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Unexpected role for perforin in regulating contraction of CD8 and CD4 T cells during prolonged *Listeria monocytogenes* infection

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Summary

After infection or vaccination, antigen-specific T cells proliferate then contract in numbers to a memory set point. T cell contraction is observed after both acute and prolonged infections although it is unknown if contraction is regulated similarly in both scenarios. Here, we show that contraction of antigen-specific CD8 and CD4 T cells is markedly reduced in TNF/perforin-double deficient (DKO) mice responding to attenuated *Listeria monocytogenes* infection. Reduced contraction in DKO mice was associated with delayed clearance of infection and sustained T cell proliferation during the normal contraction interval. Mechanistically, sustained T cell proliferation mapped to prolonged infection in the absence of TNF, however, reduced contraction required the additional absence of perforin since T cells in mice lacking either TNF or perforin (singly-deficient) underwent normal contraction. Thus, while T cell contraction after acute infection is independent of perforin, a perforin-dependent pathway plays a previously unappreciated role to mediate contraction of antigen-specific CD8 and CD4 T cells during a prolonged *L. monocytogenes* infection.

Keywords

CD8 T cell; CD4 T cells; perforin; contraction

Introduction

Infection of mice with *L. monocytogenes* results in a robust expansion in the number of antigen-specific T cells, which peak on day 7 post-infection. This expansion phase is followed by a substantial contraction in T cell numbers, ultimately resulting in survival of a population representing 5–10% the number observed at the peak of the expansion phase [1]. The remaining antigen-specific T cells then undergo further differentiation into long-lived memory populations.

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Conflict of interest

The authors declare no financial conflicts of interest.

Recent work has begun to identify important molecules that regulate contraction of T cells. Bim, which is a pro-apoptotic Bcl-2 family member [2], has been implicated in the contraction of antigen-specific CD8 T cells following both acute (*L. monocytogenes* and LCMV) and chronic infections (LCMV) [3–5], and the absence of both Bim and Fas results in markedly reduced CD8 T cell contraction in lymph nodes following infection with LCMV [6, 7]. In addition to these pro-apoptotic molecules, inflammatory cytokines also appear to regulate the contraction of antigen-specific CD4 and CD8 T cells. The amount of IL-12 present at the time of CD8 T cell priming can dictate the expression levels of T-bet, and effector T cells with high T-bet appear to be short lived [8]. Furthermore, in the absence of IFN- γ , or its receptor, antigen-specific T cells exhibit reduced contraction following infection with an attenuated strain of *L. monocytogenes* [9–11]. Recent work has also demonstrated a potential role for TNF in regulating antigen-specific CD8 T cell homeostasis. Mice deficient in either TNF, or its receptors, exhibit a modest increase in the number of LCMV-specific CD8 T cells at memory time points following either a chronic or acute LCMV infection [12–14]. These results were interpreted as reduced contraction, however, since these mice had an increase in the expansion of LCMV-specific CD8 T cells it is not known if TNF does indeed regulate CD8 T cell contraction.

Previous studies have demonstrated that CD8 T cell contraction occurs after the expansion phase in response to both acute and chronic infections [15, 16] and that truncating infections had minimal impact on the onset or degree of contraction in both CD4 and CD8 T cells [15, 17]. These data suggested that T cell contraction was programmed by early events after infection [15]. Additionally, antigen-specific CD8 T cells expand and undergo initial contraction similarly in WT mice after an acute or prolonged infection, however, it is not known if the mechanisms that mediate contraction are the same in both situations.

In this report we address the mechanisms regulating CD8 and CD4 T cell contraction in response to acute versus prolonged infection. We show that perforin regulates CD8 T cell contraction during prolonged infection but plays no discernable role in contraction after acute infection. We also demonstrate a novel role for perforin in regulating CD4 T cell contraction during prolonged infection. These data show that different molecular mechanisms regulate T cell contraction in acute versus prolonged infections. This perforin-dependent mechanism to regulate contraction may be vitally important to prevent T cell-dependent immunopathology during a chronic infection where antigen-specific T cells are repeatedly stimulated.

