2 on CD8 T cells from the lungs of young and old mice and found no significant differences in surface expression (Fig 1c). These data illustrate that pulmonary CD8 T cells from old mice express low levels of IL-12Rβ2 on their surface, and an equivalent amount to that seen on cells from young mice.

STAT-4 activation in CD8 T cells

An increase in IL-12Rβ2 expression on CD8 T cells from old mice was not observed, indicating that increased receptor expression was not responsible for the increased proportion of CD8 T cells that can produce IFN-γ in response to IL-12 [13]. We therefore determined whether the increase in proportion of CD8 T cells from old mice that could respond to IL-12 was a consequence of enhanced signaling downstream of the IL-12 receptor. Lung cells were cultured in the presence of IL-12 for 4 hours and STAT-4 phosphorylation was determined by flow cytometry. Phosphorylated STAT-4 (pSTAT-4) was detected in CD8 T cells isolated from the lung (Fig. 2) and spleen (data not shown) of young and old mice. Figure 2a shows representative dot plots and histograms illustrating gating strategies and pSTAT-4 expression, respectively. Phosphorylation of STAT-4 was dose-dependent in both age groups (Fig. 2b-d), confirming that signaling was specific for IL-12. Furthermore, there was an increased proportion (Fig. 2b) and absolute number (Fig. 2c) of CD8 T cells from old mice that contained phosphorylated STAT-4 compared to young mice, indicating that, despite equivalent IL-12Rβ2 receptor expression between young and old mice, old mice had more CD8 T cells with the potential to secrete IFN-γ

