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Defects In Apoptosis Increase Memory CD8⁺ T Cells Following Infection Of Bim^{-/-}Fas^{lpr/lpr} Mice

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Abstract

During many infections, large numbers of effector CD8⁺ T cells are generated. After pathogen clearance, the majority of these cells undergo apoptosis, while the survivors differentiate into memory CD8⁺ T cells. Although loss of both Bim and Fas function dramatically increased antigen-specific CD8⁺ T cells in the lymph nodes following acute lymphocytic choriomeningitis virus (LCMV) infection, it was unclear whether they were pardoned effector or true memory CD8⁺ T cells. In this study, we demonstrate they are bona fide memory T cells as characterized by surface marker expression, cytokine production, homeostatic proliferation, and ability to clear a secondary challenge of pathogen. Loss of both Bim and Fas also increased the number of virus-specific CD4⁺ T cells found in the lymph nodes compared to the parental genotypes or wildtype mice. These studies illustrate that decreasing apoptosis increases the number of memory T cells and therefore could increase the efficacy of vaccines.

Keywords

apoptosis; viral infection; cytolytic T cells

1. Introduction

CD8⁺ T cells play a prominent role in the clearance of intracellular pathogens and tumors. Following positive and negative selection, CD8⁺ T cells egress from the thymus and exist in a quiescent state in secondary lymphoid tissues. In this naïve state, CD8⁺ T cells undergo minimal proliferation [1]. However, upon TCR recognition of cognate antigen presented in the context of MHC proteins on the surface of APCs, a new program of gene expression is initiated. It culminates in rapid clonal expansion and differentiation into effector cells that secrete cytokines such as interferon gamma (IFN γ), tumor necrosis factor alpha (TNF α) and interleukin 2 (IL-2). They also lyse infected cells through perforin and Fas mediated pathways [2, 3]. Recently, the generation of two effector cell populations have been

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characterized following LCMV infection. Short-lived effector cells (SLECs) arise early following infection and are eliminated following viral clearance. In contrast, memory precursor effector cells (MPECs) are maintained at later time points postinfection and further differentiate into long-lived memory cells[4]. These two populations can be identified based on surface marker expression. Earlier studies utilized IL-7R (CD127) as a marker to characterize previously activated T cells as committed to differentiate into memory cells[5]. However, this marker alone does not distinguish SLECs from MPECs. Instead, the killer cell lectin-like receptor G1 (KLRG1) marks terminally differentiated cells and correlates with effector cells adopting a SLEC fate. By utilizing both markers, SLECs are identified as IL-7R^{Low}KLRG1^{High}, while MPECs are IL-7R^{High}KLRG1^{Low}. Upon pathogen clearance, the immune system returns to homeostasis through apoptosis of the majority of effector cells, while surviving cells further differentiate into long lived memory cells. These cells provide long term protection by maintaining cell numbers through homeostatic proliferation and by their ability to rapidly respond to antigen re-encounter[6, 7].

Previously we have shown that loss of Bim, a proapoptotic member of the Bcl-2 superfamily that regulates apoptosis in lymphocytes following growth factor withdrawal [8], resulted in a block in apoptosis of CD8⁺D^bNP396-404⁺ T cells following chronic LCMV infection. In addition, loss of Bim resulted in a transient delay in the contraction of multiple antigen-specific CD8⁺ T cell populations following acute LCMV infection [9, 10]. However, the delay rather than block on contraction suggested that Bim alone does not regulate the apoptosis of activated CD8⁺ T cells during acute infection. Our subsequent study of mice lacking both Bim and Fas demonstrated that mutating components of both the intrinsic and extrinsic pathways of cell death resulted in accelerated development of autoimmunity and increased antigen-specific CD8⁺ T cells in the lymph nodes following acute LCMV infection compared to mutation of each pathway individually[11]. However, it was unknown if these surviving cells were memory cells and how loss of both death pathways would affect antigen-specific CD8⁺ T cell numbers during chronic viral infection.

Here we demonstrate that following acute LCMV infection, antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} mice exhibit cardinal properties of memory cells such as homeostatic proliferation, the production of multiple cytokines following antigen re-stimulation, and viral clearance upon rechallenge. Loss of Bim and Fas function also increased the number of virus-specific CD4⁺ T cells in the lymph nodes at later timepoints. In contrast to the increase in antigen-specific CD8⁺ T cells observed following acute LCMV infection, chronic LCMV infection of Bim^{-/-}Fas^{lpr/lpr} animals resulted in similar numbers of antigen-specific CD8⁺ T cells as the Bim^{-/-} parental genotype. These findings demonstrate that the antigen-specific CD8⁺ T cells remaining in Bim^{-/-}Fas^{lpr/lpr} at later time points postinfection with LCMV Armstrong are “bona fide” memory cells. On the contrary, the lack of an effect by loss of Fas function in Bim^{-/-} mice suggests that other components function with Bim during chronic LCMV infection to regulate antigen-specific CD8⁺ T cell numbers.