Results

Aberrant CD8 and CD4 T cell contraction in TNF/perforin-deficient mice

Perforin and TNF have been implicated in regulating various aspects of the CD8 T cell response to infections or immunization [9, 12–14, 18, 19]. To determine how these molecules influence the T cell response to bacterial infection we generated C57BL/6 TNF/perforin-double deficient (DKO) mice by crossing single knockout parental mice (all on the C57BL/6 background). TNF-deficient mice are very susceptible to virulent *L. monocytogenes* [20], thus C57BL/6 (WT) and DKO mice were infected with 5×10^6 attenuated *actA*-deficient *L. monocytogenes* expressing ovalbumin (here on referred to as *actA*-deficient LM-OVA), which allowed us to analyze the response of endogenous OVA_{257–264}-specific CD8 T cells [21] (Fig. 1) and LLO_{190–201}-specific CD4 T cells [22] (Fig. 2). Following infection, OVA-specific CD8 T cells and LLO-specific CD4 T cells in the spleen of WT mice expanded both in frequency and in total number with a peak at day 7 p.i. This expansion was followed by a rapid contraction in both frequency and total T cell numbers between day 7 and 14 (Fig. 1A–B and Fig. 2A–B) with a relatively stable memory set point for CD8 T cells and further decline for CD4 T cells (Fig. 1C and Fig. 2C). Infection

of DKO mice with *actA*-deficient LM-OVA resulted in a modest but significant increase in the total number of OVA-specific CD8 T cells (~1.3-fold, $p=0.008$) and LLO-specific CD4 T cells (2.6-fold, $p=0.0006$) on day 7 as compared to WT mice (Fig. 1B and 2B). However, there was a striking and significant ($p<0.001$) reduction in the contraction of OVA-specific CD8 T cells between day 7 and day 41 (Fig. 1A–C) in DKO mice (~65% survival in total numbers from the peak) as compared to WT mice (~5% survival from the peak) (Fig. 1C) resulting in a significantly ($p<0.001$) higher number (~60-fold) of memory CD8 T cells in DKO mice (Fig. 1B). The reduced contraction and increased number of OVA-specific CD8 T cells observed in DKO mice was evident out to day 440 p.i. (Fig. 1A–C). To demonstrate reduced contraction was occurring in multiple tissues we quantified OVA-specific CD8 T cells in the blood, spleen, inguinal lymph nodes, liver, and lung from WT and DKO mice on day 7 and 14 post infection. Since ~95% of contraction is complete by day 14 (7.3% survival) compared to day 41 (2.8% survival) in WT mice, we chose to analyze contraction at day 14. Importantly, in every tissue analyzed OVA-specific CD8 T cells exhibited substantially less contraction in DKO mice compared to WT mice (Fig. 1D–H).

Previous work demonstrated the absence of IFN- γ resulted in reduced contraction of LLO190-specific CD4 T cells following infection with *L. monocytogenes* [11]. Therefore, we wanted to determine if the absence of both perforin and TNF also affected the contraction of LLO190-specific CD4 T cells following infection with *actA*-deficient LM-OVA. Similar to OVA-specific CD8 T cells, there was a significant ($p<0.0001$) decrease in the contraction of LLO-specific CD4 T cells between day 7 and day 41 (Fig. 2A–C) in DKO mice (~87% survival in total numbers from the peak) as compared to WT mice (~8% survival from the peak) (Fig. 2C). This resulted in a significantly ($p<0.0001$) higher number (~30-fold) of memory CD4 T cells at day 41 in DKO mice compared to WT mice (Fig. 2B). Although LLO-specific CD4 T cells continually decline between day 41 and day 440 in both WT and DKO mice (Fig. 2A–B) there was still a significant ($p=0.0355$) decrease in the contraction of LLO-specific CD4 T cells in DKO mice compared to WT (Fig. 2C). This resulted in significantly ($p=0.0055$) more LLO-specific CD4 T cells in the spleens of DKO mice compared to WT mice (Fig. 2B). Collectively, these data suggest that TNF and/or perforin play a critical role in regulating the contraction of antigen-specific CD8 and CD4 T cells after *actA*-deficient LM infection of C57BL/6 mice.

Absence of TNF and perforin results in a prolonged infection and antigen-display

TNF and perforin are important effector molecules utilized by the innate and adaptive immune system to control *L. monocytogenes* infections [23]. Consistent with this notion, WT mice cleared *actA*-deficient LM-OVA by day 8 p.i. in the spleen, however, *actA*-deficient LM-OVA were still detectable in DKO mice on day 8 and 11 p.i. (Fig. 3A–B). One way to determine if the prolonged infection also results in prolonged antigen-display is to adoptively transfer CFSE labeled TCR transgenic (Tg) T cells into the infected mouse and monitor proliferation of the transferred cells [24]. Therefore, we labeled naïve Thy1.1+ OT-I TCR Tg CD8 T cells that recognize OVA_{257–264} [25, 26] with CFSE and injected them into naïve or infected WT and DKO mice (both Thy1.2+) on day 11 p.i. and analyzed proliferation (CFSE dilution) three days later in the spleen. Proliferation of naïve OT-I TCR Tg CD8 T cells did not occur in naïve WT or DKO mice or mice infected with *actA*-deficient LM that lack the OVA_{257–264} epitope (Fig. 3C). Whereas OT-I TCR Tg CD8 T cells exhibited modest CFSE dilution between days 11 and 14 in WT mice (on average only $14\pm 7.2\%$ of OT-I TCR Tg CD8 T cells divided at least once), OT-I TCR Tg CD8 T cells in DKO mice proliferated extensively (on average $46\pm 13.3\%$ of OT-I TCR Tg CD8 T cells divided at least once) during this interval (Fig. 3C). These data indicate that the absence of TNF and perforin results in a prolonged *actA*-deficient LM-OVA infection and prolonged antigen-display compared to WT mice.