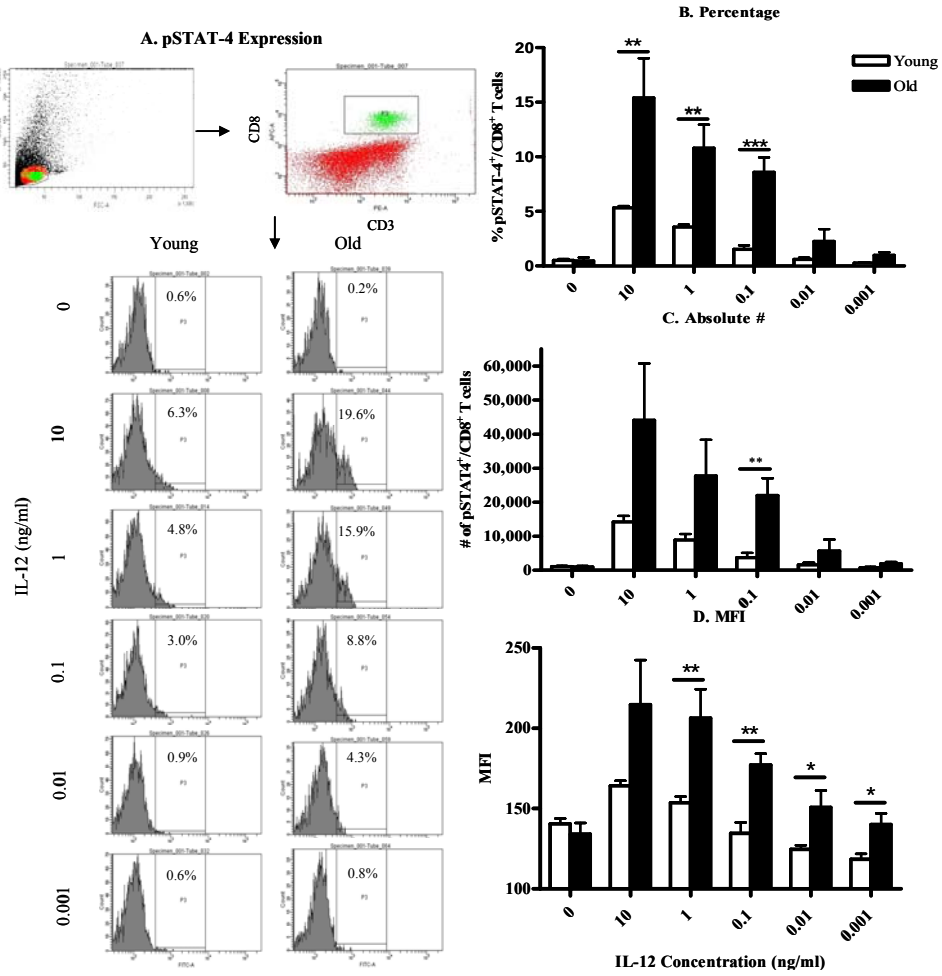

Figure 2: CD8 T cells from old mice have increased pSTAT-4 levels in response to IL-12
Single cell suspensions were isolated from the lungs of young (open bars) and old (closed bars) mice and cultured with tissue culture media (TCM) or IL-12 for 4 hours. Cells were fixed in media containing sodium azide, permeabilized, and flow cytometry performed using antibodies against CD3, CD8, and phosphorylated STAT-4 (pSTAT-4). Lymphocytes were gated according to their characteristic forward and side scatter and CD8 T cells were identified by CD8+/CD3+ double staining. An isotype control antibody for pSTAT-4 was used to set the positive gates A.) Representative dot plots showing gating strategies and histograms illustrating pSTAT-4 expression. B.) Percentage of CD8 T cells expressing pSTAT-4. C.) Percentages were used to calculate the absolute number of CD8 T cells expressing pSTAT-4. D.) Mean fluorescence intensity (MFI) of pSTAT4 in CD8 T cells. Data represent the mean +/- SE of 4-5 mice and are representative of 2 independent experiments. Significance was determined by unpaired Student's *t*-test * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

in response to IL-12 alone. Perhaps more significant, determination of the MFI of pSTAT-4 in pulmonary CD8 T cells showed that at each concentration of IL-12 tested, CD8 T cells from old mice contained significantly more pSTAT-4 per cell compared to young CD8 T cells (Fig 2d). Therefore, at the single cell level, CD8 T cells from old mice were more responsive to IL-12 than similar cells from young mice. The increase in pSTAT-4 observed in CD8 T cells from old mice was not a result of increased basal levels of pSTAT-4, as pSTAT-4 levels remained equivalent between young and old CD8 T cells in the absence of IL-12 (Fig. 2). To confirm that the increased phosphorylation of STAT-4 in CD8 T cells from old mice was not simply a consequence of altered kinetics compared to young, a time course was performed, beginning at 10 min and extending to 24 hours. These experiments revealed that there were more CD8 T cells

from old mice that could phosphorylate STAT-4 in response to IL-12 at all time points tested however, the kinetics of phosphorylation remained equivalent to that seen in CD8 T cells from young mice with a similar peak in phosphorylation at 4 hours (data not shown). Together, these data suggest that as a population, and on an individual cell basis, CD8 T cells from the lungs of old mice have increased IL-12 signaling compared to their younger counterparts.

STAT-4 phosphorylation is enhanced in purified $CD8^+$ cells from old mice

To confirm that STAT-4 activation within CD8 T cells was a direct consequence of IL-12 stimulation and not due to an intermediate cell or cytokine present in whole lung cultures, we purified $CD8^+$ cells from the lungs and spleens of young and old mice, and cultured purified cells with IL-12. STAT-4 phosphorylation was detected in purified $CD8^+$ cells from the lungs (Fig 4a-b) and spleens (Fig 4c-d) of both young and old mice, illustrating that IL-12 could act directly on $CD8^+$ cells. Importantly, the proportion of $CD8^+$ cells that could phosphorylate STAT-4 was significantly greater in lung (Fig 4a) and spleen (Fig 4c) cultures from old mice. Furthermore, STAT-4 phosphorylation was increased within individual $CD8^+$ cells purified from the lungs (Fig 4b) and spleens (Fig 4d) of old mice compared to those purified from young mice.