2. Materials and Methods

2.1 Mice, Virus, and Infections

Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-}Fas^{lpr/lpr}, and wildtype mice have been described previously[11]. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Wake Forest University School of Medicine. Six to eight week old mice were infected with 2×10⁵ p.f.u. of LCMV-Armstrong intraperitoneally or 2×10⁶ p.f.u. LCMV-Clone 13 intravenously and used at the indicated time points. Virus was grown and quantitated as described previously [12].

2.2 Cell isolation

Spleens were removed from mice following cervical dislocation. After being teased apart on a wire meshscreen, red blood cells were removed by osmotic lysis in ACK buffer (NH_4Cl , KHCO_3 , and EDTA). Splenocytes were then resuspended in RPMI1640 supplemented with 10% FCS and L-glutamine, penicillin-streptomycin, and β -mercaptoethanol (complete media). Superficial cervical, axillary, brachial, and inguinal lymph nodes were excised from mice following euthanization, teased apart on a wire mesh screen, then resuspended in complete media. To isolate lymphocytes from nonlymphoid tissues, the abdomen was opened and the gallbladder was removed, the hepatic vein was cut, and 5 ml ice-cold PBS was injected directly into the hepatic artery to perfuse the liver. The entire liver was removed and teased apart on a wire mesh screen followed by incubation at 37°C with 0.25 mg/ml collagenase B (Boehringer Mannheim, Federal Republic of Germany) and 1 U/ml DNase (Sigma, St. Louis, MO) for 60 minutes with vortexing every 15 minutes. Following digestion of the liver the sample was centrifuged and the pellet was resuspended in 5 ml 44% Percoll (Sigma). The solution was then under laid with 56% Percoll and centrifuged at 2000 rpm for 20 minutes at 20°C . The interface containing lymphocytes was then removed and washed to remove residual Percoll. Red blood cells were lysed using 0.83% ammonium chloride, and then lymphocytes were washed and counted.

2.3 Surface and intracellular staining

In this study the following antibodies from were used: rat anti-mouse CD8 α -FITC, rat anti-mouse CD8 α -PE, rat anti-mouse CD8 α -PerCP, rat anti-mouse CD8 α -APC, rat anti-mouse CD4-PE, rat anti-mouse CD44-FITC, rat anti-mouse CD44-APC, rat anti-mouse CD127-FITC, rat anti-mouse KLRG1-PE, rat anti-mouse CD62L-FITC, hamster anti-mouse CD27-PE, rat anti-mouse TNF α -APC, rat anti-mouse IFN γ -FITC. All of the antibodies were purchased from BD Pharmingen except for CD127 (eBiosciences) and KLRG1 (Abcam). D^bGP33-41, D^bNP396-404, D^bGP276-286 MHC Class I tetramers were generated as previously described [6]. Surface staining was performed by incubating cells with antibodies at a 1:100 dilution in FACS buffer (2% fetal calf serum in phosphate buffered saline) for 30 minutes on ice. Cells were then washed 3 times with FACS buffer and resuspended in 2% paraformaldehyde. After three washes, intracellular staining was performed using the BD Biosciences Cytofix/Cytoperm kit according to the manufacturer's instructions. Samples were acquired on a FACSCalibur instrument and analyzed using FloJo software (TreeStar, San Francisco, CA).

2.4 Bromodeoxyuridine Labeling

Mice were administered 0.8mg/ml BrdU in the drinking water daily beginning on day 90 post infection with LCMV Armstrong until day 97. BrdU staining was performed as described by Tebo et. al [13]. Samples were acquired on a FACSCalibur or FACSria instrument and analyzed using FlowJo software.

2.5 Antibody titer

At the indicated timepoint serum was collected from mice by retro orbital bleeding and LCMV-specific antibody titers were determined by ELISA as described previously[12]. The LCMV-specific antibody titer is expressed as the reciprocal of the reading highest dilution showing an OD₄₉₂ reading greater than 3 times the average OD₄₉₂ of serum samples from uninfected mice of each genotype.

2.6 Statistical analysis

Data from wildtype, *Bim*^{-/-}, *Fas*^{lpr/lpr}, and *Bim*^{-/-}*Fas*^{lpr/lpr} mice were analyzed using an ordinary ANOVA with a Student-Newman-Keuls multiple comparison test. All calculations were performed using InStat software and a p value of <0.05 was considered significant.