Reduced T cell contraction results from prolonged infection

Reduced contraction of OVA-specific CD8 T cells (Fig. 1) and LLO-specific CD4 T cells (Fig. 2) could result from the prolonged infection and antigen-display observed in DKO mice (Fig. 3). As previously observed, antibiotic treatment to completely eliminate LM-OVA infection at 4 days p.i. had no impact on T cell contraction in WT mice [10, 11] (Fig. 4). In contrast, treatment of DKO mice with ampicillin at day 4 to eliminate the infection restored OVA-specific CD8 T cell contraction (Fig. 4A–B) and LLO-specific CD4 T cell contraction (Fig. 4C–D) to a similar level as seen in WT mice. These data strongly suggest the reduced contraction of OVA-specific CD8 T cells and LLO-specific CD4 T cells in DKO mice resulted from the prolonged *actA*-deficient LM-OVA infection.

Prolonged infection results in sustained T cell proliferation

Two potential mechanisms may explain the reduced contraction of OVA-specific CD8 T cells and LLO-specific CD4 T cells in DKO mice. First, antigen-specific T cells may undergo a lower rate of apoptosis in DKO mice compared to WT mice. Alternatively, antigen-specific T cells in DKO mice may exhibit increased proliferation compared to their response in WT mice, thus offsetting the death of T cells during the contraction phase.

To determine if increased proliferation during the contraction phase explained the reduced contraction of antigen-specific T cells in DKO mice we performed BrdU labeling during the expansion, contraction, and early memory phases. Nearly 100% of the OVA-specific CD8 T cells and LLO-specific CD4 T cells in WT mice incorporated BrdU during the expansion phase (day 4–7) (Fig. 5A–B and D–E, respectively), which was followed by a precipitous drop in proliferation throughout the contraction phase (Fig. 5A–B and D–E, respectively). Similarly, nearly 100% of OVA-specific CD8 T cells and LLO-specific CD4 T cells in DKO mice incorporated BrdU during the expansion phase (Fig. 5A–B and D–E, respectively). However, and in contrast to WT mice, OVA-specific CD8 T cells and LLO-specific CD4 T cells in DKO mice exhibited sustained BrdU incorporation throughout the contraction phase (Fig. 5A–B and D–E, respectively). Importantly, sustained BrdU incorporation in OVA-specific CD8 T cells and LLO-specific CD4 T cells was not observed in antibiotic-treated DKO mice (Fig. 5C and F, respectively), demonstrating that the sustained proliferation of antigen-specific T cells observed in DKO mice resulted from the prolonged infection and antigen display. Importantly, the rate of BrdU incorporation in OVA-specific CD8 T cells and LLO-specific CD4 T cells were similarly low in WT and DKO mice during an early (days 33–41) memory interval (Fig. 5A–B and D–E, respectively). These data are consistent with the gradual decrease in antigen load in DKO mice (Fig. 3) and the eventual clearance of *actA*-deficient LM-OVA in DKO mice (no bacteria could be detected in organ homogenates at day 41 p.i., data not shown). Together, these data suggest that prolonged antigen-display stimulates sustained antigen-specific T cell proliferation throughout the contraction phase in DKO mice. Thus, TNF and/or perforin are required to mediate contraction of OVA-specific CD8 T cells and LLO-specific CD4 T cells in the context of sustained proliferation during the contraction phase.