To validate the flow cytometry data, and to verify that the increased proportion of pSTAT4 observed within $CD8^+$ cells from old mice was due to increased phosphorylation of STAT-4 and not an increased amount of STAT-4 protein within the cells, we determined STAT-4 and pSTAT-4 expression via Western blotting. Western blotting requires a substantially greater number of purified $CD8^+$ cells than we could readily obtain from the lungs of mice, and we therefore performed Western blots on $CD8^+$ cells purified from the spleen. Samples were normalized for cell number and protein band densities were normalized to an internal control, GAPDH. Equivalent amounts of STAT-4 were detected in $CD8^+$ cells from young and old mice

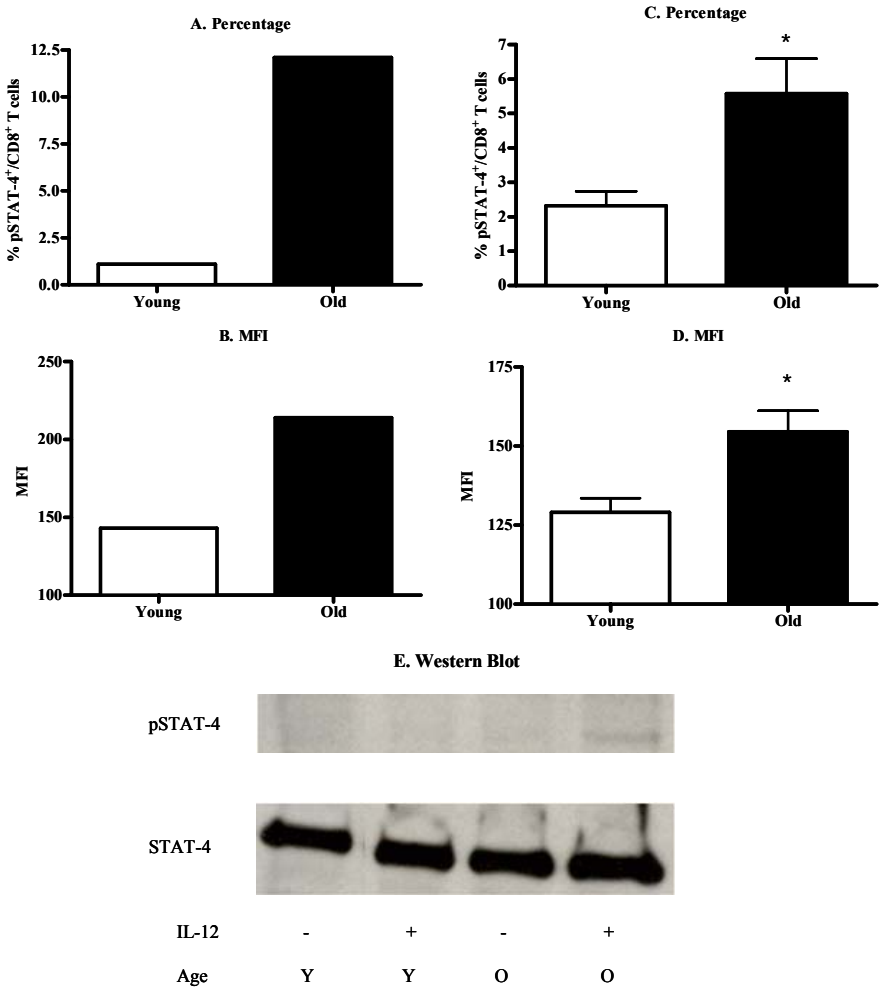

Figure 4: IL-12 stimulation leads to STAT-4 phosphorylation in purified CD8+ cells