3. Results

3.1 Antigen-specific CD8⁺ T cells in *Bim*^{-/-}*Fas*^{lpr/lpr} animals exhibit phenotypic markers of memory cells

Previously, we demonstrated an increase in antigen-specific CD8⁺ T cells in mice lacking both *Bim* and *Fas* at later time points following acute LCMV infection [11]. To determine if these cells were memory cells or if they were effector cells that had been rescued from death, we sacrificed mice at various time points postinfection and examined the surface marker phenotype of the virus-specific CD8⁺ T cell populations. Mice of each genotype (wildtype, *Bim*^{-/-}, *Fas*^{lpr/lpr}, and *Bim*^{-/-}*Fas*^{lpr/lpr}) were infected with LCMV Armstrong and sacrificed on day 8 and day 97 postinfection. Lymph node lymphocytes (Fig. 1A) were stained with anti-CD8 α , anti-KLRG1, anti-CD127 (IL-7R), and D^bNP396-404 MHC class I tetramer to determine the percentage of short-lived effector cells (SLECs, KLRG1^{High}CD127^{Low}) and memory precursor effector cells (MPECs, KLRG1^{Low}CD127^{High}) for each of the antigen-specific CD8⁺ T cell populations on day 8 and day 97 postinfection. Previous studies have demonstrated that high levels of KLRG1 are expressed on cells committed to a SLEC fate [4]. These cells are present at early time points postinfection, however they decline rapidly during the contraction phase following antigen clearance and continue to gradually decline over time. In contrast, most of the antigen-specific CD8⁺ T cells during the memory phase bear a MPEC phenotype. On day 8 postinfection with LCMV Armstrong, the percentage of antigen-specific SLECs (upper left quadrant) was similar between wildtype, *Bim*^{-/-}, and *Fas*^{lpr/lpr} mice (Fig. 1A). When *Bim*^{-/-}*Fas*^{lpr/lpr} mice were examined the percentage of SLECs was slightly decreased. This contrasted with the percentage of MPECs which was similar across all genotypes at day 8. Thus, it does not appear the mutating both the intrinsic and extrinsic pathways of cell death causes a major increase in the percentage of either antigen-specific population at the peak of the response. Although the percentages of SLEC and MPECs were similar, the percentage of antigen-specific KLRG1^{Low}CD127^{Low} CD8⁺ T cells (lower left quadrants in panel A) at this time point was significantly increased in *Bim*^{-/-} and *Bim*^{-/-}*Fas*^{lpr/lpr} animals compared to wildtype and *Fas*^{lpr/lpr} animals (p value < 0.05). On day 97 postinfection, the percent of MPECs in the lymph nodes of *Bim*^{-/-}*Fas*^{lpr/lpr} animals was similar to both the *Bim*^{-/-} and *Fas*^{lpr/lpr} parental genotypes for each of the antigen-specific populations examined (Fig. 1B, left panel). Additionally, the percentage of MPECs was similar to the parental genotypes in the spleens of *Bim*^{-/-}*Fas*^{lpr/lpr} animals compared to both parental strains at this time point, with the predominate population being a MPEC phenotype (Fig. 1B, right panel). In addition to expressing high levels of CD127 and low levels of KLRG1, memory cells can be further subdivided based on expression of CD62L and CD27. Central memory cells (T_{CM}), have increased proliferative potential and are found in lymphoid organs expressing high levels of both markers. This contrasts with effector memory cells (T_{EM}) that have low levels of each marker and reduced proliferative potential and are enriched in nonlymphoid organs [14, 15]. When we measured the percentage of T_{cm} we found that *Bim*^{-/-}*Fas*^{lpr/lpr} animals had similar percentages in both the lymph nodes (Fig. 1C, left panel) and spleen (right panel) as the parental genotypes. These results demonstrate that the increased numbers of antigen-specific CD8⁺ T cells we observe in the *Bim*^{-/-}*Fas*^{lpr/lpr} animals exhibit a surface marker phenotype at later time points postinfection consistent with memory cell differentiation.

3.2 Bim^{-/-}Fas^{lpr/lpr} mice have increased numbers of functional antigen-specific CD8⁺ T cells in the lymph nodes on day 97 postinfection with LCMV Armstrong

Although the antigen-specific CD8⁺ T cells present in the lymph nodes of Bim^{-/-}Fas^{lpr/lpr} animals display surface marker expression characteristic of “bona fide” memory cells, it was unclear if these cells were functional and capable of rapidly responding to re-stimulation. Therefore, we determined the ability of CD8⁺ T cells from the spleen and lymph nodes of Armstrong infected mice of each genotype to produce IFN γ and TNF α following peptide re-stimulation in vitro on day 97 postinfection. The majority of CD8⁺ T cells from the lymph nodes of each genotype produced both IFN γ and TNF α following peptide stimulation with the indicated peptides (Fig. 2A). When CD8⁺IFN γ ⁺T cells were enumerated in the lymph nodes at day 97 postinfection (Fig. 2B), the number of functional cells in Bim^{-/-}Fas^{lpr/lpr} animals was significantly increased compared to the parental genotypes. In contrast, similar numbers were observed in the spleen as the Bim parental genotype (Fig. 2C). While the number of functional cells was increased in the lymph nodes it was critical to determine their ability to produce multiple cytokines. Previous studies have demonstrated that memory CD8⁺ T cells rapidly produce both IFN γ and TNF α [3]. When the percentage of IFN γ producing cells that also made TNF α was determined it was similar to the parental genotypes in both the lymph nodes (Fig. 2D) and spleen (Fig. 2E). Thus, the antigen-specific CD8⁺ T cells in Bim^{-/-}Fas^{lpr/lpr} animals, similar to bona fide memory cells, are capable of rapidly producing both IFN γ and TNF α following peptide stimulation.