Memory T cells have an effector memory-like phenotype in DKO mice

When T cells are repetitively stimulated by antigen they develop an effector memory phenotype, consistent with low expression of CD62L and IL-2 [27–29]. Furthermore, repetitive antigen stimulation during chronic infections leads to functional exhaustion, which is associated with both, impaired cytokine production (IL-2, TNF, and IFN- γ) and the expression of inhibitory receptors (e.g. PD-1 and LAG-3) [30, 31]. Since DKO mice exhibit a prolonged infection and antigen display we analyzed the phenotype of antigen-specific memory T cells in WT and DKO mice. Consistent with their generation in an environment with prolonged antigen-display, we observed a decrease in the expression of CD62L and

CD127 as well as reduced IL-2 production in OVA-specific CD8 T cells in DKO mice compared to WT mice (Fig. 6A–B). We also observed a decrease in CD127 expression and IL-2 production by LLO-specific CD4 T cells in DKO mice compared to WT, however, there was no difference in CD62L expression (Fig. 6C). Interestingly, in spite of prolonged antigen stimulation (Fig. 3C) and increased proliferation (Fig. 5) in DKO mice OVA-specific CD8 T cells in DKO mice did not express the inhibitory receptor PD-1 (Fig. 6D–E). Likewise there was little to no expression of the inhibitory receptor LAG-3 on day 14 in DKO mice (Fig. 6E). Collectively, these data suggest that in spite of prolonged antigen stimulation in DKO mice antigen-specific T cells do not become exhausted, but develop into effector memory-like cells.

Perforin enforces T cell contraction during a prolonged *Listeria* infection

Analysis of IFN- γ /perforin-double deficient BALB/c mice revealed an increased expansion of antigen-specific CD8 T cells and reduced contraction following infection with *actA*-deficient *L. monocytogenes*, which tracked to perforin-deficiency and IFN- γ -deficiency respectively [9]. To determine if the reduced contraction and increased proliferation observed in DKO mice tracked to either TNF- or perforin-deficiency we analyzed OVA-specific CD8 T cells and LLO-specific CD4 T cells after infection in TNF-single deficient (TKO) and perforin-single deficient (PKO) mice. Surprisingly, contraction of OVA-specific CD8 T cells (Fig. 7A–B) and LLO-specific CD4 T cells (Fig. 7C–D) in TKO and PKO mice was not significantly reduced compared to WT mice. This suggests reduced contraction of OVA-specific CD8 T cells and LLO-specific CD4 T cells observed in DKO mice requires the absence of both TNF and perforin.

We next determined if the sustained proliferation of OVA-specific CD8 T cells and LLO-specific CD4 T cells observed during the normal contraction interval in DKO mice (Fig. 5) tracked to TNF- or perforin-deficiency. Consistent with both TNF and perforin functioning as effector molecules in the clearance of *L. monocytogenes* [32], we observed delayed clearance of *actA*-deficient LM-OVA in the liver of DKO, TKO, and PKO mice compared to WT mice (Fig. 7E). Since perforin has been shown to be involved in the elimination of antigen-presenting dendritic cells [19], we hypothesized that sustained proliferation would also track to perforin-deficiency. Contrary to our hypothesis, OVA-specific CD8 T cells (Fig. 7F) and LLO-specific CD4 T cells (Fig. 7H) in perforin-deficient mice exhibited substantially reduced BrdU incorporation during the contraction interval compared to DKO mice. In contrast, OVA-specific CD8 T cells in DKO and TKO mice exhibit similarly high rates of BrdU incorporation (Fig. 7F). Similarly, LLO-specific CD4 T cells in both DKO and TKO mice exhibited significantly ($p=0.0005$ and $p=0.024$, respectively) higher rates of BrdU incorporation compared to WT mice (Fig. 7H). However, treatment with ampicillin at day 4 p.i. decreased the rate of BrdU incorporation in both OVA-specific CD8 T cells (Fig. 7G) and LLO-specific CD4 T cells (Fig. 7I) in both DKO and TKO mice, demonstrating the increased proliferation is a result of the prolonged infection/antigen-display caused by the absence of TNF.

Based on the observation that increased proliferation tracked to TNF-deficiency (Fig. 7F and H) this suggests prolonged antigen-display observed in DKO mice (Fig. 3C) would track to TNF-deficiency, whereas antigen-presentation in WT and perforin-deficient mice would be similar. Consistent with this notion, antigen-presentation, as detected by CFSE dilution in transferred naïve OT-I TCR Tg CD8 T cells, was similarly low in WT and PKO mice during days 11–14 (Fig. 7J). In contrast, OT-I TCR Tg CD8 T cell exhibited substantial CFSE dilution in TKO mice during days 11–14 p.i. (Fig. 7J), indicating that prolonged antigen-display tracks to TNF-deficiency. Collectively, these data strongly suggest that TNF-deficiency results in prolonged infection and antigen-display, which causes sustained

proliferation of antigen-specific T cells during the normal contraction interval. However, only in the absence of both TNF and perforin is T cell contraction reduced.