CD8+ cells were purified from the lungs (A-B) and spleens (C-E) of young (open bars) and old (closed bars) mice using magnetic cell separation. Purified cells were cultured with TCM or IL-12 for 2 (A-B) or 4 (C-E) hours. (A-D) Cells were fixed in media containing sodium azide, permeabilized, and stained with antibodies against CD3, CD8, and pSTAT-4. CD8 T cells were gated according to CD8+/CD3+ double staining. A.) The percentage of pulmonary CD8 T cells expressing pSTAT-4. B.) Mean fluorescence intensity (MFI) of pSTAT4 in pulmonary CD8 T cells. Positive gates were set on un-stimulated cell cultures. Data represent pools of 4-5 mice. Error bars are absent from pooled samples however the experimental data are representative of 3 independent experiments. C.) The percentage of splenic CD8 T cells expressing pSTAT-4. D.) MFI of pSTAT-4 in splenic CD8 T cells. Data represent the mean +/- SE of 4 or 5 mice. Significance was determined by unpaired Student's *t*-test. *$p<0.05$. E.) Lysates were prepared for western blot analysis and pSTAT-4 and STAT-4 expression in purified splenic CD8+ cells from young (Y) and old (O) mice detected via Western blot. Data represent pools of 10 young and 10 old mice and data are representative of 2 independent experiments.

(Fig. 4e). Furthermore, we demonstrate that in response to stimulation with IL-12, pSTAT-4 could be detected in purified CD8 cells from the spleens of old mice only (Fig 4e). The absence of pSTAT-4 in $CD8^+$ cells purified from young mice was not surprising, given the small number of cells that could phosphorylate STAT-4 observed using flow cytometry. Studies using purified $CD8^+$ cells from the lung also found equivalent amounts of STAT-4 protein however cell yield was insufficient to detect pSTAT-4 by Western blot (data not shown). These data therefore verified that the increased phosphorylation of STAT-4 within $CD8^+$ cells purified from old mice in response to IL-12 was due to increased phosphorylation of STAT-4 and not increased basal levels of STAT-4 protein.

IL-12 stimulation leads to IFN-γ production by purified CD8$^+$ cells.

To confirm that the stimulation of purified CD8$^+$ cells with IL-12 leads to IFN-γ production, we stimulated CD8$^+$ cells purified from the lungs of young and old mice for 48 hours with IL-12 and determined the frequency of IFN-γ secreting cells by the antibody-based detection assay, Enzyme Linked Immunosorbent Spot (ELISpot). In response to IL-12 stimulation, more CD8$^+$ cells purified from the lungs of old mice produced IFN-γ than CD8$^+$ cells purified from the lungs of young mice (Fig. 5). The total number of CD8$^+$ cells that could secrete IFN-γ was different to the number of CD8 T cells that could phosphorylate STAT4 (described above) however, this is likely due to differences in assay conditions, antibody affinities, and the comparison between signaling pathways and protein secretion. Old and young mice therefore possess a population of CD8 T cells that can secrete IFN-γ in response to stimulation with IL-12, mediated by activation of STAT-4. Furthermore, old mice had a significantly greater number of CD8 T cells that could respond directly to IL-12 than did young mice, leading to an increased number of cells that could secrete IFN-γ, resulting in the increased production of IFN-γ that we have previously described [13].

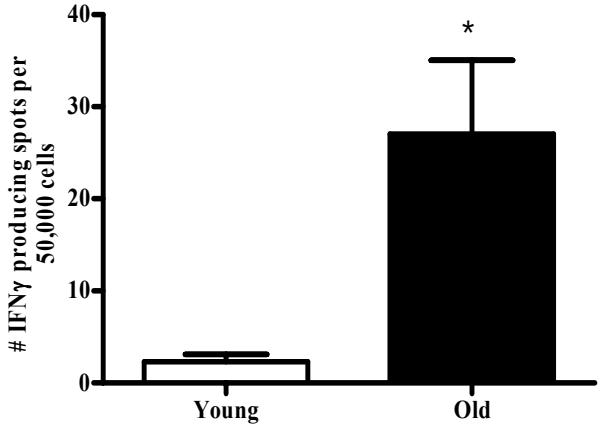

Figure 5: CD8+ cells secrete IFN-γ in response to IL-12
CD8+ cells were purified from the lungs of young and old mice using magnetic cell separation. Cells were plated on an ELISpot plate that had been pre-coated with IFN-γ capture antibody, and were stimulated with TCM or IL-12 for 48 hours at 37°C. IFN-γ producing cells were determined by ELISpot. Data are expressed as pools of 2 mice and are the combined result of 3 independent experiments. Significance was determined by unpaired Student's *t*-test. *p<0.05.