3.3 Antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} animals undergo similar rates of homeostatic proliferation as the parental genotypes and wildtype animals during the memory phase

In addition to surface marker expression and rapid production of the cytokines IFN γ and TNF α , another cardinal property of memory cells is homeostatic proliferation. Previous studies have demonstrated that memory CD8⁺ T cells have the ability to proliferate in the absence of antigen to maintain constant cell numbers [7, 16]. To determine whether antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} mice were capable of undergoing homeostatic proliferation, mice of each genotype were administered 0.8 mg/ml BrdU in the drinking water from day 90 to 97 postinfection with LCMV Armstrong. On day 97 mice were sacrificed, and the spleens, lymph nodes, and liver were harvested and stained with anti-CD8 α , D^bGP33-41 or D^bNP396-404 MHC class I tetramers, and intracellular anti-BrdU. When BrdU incorporation was quantitated for the spleen (Fig. 3A), liver (Fig. 3B), and lymph nodes (Fig. 3C), the percent of antigen-specific BrdU⁺CD8⁺ T cells was similar to the parental genotypes and the wildtype control. In the case of LCMV, ~25% of antigen-specific CD8⁺D^bNP396-404⁺ cells in the spleen have been shown to incorporate BrdU over an eight day period [17]. In the current study, the antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} underwent a similar amount of homeostatic proliferation as the wildtype in the spleen. Similar to previous findings [10], the extent of proliferation of antigen-specific CD8⁺ T cells in the Bim^{-/-} animals was reduced compared to the wildtype in all compartments examined. Therefore, antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} animals are able to undergo homeostatic proliferation following acute LCMV infection.

3.4 Bim^{-/-}Fas^{lpr/lpr} mice contain increased numbers of functional antigen-specific CD4⁺ T cells in the lymph nodes on day 97 postinfection

CD4⁺ T cells also play an important role in the generation and maintenance of CD8⁺ memory T cells following infection [18–20]. Therefore, we determined the number of functional CD4⁺ T cells in wildtype, Bim^{-/-}, Fas^{lpr/lpr}, and Bim^{-/-}Fas^{lpr/lpr} mice following GP61-80 peptide stimulation on day 97 (Fig. 4A&B) postinfection with LCMV Armstrong. In the lymph nodes (Fig. 4A) increased numbers of functional cells were observed in double mutant compared to single mutant or wildtype mice. This contrasted with the spleen (Fig.

4B) where $Bim^{-/-}Fas^{lpr/lpr}$ animals had similar numbers as $Bim^{-/-}$ mice. When the portion of $IFN\gamma$ producing cells that also made $TNF\alpha$ was determined similar results were observed in both the lymph nodes (Fig. 4C) and spleen (Fig. 4D). Notably there was a greater percentage of cells that could produce both cytokines in the spleens compared to the lymph nodes. In double mutant mice the portion of cells that could make both cytokines was reduced in the spleen compared to the other genotypes. Thus, loss of both *Bim* and *Fas* function increases antigen-specific $CD4^{+}$ T cells in the lymph nodes at late timepoints post infection similar to what we observed in the $CD8^{+}$ T cell response.

3.5 Antigen-specific $CD8^{+}$ T cells from $Bim^{-/-}Fas^{lpr/lpr}$ mice respond to secondary challenge

One of the hallmarks of memory cells is the ability to clear a secondary challenge of pathogen. Because secondary infection can be influenced by antibody levels we measured LCMV-specific IgG in all four genotypes by ELISA (Fig. 5A). By day 87 postinfection all four genotypes had reached a high titer that did not differ significantly. In order to determine if the antigen-specific $CD8^{+}$ T cells in $Bim^{-/-}Fas^{lpr/lpr}$ mice were capable of responding to secondary challenge in vivo, we infected LCMV Armstrong immune animals with LCMV Clone 13 and on day 5 post secondary challenge measured viral clearance and the number of antigen-specific T cells. Mice were sacrificed and the level of virus in the spleen was below the limit of detection for all four genotypes (Fig. 5B). In contrast naïve wildtype mice that were infected with LCMV Clone 13 reached titers of 2×10^8 p.f.u./g at the same timepoint. Virus-specific $CD8^{+}$ T cells were quantitated in the lymph nodes (Fig. 5C), spleen (Fig. 5D), and liver (Fig. 5E). In the lymph nodes the number of LCMV-specific $CD8^{+}$ T cells was increased whereas in the other tissues similar numbers were observed. Nevertheless, the total number of virus-specific $CD8^{+}$ T cells in the spleen, liver, and lymph nodes in the $Bim^{-/-}Fas^{lpr/lpr}$ animals was similar to the single mutants (data not shown). Thus, mice lacking both *Bim* and *Fas* are able to clear a secondary challenge of pathogen.