Previous research has demonstrated serglycin, which along with perforin is found in cytolytic granules, contributes to the regulation of antigen-specific CD8 T cell contraction following infection with LCMV in a T cell intrinsic mechanism [33]. Furthermore, release of granzyme B (GrB), which is also found in cytolytic granules, into the cytosol of antigen-specific CD8 T cells is important in regulating the expansion of CD8 T cells following infection with LCMV [34]. These data suggest that components of the cytolytic machinery function in a cell intrinsic mechanism to regulate antigen-specific T cell numbers following infection. It is well documented that CD8 T cells express perforin [23, 35, 36]. Therefore, we wanted to determine if *Listeria*-specific CD4 T cells also express perforin.

Unfortunately, we were unable to reliably detect perforin in effector CD8 T cells by using commercially available antibodies. Therefore, we sort purified naïve CD4 T cells (CD4⁺/CD11a^{lo}/CD49d^{lo}) and *Listeria*-specific CD4 T cells (CD4⁺/CD11a^{hi}/CD49d^{hi}) and analyzed expression of perforin mRNA five days after *actA*-deficient LM-OVA infection. Sort purification of naïve CD4 T cells (CD11a^{lo}/CD49d^{lo}) and antigen-specific CD4 T cells (CD11a^{hi}/CD49d^{hi}) was based on two recent publications [37] (Butler N, et al., *Nature Immunology*, In Press) that demonstrate up-regulation of CD11a and CD49d specifically identify antigen-specific CD4 T cells. Five days post *actA*-deficient LM-OVA infection we observed increased expression of perforin mRNA in the *Listeria*-specific CD4 T cells (CD11a^{hi}/CD49d^{hi}) compared to naïve CD4 T cells (CD11a^{lo}/CD49d^{lo}) as well as cells from a naïve spleen (Fig. 8A). Furthermore, we observed high levels of intracellular GrB expression in both OVA-specific CD8 T cells (70%-95%) (Fig. 8B) and LLO-specific CD4 T cells (25%-50%) (Fig. 8C). Thus, these data argue that a perforin-dependent pathway potentially involving GrB enforces the contraction of antigen-specific T cells in the context of prolonged infection/antigen-display and elevated proliferation.

Discussion

In this study, we demonstrate that different mechanisms regulate CD8 and CD4 T cell contraction after acute and prolonged infections in C57BL/6 mice. During prolonged *L. monocytogenes* infections and functional antigen-display (resulting from TNF-deficiency) antigen-specific T cells exhibit elevated rates of proliferation during the normal contraction interval. In the absence of perforin prolonged infection and antigen-display results in reduced contraction. In contrast, perforin-deficiency has no influence on antigen-specific T cell contraction after an acute infection. Thus, perforin plays a previously unappreciated, yet vital, role in ensuring the contraction of antigen-specific T cells during prolonged infections where T cells exhibit sustained proliferation throughout the normal contraction phase.

Perforin has been shown to regulate antigen-specific CD8 T cell responses following infection [9, 38–40]. In those reports it was shown that the absence of perforin results in increased numbers of CD8 T cells during the expansion phase, causing immunopathology during chronic infections. However, a role for perforin in regulating antigen-specific CD4 T cell responses in vivo has not been described. Here we also show that perforin enforces contraction of antigen-specific CD4 T cells during prolonged infection. Failure to ensure CD4 T cell contraction could have equally dire consequences to the host given the ability of these cells to orchestrate inflammatory responses and tissue destruction.

A previous study suggested that leakage of granzyme B into activated T cells could be toxic in the absence of the Spi6 serpin molecule that inactivates granzyme B [34]. In that study, the absence of Spi6 resulted in reduced proliferative expansion of CD8 T cells after infection presumably due to increased death. Furthermore, serglycin-deficient mice exhibit

delayed contraction of antigen-specific CD8 T cells following viral infection [33]. Collectively, these results suggest that components of cytolytic granules function to control antigen-specific T cell numbers following infection. Our results suggest the possibility this is a perforin-dependent pathway, perhaps involving leakage of granule contents into the cytosol of repeatedly stimulated CD8 and CD4 T cells. Collectively, this mechanism functions to mediate the contraction of repeatedly stimulated T cells during prolonged infection. Consistent with this possibility we have observed elevated levels of intracellular granzyme B not only in OVA-specific CD8 T cells but also in LLO-specific CD4 T cells after infection with *actA*-deficient LM-OVA (Fig. 8B–C). Furthermore, previous studies have also demonstrated that human CD4 and CD8 T cells differentially express granzymes A and B [41]. In that report it was shown that following stimulation with conA and IL-2 human CD4 and CD8 T cells up-regulated granzyme B, while granzyme A remained low (CD4 T cells) or was down-regulated (CD8 T cells). While most CD4 T cells became granzyme B positive only about 10% of those cells were also granzyme A positive. This raises the intriguing possibility that memory T cells may be selected for survival during a prolonged infection based on differential granzyme expression. It will be interesting to explore this possibility as the appropriate reagents come available.