Discussion:

In this study we evaluated the capacity of CD8 T cells from old mice to respond directly to IL-12 stimulation in the absence of TCR activation. The rationale for such a study was based on previous observations by our group showing that more CD8 T cells from the lungs of old mice, compared to young, were capable of secreting IFN-γ in response to IL-12 in a T cell receptor-independent manner [13]. We hypothesized that this was a result of enhanced IL-12 receptor expression, leading to increased IL-12 signaling and IFN-γ production. Contrary to our original hypothesis we determined that the expression of IL-12Rβ2 was equivalent on CD8 T cells from young and old mice. Analysis of the IL-12 signaling pathway however, showed that individual CD8 T cells from old mice could phosphorylate STAT-4 at a higher level than CD8 T cells from young mice, and that old mice possessed significantly more CD8 T cells that phosphorylated STAT-4 in response to IL-12 than young mice. The kinetics of phosphorylation was equivalent between CD8 T cells from young and old mice, indicating that the signaling pathway functioned similarly in each age group. Taken together, these data demonstrate that old mice possess a substantial population of CD8 T cells in the lung (and spleen) that have increased STAT-4 phosphorylation in response to IL-12 stimulation, which ultimately leads to enhanced IFN-γ production.

The stimulation of CD8 T cells to secrete IFN-γ in response to T_H1 cytokines has been described in young animals and this ability to bypass TCR-MHC interactions is a characteristic of differentiated effector/memory cells, a population that can be identified by the high surface expression of CD44 [21]. We have previously shown that CD8 T cells from old mice that can be stimulated to secrete IFN-γ are of a similar phenotype [13], and have recently demonstrated that the vast majority of $pSTAT4^+$ CD8 T cells from old mice also express high levels of CD44 (data

not shown). CD8 CD44^{hi} T cells that can secrete IFN-γ in a TCR-independent manner serve as a population of cells that can rapidly respond to infection and there is growing evidence that CD8 CD44^{hi} T cells can contribute to the early immune response to pathogens such as *Listeria monocytogenes* and *Burkholderia pseudomallei* [21-24]. The biological relevance of our findings in old mice can be clearly linked to studies of infection with the intracellular pathogen *M. tuberculosis*, where the secretion of IL-12 is a critical event for early immune activation [15]. Old mice can express an early resistance to infection with *M. tuberculosis* which is associated with increased IL-12 within the lung [13, 17, 25-27] and our current data supports a role for CD8 T cells in this early control.

We have therefore demonstrated that the increased responsiveness of CD8 T cells from old mice to IL-12 is a consequence of an increased number of CD8 T cells in old mice that can respond to IL-12, in combination with enhanced IL-12 signaling within each individual cell. The net outcome is an expanded population of CD8 T cells that can produce IFN-γ in a T cell receptor independent manner which we believe leads to an enhanced early resistance to infection with *M. tuberculosis* [17, 26], and perhaps to other pathogens. These studies provide evidence that post-exposure therapies or novel vaccine designs that incorporate IL-12 could be developed to reduce the morbidity and mortality associated with infectious diseases in the elderly.