3.6 $Bim^{-/-}Fas^{lpr/lpr}$ mice generate secondary effector $CD8^{+}$ T cells following rechallenge

In order to determine if the antigen-specific $CD8^{+}$ T cells from the different genotypes were functional, we restimulated splenocytes and lymph node lymphocytes from wildtype, $Bim^{-/-}$, $Fas^{lpr/lpr}$, and $Bim^{-/-}Fas^{lpr/lpr}$ animals with LCMV MHC Class I restricted epitopes and determined the number of cytokine-producing cells. Similar to primary effector cells, secondary effectors contained a mix of $IFN\gamma^{+}$ and $IFN\gamma^{+}TNF\alpha^{+}$ cells (Fig. 6A). The number of $CD8^{+}IFN\gamma^{+}$ T cells in the spleens (Fig. 6B) of $Bim^{-/-}Fas^{lpr/lpr}$ animals was similar to the parental genotypes. However, we observed increased numbers of $CD8^{+}IFN\gamma^{+}$ T cells in the lymph nodes (Fig. 6C) of $Bim^{-/-}Fas^{lpr/lpr}$ animals. When the percentage of $IFN\gamma^{+}$ cells that produced $TNF\alpha$ was determined similar results were observed in the spleen regardless of epitope (Fig. 6D). In the lymph nodes (Fig. 6E) the percentage of double cytokine producing cells was increased in the $Fas^{lpr/lpr}$ and $Bim^{-/-}Fas^{lpr/lpr}$ mice compared to wildtype or $Bim^{-/-}$ animals. Thus, memory cells from all four genotypes are capable of differentiating into functional secondary effector cells.

3.7 Chronic LCMV infection results in similar numbers of antigen-specific $CD8^{+}$ T cells in $Bim^{-/-}Fas^{lpr/lpr}$ and $Bim^{-/-}$ animals

Our results with acute LCMV infection prompted us to examine how the antigen-specific $CD8^{+}$ T cells responses following chronic LCMV infection would be affected by mutations in both *Bim* and *Fas*. LCMV Clone 13 induces a chronic viral infection that is eliminated from most organs between day 60 and 90 postinfection, however virus persists in the kidneys and brain. Mice of each genotype were infected with LCMV Clone 13 and the antigen-specific responses at various days postinfection were determined by staining splenocytes, and liver and lymph node lymphocytes with anti- $CD8\alpha$ and MHC class I

tetramers D^bGP33-41 (Fig. 7A), D^bNP396-404 (Fig. 7B), and D^bGP276-286 (Fig. 7C). On day 8 post infection, we observed increased numbers of D^bGP33-41, D^bNP396-404, and D^bGP276-286 antigen-specific CD8⁺ T cells in both Bim^{-/-} and Bim^{-/-}Fas^{lpr/lpr} animals in the spleen. This increase in cell numbers was also observed in the liver for D^bNP396-404 specific CD8⁺ T cells. In contrast to acute infection, we did not observe an increase in the number of D^bGP33-41 and D^bGP276-286 specific CD8⁺ T cells in the lymph nodes of Bim^{-/-}Fas^{lpr/lpr} animals compared to the parental genotypes on day 8 postinfection. However, the number of D^bNP396-404 specific CD8⁺ T cells was increased in the lymph nodes of Bim^{-/-}Fas^{lpr/lpr}, Bim^{-/-}, and Fas^{lpr/lpr} animals compared to wildtype. By day 35 postinfection, the number of D^bGP33-41 and D^bGP276-286 specific CD8⁺ T cells declined in the spleen and liver in all genotypes. The number of D^bNP396-404 specific CD8⁺ T cells remained elevated in both the spleen and liver of both Bim^{-/-} and Bim^{-/-}Fas^{lpr/lpr} animals until day 111. Although the number of D^bNP396-404⁺ CD8⁺ T cells in the spleen and liver remains elevated, contraction occurred in the lymph nodes of all genotypes by day 35 postinfection. When the total number of antigen-specific CD8⁺ T cells in the spleen, liver and lymph node was calculated for each epitope there was no difference between Bim^{-/-} and Bim^{-/-}Fas^{lpr/lpr} mice (Fig. 7D).

Because differences in antigen-specific CD8⁺ T cells could be due to variation in viral load, we measured levels of LCMV in all four genotypes by plaque assay. The viral titers in the spleen, liver, serum, and kidney on days 8 (Fig. 8A), 35 (Fig. 8B) and 111 (Fig. 8C) post infection were determined. On days 8 and 35 viral titers were not significantly different between Bim^{-/-}Fas^{lpr/lpr} mice and the parental genotypes. By day 111 virus was below the detection limit in the spleen, liver and sera of wildtype and Bim^{-/-} mice, while several Fas^{lpr/lpr} and Bim^{-/-}Fas^{lpr/lpr} mice had detectable virus. When titer in the kidney was examined all four genotypes had similar amounts of virus. Taken together these results demonstrate that additional loss of both Bim and Fas does not significantly alter viral replication or antigen-specific CD8⁺ T cells compared to Bim deficient mice.