Several studies evaluating the changes in numbers of antigen-specific CD8 T cells following acute and chronic infections in WT hosts have shown that contraction is similar between the two types of infections [15, 16, 40], leading to the conclusion that contraction is “programmed” early after activation. It has also been shown Bim regulates contraction of antigen-specific CD8 T cells following acute *L. monocytogenes* and LCMV Armstrong or chronic LCMV clone 13 infection [3, 4]. However, the failure to contract in the absence of Bim clearly occurs without the sustained proliferation we observe in a prolonged *L. monocytogenes* infection [5]. This suggests that perforin- and Bim-dependent contraction act at fundamentally different levels. Collectively, the preceding data provided no reason to believe the mechanisms regulating contraction of antigen-specific CD8 T cells following acute or chronic infections would be different, supporting the notion that contraction in the CD8 T cell compartment was programmed by early events after infection. However, our new results suggest that the mechanisms regulating antigen-specific T cell contraction after acute and prolonged infection are differentially dependent on perforin.

Maintenance of memory CD8 T cells after acute infection depends on cytokines such as IL-7 and IL-15 that drive basal proliferation and confer long-term antigen independent survival to these populations [42]. In contrast, recent data reveal that maintenance of memory CD8 T cells during chronic LCMV clone 13 infection is antigen-dependent [43]. Memory antigen-specific CD8 T cells following a chronic infection respond poorly to IL-7 and IL-15, have reduced expression of the IL-7 and IL-15 receptors, and fail to undergo cytokine-dependent basal proliferation and thus do not survive in the absence of antigen. Instead memory CD8 T cells in chronically infected mice are dependent on antigen to maintain their memory cell numbers [44]. Additionally, memory CD8 T cells generated during a chronic infection exhibit sustained proliferation during the memory phase that is much faster than the cytokine-driven basal turnover of memory CD8 T cells formed after an acute infection [44]. Our results show that antigen-specific CD8 T cells exhibit sustained proliferation throughout the normal contraction interval during prolonged infections. These data suggest that antigen-dependency of memory CD8 T cells formed during chronic infection may be selected for during the contraction phase. Interestingly, the IL-7 receptor begins to be upregulated on some antigen-specific CD8 T cells (often called memory precursors) during the contraction phase after an acute infection, thus providing an important receptor for transitioning memory CD8 T cells to antigen-independent maintenance [8, 45]. However, continuous stimulation through the TCR during chronic infections may result in a population of memory precursor CD8 T cells that have reduced IL-7 receptor expression.

This may result in memory populations that instead have become dependent on continued antigen-stimulation for their maintenance. However, the memory populations in LM-infected DKO mice that eventually clear LM exhibit a normal rate of basal proliferation, suggesting the intriguing notion that sustained proliferation during the normal contraction phase does not invariably result in a memory population that is antigen-dependent. Perhaps long-term antigen persistence during chronic infection is required to elicit the inhibitory receptor changes associated with persistence of an antigen-dependent memory population in the absence of overt mortality [31].

Following an acute infection pathogens are eliminated and antigen is cleared. On the other hand, the immune system is faced with a different situation during prolonged infections where antigen persists during the contraction phase and is capable of driving sustained T cell proliferation and immunopathology. In this latter situation perforin has been utilized by the immune system to ensure continuously stimulated antigen-specific T cells are eliminated, thus allowing the host time to impose functional constraints on T cell responses that permit host survival during chronic infection.

Materials and methods

Mice

C57BL/6J mice and C57BL/6J-perforin-deficient (PKO) mice were obtained from the National Cancer Institute (Frederick, MD) and The Jackson Laboratory (Bar Harbor, ME) respectively. TNF-deficient mice were originally obtained from a 129/SVxB6 background from George Kollias [20, 46] and were backcrossed to C57BL/6J mice >12 generations to generate C57BL/6J-TNF-deficient mice (TKO). TNF/perforin-double deficient (DKO) mice were generated by crossing C57BL/6J-TNF-deficient mice to C57BL/6J-perforin-deficient mice. DKO, TKO, and PKO mice were maintained by brother-sister mating and housed under SPF conditions at the University of Iowa (Iowa City, IA) animal care unit until the initiation of experiments with LM at which point the mice were transferred to housing at the appropriate biosafety level. Animal experiments were approved by the Institution Animal Care and Use Committee.