M**aterials and Methods:**

Cell isolation. The lungs of young (2 months) and old (18 months) C57BL/6 or BALB/c mice were perfused with PBS containing 50U/ml of heparin and placed in supplemented Dulbecco's modified Eagle's medium (DMEM). The lungs were minced and digested for 30 min at 37°C in 4ml DMEM containing collagenase XI and type IV bovine pancreatic DNase and subsequently

passed through a 70μm cell strainer to achieve a single cell suspension. Residual red blood cells were lysed using ACK lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$) and re-suspended in DMEM. To achieve a single cell suspension of splenocytes, the spleens of young or old mice were harvested and passed through a 70μm cell strainer and red blood cells subsequently lysed with ACK lysis buffer, and re-suspended in DMEM. No significant differences between the total number of cells, or CD8 T cells, in the lung were found between old and young mice.

CD8 purification. Splenocytes from individual mice, or pooled lung cells from 2-5 young or old mice were placed in a tissue culture grade petri dish for 1 hour at 37°C. Non-adherent cells were collected and $CD8^+$ cells isolated using CD8a MicroBeads or $CD8^+$ cells or BD™ IMag anti-mouse CD8a particles according to the manufacturer's instructions. To obtain maximum purity (over 90% for all samples) using the Miltenyi MACS system, cells were passed over two LS columns.

***In vitro* cell culture.** $5x10^5$ cells from individual whole lung cultures or $1\text{-}2x10^5$ cells from pooled cultures of purified $CD8^+$ cells were plated in 96-well tissue culture plates and, unless otherwise stated, cultured for 4 hours at 37°C with supplemented DMEM with or without 10ng/ml of recombinant IL-12.

Flow cytometry. Flow cytometric analysis of the IL-12Rβ2 was carried out by suspending cells isolated from the lungs of BALB/c mice in deficient RPMI (dRPMI) supplemented with 0.1% sodium azide. $5x10^5$ cells were incubated with 0.3μg of Fc block™ for 5 min at 4°C. Cells were subsequently labeled with 2μg of purified anti-IL-12Rβ2 or rabbit IgG and 0.3μg of anti-CD3 and anti-CD8 and incubated for 20 min at 4°C. The cells were washed twice in dRPMI and labeled with 0.3μg of anti-rabbit IgG for 20 min at 4°C. Cells were washed twice and read using a BD Biosciences LSRII flow cytometer and analyzed with FACS DIVA software. Intracellular

labeling of phosphorylated STAT-4 was carried out in accordance with the BD™ Phosflow Protocol III for mouse splenocytes or thymocytes.

IFN-γ ELISpot. $CD8^+$ cells were purified from C57BL/6 mice using the Miltenyi MACS system as described previously and $5x10^4$ $CD8^+$ cells were plated on a multiscreen-IP filter plate that had been pre-coated with IFN-γ capture antibody. Cells were cultured in the presence or absence of IL-12 (10ng/ml) and incubated at 37°C, 5% CO_2 for 48 hours. The number of IFN-γ producing cells was determined using the Ready-Set-Go! mouse IFN-γ enzyme linked immunosorbent spot (ELISpot) kit from ebioscience. Spot density was determined by counting the number of spots per well using a dissecting microscope and data were expressed as the number of spots per 50,000 cells. For negative control wells, the spot number did not exceed 7 spots/well.

Western blot. $CD8^+$ cells were purified from the spleens of 10 young and 10 old C57BL/6 mice using the BD IMag system as previously described, and were plated at $8x10^6$-$1x10^7$ cells per well in a 24-well plate and cultured with supplemented DMEM or IL-12 (10ng/ml) for 4 hours at 37°C. Lysates were prepared using TN1 lysis buffer and were subjected to electrophoresis on a 10% Tris-HCl gel. Proteins were transferred to a PVDF membrane and subsequently labeled with anti-phosphorylated (Y693) STAT-4 (ST4P) or anti-STAT-4 (Z-17S). Lysate preparation, electrophoresis, and western blotting were carried out using standard methodology.

Statistical analysis. Statistical significance was determined using PRISM 4 software. The unpaired two-tailed Student *t* test was used for comparisons between young and old mice.

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