4. Discussion

In this study, we examined the effects of mutating components of both the intrinsic and extrinsic pathways of cell death on the generation of memory CD8⁺ T cells and the survival of CD8⁺ T cells during chronic viral infection. Following mutation of both the pro-apoptotic BH3-only protein Bim [21] and the extrinsic death receptor Fas [22], we observed increased numbers of antigen specific CD8⁺ T cells at late time points postinfection compared to parental genotypes following acute LCMV infection[11]. Here we report six novel observations. First, these cells exhibited a memory surface marker phenotype. Second, they were capable of undergoing homeostatic proliferation for self-renewal. Third, upon restimulation they were polyfunctional producing both IFN γ and TNF α . Fourth, upon rechallenge they regained effector function and cleared virus. Fifth, loss of Bim and Fas also increases the number of virus-specific CD4⁺ T cells in the lymph nodes at later timepoints. Sixth, in contrast to the increased antigen specific T cell numbers observed following acute infection, chronic LCMV infection resulted in similar numbers of antigen specific CD8⁺ T cells in Bim^{-/-}Fas^{lpr/lpr} animals compared to the Bim parental genotype. Together, these results demonstrate that the long-term surviving antigen-specific CD8⁺ T cells in mice lacking functional Bim and Fas are true memory cells. Additionally, they highlight differences in the cell death pathways used to regulate shutdown of immune responses following acute and chronic viral infection.

How do memory T cells accumulate in the lymph nodes of Bim^{-/-}Fas^{lpr/lpr} animals? Increased survival of antigen-specific CD8⁺ T cells could be due to autonomous and non-autonomous effects. Prlic and Bevan[23], demonstrated that Bim functions in a CD8⁺ T cell

autonomous manner through the adoptive transfer of Bim^{-/-} mutant OT-I CD8⁺T cells followed by infection with *Listeria monocytogenes* expressing ovalbumin. During the early contraction phase the number of Bim^{-/-} OT-I cells remained elevated. Additionally, these cells expanded upon secondary challenge similar to our observations. The manner by which Fas functions is less clear. For Fas to function autonomously in T cells its expression or sensitivity to signaling must be different in the lymph nodes compared to the spleen and liver. Alternatively Fas and Fas Ligand function could be critical in cell types besides T cells. Prior studies have shown that inflammation can induce Fas Ligand in nonlymphoid cells [24, 25] and that the high endothelial venules (HEV) of the lymph nodes express Fas Ligand [26]. A tantalizing possibility is that lack of Bim and Fas extends the survival of dendritic cells in the lymph nodes similar to that observed in Bim^{-/-} animals, which could provide increased cytokines such as IL-7 or IL-15 or persistent antigenic stimulation [27]. However, increased antigen presentation is unlikely since the proliferation of antigen-specific CD8⁺ T cells is actually decreased in Bim^{-/-}Fas^{lpr/lpr} compared to wild-type and Fas^{lpr/lpr} mice at both early [11] and late time points (Fig. 3). Instead, these findings provide evidence that rather than recruiting new cells into the response, the surviving SLECs are differentiating into functional memory cells. In the spleen and liver, other mechanisms must function in the absence of Bim to kill antigen-specific CD8⁺ T cells as they reexpress CD127 [10]. Our observation that these are “bona fide” memory cells is a critical finding, as it demonstrates that increasing effector cell survival may decrease the number of boosts required to generate protective immunity following vaccination.

In contrast to protective immunity observed following acute infection, memory cells are not generated during chronic infection and antigen is necessary for their maintenance [28]. Although the differentiation of the antigen-specific CD8⁺ T cells is distinct between acute and chronic infection, shutdown of the immune response following both types of infection still involves apoptosis [9]. Our observations demonstrate that mutation of Fas does not result in differences in contraction of the antigen-specific CD8⁺ T cells following chronic LCMV infection. Previous studies by Zhou et. al. demonstrated that the decline of D^bNP396-404⁺CD8⁺ T cells in FasL mutant mice was delayed compared to wildtype [29]. The discrepancy between our studies could be due to the different strains of LCMV used in these two studies (Clone 13 vs. Docile) or that we examined mice that contained a mutation Fas as opposed to Fas Ligand. A previous report by Hughes et. al. found that Fas and Bim function in a cooperative manner to limit antigen-specific CD8⁺ T cell numbers during chronic mouse γ -herpes virus (MHV) infection[30]. Recently these results have been extended by Masson and colleagues who demonstrated that one of the downstream targets of Fas signaling, Bid, is required with Bim to downsize responses following MHV infection. How can the differences between our results during chronic LCMV infection and those with MHV be reconciled? Following intranasal MHV infection, the primary site of productive viral replication is the lung and virus is rarely found in lymphoid tissue at early time points postinfection [31, 32]. However, once the virus establishes latency, it can be detected in the spleen where the primary reservoir is B cells [33]. Hao et al. recently demonstrated the importance of an intact Fas/FasL pathway in germinal center B cells in maintaining both T and B cell homeostasis, which could explain the differences in results observed between these studies [34]. In contrast, chronic LCMV infection results in a systemic infection with high viral replication occurring in numerous tissues including the spleen, liver, lung, kidney, and brain [9, 35]. The extent and duration of antigen presentation and cytokine production could be driving the combinatorial expression of various death receptors and their ligands in a manner analogous to inhibitory receptors[36].