Bacteria Infections

Listeria monocytogenes expressing ovalbumin (virulent LM-OVA) was a gift from Hao Shen and Leo Lefrancois [21]. An attenuated version of this strain was generated by inducing an in-frame deletion in the *actA* gene as previously described [47] (referred to as *actA*-deficient LM-OVA). We also used an *actA*-deficient strain of *L. monocytogenes* that does not express ovalbumin, DPL-1942 [48] (received from Dan Portnoy) (referred to as ActA- LM). Mice were infected through the tail vein with 5×10^6 *actA*-deficient LM-OVA or ActA- LM.

Detection of tissue bacteria

Spleens and livers were collected from mice at the indicated days post infection with *actA*-deficient LM-OVA. Numbers of CFU per spleen were determined by homogenizing spleens or livers. Tissue homogenates were serially diluted in 0.2% IGEPAL (Sigma Aldrich, St. Louis, MO) and plated on tryptic soy agar (TSA) supplemented with 50 µg/ml streptomycin for 18–24 hours at 37°C. Colonies were counted and CFU/tissue was calculated.

Quantification and phenotypic analysis of antigen-specific T cells

Mice were perfused with cold PBS. Lungs were digested with collagenase II (100 units/ml) + DNaseI (1 µg/ml) for 60 minutes at 37°C. Spleens, livers and lungs were disrupted into single cell suspensions. Liver and lung mononuclear cells were collected by spinning cells in

35% Percoll/HBSS. Spleen, liver, and lung cells were treated with Tris-ammonium chloride to lyse RBC. PBL were obtained by treating blood with Tris-ammonium chloride to lyse RBC. Tissues were harvested at the indicated day post *actA*-deficient LM-OVA infection. Total tissue antigen-specific T cells were determined by intracellular cytokine staining (ICS) for IFN- γ after 5 hrs of incubation in brefeldin A (Biolegend, San Diego, CA), in the presence or absence of 200 nM OVA_{257–264} or 5 μ M LLO_{190–201}. For MHC I tetramer staining, cells were incubated 45 minutes at 4°C with MHC I tetramers loaded with OVA_{257–264} followed by anti-CD8 mAb for 20 min at 4°C then fixed and permeabilized with Cytofix/CytoPerm solution (BD Pharmingen, San Diego, CA). For phenotypic analysis after incubation cells were surface stained with the indicated antibodies then fixed and permeabilized with BD Cytofix/CytoPerm™ (BD Biosciences, San Diego, CA). Intracellular staining for IFN- γ , IL-2, and granzyme B were done in the presence of BD Perm/Wash™ (BD Biosciences). Cell surface expression of CD62L was detected by incubating cells with 0.1 mM TAPI-2 (Peptides International Inc., Louisville, KY) for 30 minutes prior to and during stimulation with peptides.

OT-I TCR Tg CD8 T cell detection of OVA-antigen

Thy1.1 OT-I TCR Tg CD8 T cells were enriched using a negative selection Mouse CD8+ T Cell Enrichment Kit (StemCell Technologies Inc., Vancouver, British Columbia). Enriched OT-I TCR Tg CD8 T cells were labeled with 1 μ M CFSE prior to i.v. injection (0.6–2 $\times$ 10⁶ cells/mouse) into naïve or infected Thy1.2 mice. CFSE labeled OT-I TCR Tg CD8 T cells were injected on day 11 p.i. CFSE fluorescence was analyzed by gating on Thy1.1+/V α 2+ CD8 T cells three days after injection (day 14).

Ampicillin and BrdU treatment

Mice were treated with ampicillin (2 mg/ml) given in drinking water beginning on day 4 post-infection and ending on day 7 post-infection. BrdU treatment began at various days post-infection with a 0.2 ml injection (i.p.) of 10 mg/ml BrdU (BD Pharmingen) resuspended in PBS. Mice were then provided drinking water with 0.8 mg/ml BrdU (Sigma Aldrich), which was changed daily, until the indicated day of analysis. Incorporation of BrdU into the DNA of dividing cells was done according to manufacture's recommendations (BD Pharmingen).