In this study, we demonstrate that increasing antigen specific T cell survival after clearance of an acute infection augments the number of true memory cells at later time points postinfection. Many vaccines including MMR and varicella require multiple boosts [37]. By

increasing T cell survival, it may be possible to limit the number of boosts required to generate protective immunity. Our results also demonstrate that mutating both Bim and Fas does not significantly increase antigen specific T cell numbers or accelerate viral clearance from immune privileged sites following chronic LCMV infection. This suggests there are multiple components of viral infection influencing apoptosis and the outcome of the immune response. In the future, it will be necessary to consider not only how the immune system maintains homeostasis, but the influence of pathogens upon apoptosis and function of the immune response. Together, this study demonstrates that understanding the regulation of apoptosis has implications for optimizing vaccine development potentially through the development of small molecule inhibitors of cell death that could be administered during the contraction phase to increase the number of memory cells.

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- In this study we examined antiviral immune responses in $\text{Bim}^{-/-}\text{Fas}^{\text{lpr/lpr}}$ mice.
- Virus-specific CD8^{+} T cells in the lymph nodes appear to be memory cells.
- Loss of Bim and Fas increases virus-specific CD4^{+} T cells in the lymph nodes.
- During chronic infection further loss of Fas did not affect responses in $\text{Bim}^{-/-}$ mice.

Figure 1.

Antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} animals exhibit phenotypic markers characteristic of memory cells at later times postinfection. Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-}Fas^{lpr/lpr}, and wildtype mice were infected with LCMV Armstrong and sacrificed at day 8 and day 97 postinfection and splenocytes and lymph node lymphocytes were stained with anti-CD8 α , anti-KLRG1, anti-CD127, anti-CD62L, anti-CD27 and MHC class I tetramers. The dot plots are representative samples of lymph node lymphocytes (A) from days 8 and 97 postinfection and are gated on CD8⁺D^bNP396-404⁺ T cells. The numbers indicate the percentage of short lived effector cells (SLECs, top left quadrant) and memory precursor effector cells (MPECs, lower right quadrant) in that quadrant. (B) The percentage of tetramer positive MPEC phenotype cells was determined at day 97 postinfection in the lymph nodes and spleen. (C) The percentage of tetramer positive central memory (CD62L^{high}CD27^{high}) phenotype cells was determined at day 97 postinfection in the lymph

nodes and spleen. The average and standard deviation are plotted. Six to seven mice of each genotype were analyzed at each time point.

Figure 2.

Acute viral infection of Bim^{-/-}Fas^{lpr/lpr} mice results in increased numbers of functional antigen-specific CD8⁺ T cells in the lymph nodes at day 97 postinfection. Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-}Fas^{lpr/lpr}, and wildtype mice were infected with LCMV Armstrong. (A) Mice were sacrificed on day 97 postinfection and the lymph nodes were removed and stimulated with the indicated peptide and then stained with anti-CD8α, anti-IFNγ, and anti-TNFα. The dot plots are gated on CD8⁺ T cells and the numbers indicate the percent of CD8⁺ T cells that are present in that quadrant. The number of IFNγ⁺ CD8⁺ T cells was quantitated for both the lymph nodes (B) and spleen (C). For each virus-specific CD8⁺ T cell population the percent of IFNγ⁺ cells that produced TNFα was determined in the lymph nodes (D) and spleen (E). The averages and standard deviations are plotted. Six to seven mice of each genotype were

analyzed with an ordinary ANOVA with a Student-Newman-Keuls multiple comparison test. *, significant increase compared to both $Bim^{-/-}$ and $Fas^{lpr/lpr}$ with a p-value ≤ 0.05 .

Figure 3.

Antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} animals are able to undergo homeostatic proliferation following acute LCMV infection. Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-}Fas^{lpr/lpr}, and wildtype mice were infected with LCMV Armstrong. Mice were administered 0.8 mg/ml BrdU in their drinking water from day 90 to 97 postinfection. Mice were sacrificed on day 97 postinfection and splenocytes, lymph node lymphocytes, and liver lymphocytes were stained with anti-CD8 α and the MHC class I tetramers D^bGP33-41 and D^bNP396-404 in addition to anti-BrdU so that the number of antigen-specific cells in S phase could be determined. The percentage of D^bGP33-41 and D^bNP396-404 specific CD8⁺ T cells that incorporated BrdU was quantitated in the spleen (A), liver (B), and lymph nodes (C) and the averages and standard deviations are shown. Six to seven mice of each genotype were analyzed with an ordinary ANOVA with a Student-Newman-Keuls multiple comparison test. *, significant increase compared to both Bim^{-/-} and Fas^{lpr/lpr} with a p-value ≤ 0.05 .

Figure 4.

Bim^{-/-}Fas^{lpr/lpr} mice contain increased numbers of functional antigen-specific CD4⁺ T cells in the lymph nodes at day 97 postinfection. Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-}Fas^{lpr/lpr}, and wildtype mice were infected with LCMV Armstrong. Mice were sacrificed on day 97 postinfection and the lymph nodes were removed and stimulated with GP61-80 peptide and then stained with anti-CD4, anti-IFN γ , and anti-TNF α . The number of IFN γ ⁺ CD4⁺ T cells was quantitated for both the lymph nodes (A) and spleen (B). For each virus-specific CD4⁺ T cell population the percent of IFN γ ⁺ cells that produced TNF α was determined in the lymph nodes (C) and spleen (D). The averages and standard deviations are plotted. Six to seven mice of each genotype were analyzed with an ordinary ANOVA with a Student-Newman-Keuls multiple comparison test. *, significant increase compared to both Bim^{-/-} and Fas^{lpr/lpr} with a p-value ≤ 0.05 .

Figure 5.

Antigen-specific CD8⁺ T cells from Bim^{-/-}Fas^{lpr/lpr} animals are able to respond to rechallenge. Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-}Fas^{lpr/lpr}, and wildtype mice were infected with LCMV Armstrong. Prior to rechallenge, LCMV-specific IgG (A) was determined by ELISA. On day 87 postinfection, LCMV Armstrong immune mice were challenged with LCMV Clone 13. Mice were sacrificed on day 5 post challenge and viral titer (B) was determined in the spleen by plaque assay. Spleens were also collected from naïve wildtype mice that were infected with Clone 13 five days earlier. Lymph node lymphocytes (C), splenocytes (D) and liver lymphocytes (E) were stained with anti-CD8 α and the MHC class I tetramers D^bGP33-41, D^bNP396-404, and D^bGP276-286 and the averages and standard deviations are shown. Six mice of each genotype were analyzed with an ordinary ANOVA with a Student-Newman-Keuls multiple comparison test. *, significant increase compared to both Bim^{-/-} and Fas^{lpr/lpr} with a p-value ≤ 0.05 .

Figure 6.

Bim^{-/-}Fas^{lpr/lpr} animals contain similar numbers of functional CD8⁺ T cells as the parental genotypes following secondary challenge. Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-}Fas^{lpr/lpr}, and wildtype mice were infected with LCMV Armstrong. On day 87 postinfection, LCMV Armstrong immune mice were challenged with LCMV Clone 13. Mice were sacrificed on day 5 post challenge and the spleen was removed and stimulated with the indicated peptide and then stained with anti-CD8α, and anti-IFNγ, and anti-TNFα. The dot plots are gated on CD8⁺ T cells and the numbers indicate the percent of CD8⁺ T cells that are present in that quadrant (A). The number of IFNγ⁺ CD8⁺ T cells was quantitated for both the spleen (B) and lymph nodes (C). For each virus-specific CD8⁺ T cell population the percent of IFNγ⁺ cells that produced TNFα was determined in the spleen (D) and lymph nodes (E). The averages and standard deviations are plotted. Six mice of each genotype were analyzed at each time point.

with an ordinary ANOVA with a Student-Newman-Keuls multiple comparison test. *, significant increase compared to both $Bim^{-/-}$ and $Fas^{lpr/lpr}$ with a p-value ≤ 0.05 .

Figure 7.

Chronic LCMV infection results in similar numbers of antigen-specific CD8⁺ T cells in Bim^{-/-} Fas^{lpr/lpr} and Bim^{-/-} mice. Bim^{-/-}, Fas^{lpr/lpr}, Bim^{-/-} Fas^{lpr/lpr}, and wildtype mice were infected with LCMV clone 13. Mice were sacrificed at the indicated time points and splenocytes, liver lymphocytes, and lymph node lymphocytes were stained with anti-CD8α, anti-CD44, and MHC class I tetramers so that the number of antigen-specific CD8⁺ T cells could be determined. The numbers of D^bGP33-41 (A), D^bNP396-404 (B), and D^bGP276-286 (C) specific CD8⁺ T cells were quantitated, and the averages and standard deviations are shown. The total number of antigen-specific CD8⁺ T cells was quantitated on day 111 postinfection for each epitope in all three tissues (D) and the averages and standard deviations are shown. Three to seven mice of each genotype were analyzed at each time point with an ordinary ANOVA with a Student-Newman-Keuls multiple comparison test. *, significant increase compared to both Bim^{-/-} and Fas^{lpr/lpr} with a p-value ≤ 0.05.

Figure 8.

Clone 13 infection generates similar viral titers in $Bim^{-/-} Fas^{lpr/lpr}$ animals compared to the parental genotypes. $Bim^{-/-}$, $Fas^{lpr/lpr}$, $Bim^{-/-} Fas^{lpr/lpr}$, and wildtype mice were infected with LCMV clone 13. Mice were sacrificed on days 8 (A), 35 (B), and 111 (C) postinfection and virus was quantitated in the spleen, liver, serum, and kidneys by plaque assay. Each symbol indicates the value for one mouse. Dashed lines in the graphs indicate the limit of detection. Three to nine mice were analyzed at each time point. *, significant increase compared to both $Bim^{-/-}$ and $Fas^{lpr/lpr}$ with a p-value ≤ 0.05