Antibodies

The following antibodies were used from eBioscience (San Diego, CA) CD8 (53-6.7), CD4 (RM4-5), Thy1.1(CD90.1) (HIS51), LAG-3-PE (eBioC9B7W), and rat IgG1-PE. The following antibodies were used from BD Pharmingen, IFN- γ (XMG1.2), CD62L-PE (MEL-14), PD-1-PE (J43), and Syrian Hamster IgG2-PE. The following antibodies were used from Biolegend (San Diego, CA) IgG2A-PE (RTK2758), CD127-PE (SB/199), and IL-2-PE (JES6-5H4). The following antibodies were used from Invitrogen (Carlsbad, CA) mouse anti-human granzyme B-PE and mouse IgG-PE isotype control.

Perforin Quantitative RT-PCR

C57BL/6 mice were infected with 5 $\times$ 10⁶ *actA*-deficient LM-OVA. Five days p.i. splenocytes were depleted of CD19+ and CD8+ cells using anti-PE magnetic beads (Miltenyi Biotec). The CD4+ enriched cell suspensions were then sort purified into activated CD4+/CD11a^{hi}/CD49d^{hi} and naïve CD4+/CD11a^{lo}/CD49d^{lo} T cell populations [37] (Butler N, et al., *Nature Immunology*, In Press) using a FACSDiva (BD Pharmingen). Total RNA was isolated from activated and naïve CD4 T cells with RNAeasy Kit (Qiagen). cDNA was generated and relative quantitative (2^{- $\Delta\Delta$ CT}) RT-PCR was performed using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) in triplicate on an Applied Biosystems

Model 7000 using the manufacturers recommended protocols. Primers; perforin FWD, 5'-GAGAAGACCTATCAGGACCA -3'; perforin REV, 5'- AGCCTGTGGTAAGCATG -3' (as previously mentioned [49]); HPRT FWD, 5'- CCTCATGGACTGATTATGGACA -3'; HPRT REV, 5'- TATGTCCCCCGTTGACTGAT -3'.

Statistical Analysis

Data were analyzed as indicated using Prism 4.0b software.

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Abbreviations

LM-OVA	<i>actA</i> -deficient <i>L. monocytogenes</i> expressing ovalbumin
OVA	chicken ovalbumin
LLO	listeria lysine O
WT	wild-type
DKO	TNF/perforin-double deficient
TKO	TNF-deficient
PKO	perforin-deficient
Amp	ampicillin
AICD	activation-induced cell death
ICS	intracellular cytokine staining
Tg	transgenic

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Figure 1.

Aberrant contraction of OVA-specific CD8 T cells in mice lacking perforin and TNF. WT and DKO mice were infected with *actA*-deficient LM-OVA at day 0. (A) Frequency of spleen CD8 T cells that are OVA-specific as detected by peptide stimulated ICS for IFN- γ at the indicated days after infection. Representative contour plots are shown. (B) Total number of OVA-specific CD8 T cells in the spleen. Data (mean $\pm$ S.E.M.) are from 10 independent experiments with 3–27 mice per time point per genotype. (C) Percent survival (mean $\pm$ S.E.M.) of OVA-specific CD8 T cells on day 41 or day 440 p.i. relative to day 7 of the respective genotype. Frequency or total number of OVA-specific CD8 T cells in the blood (D), spleen (E), liver (F), and lung (G) as detected by staining with K^b-OVA_{257–264} tetramers. (D–G) Data (mean $\pm$ S.D.) are from 3 mice per time point per genotype. (H) Percent survival (mean $\pm$ S.D.) of K^b-OVA-specific CD8 T cells (from D–G) on day 14 p.i. relative to day 7 of the respective genotype. All data were analyzed by unpaired two-tailed t Test.

Figure 2.

Aberrant contraction of LLO-specific CD4 T cells in mice lacking perforin and TNF. WT and DKO mice were infected with *actA*-deficient LM-OVA at day 0. (A) Frequency of spleen CD4 T cells that are LLO-specific as detected by peptide stimulated ICS for IFN- γ at the indicated days after infection. Representative contour plots are shown. (B) Total number of LLO-specific CD4 T cells in the spleen. Data (mean $\pm$ S.E.M.) are from 10 independent experiments with 3–27 mice per time point per genotype. (C) Percent survival (mean $\pm$ S.E.M.) of LLO-specific CD4 T cells on day 41 or day 440 p.i. relative to day 7 of the respective genotype. All data were analyzed by unpaired two-tailed t Test.

Figure 3.

Absence of perforin and TNF results in a prolonged LM-OVA infection and antigen display. (A–B) LM-OVA burden was detected in the spleen on day 8 (A), and day 11 (B) post-infection. (C) WT and DKO mice were untreated or infected with either *actA*-deficient LM or *actA*-deficient LM-OVA. Presentation of OVA_{257–264} was detected by injection of CFSE labeled OT-I TCR Tg CD8 T cells on day 11. CFSE dilution in OT-I TCR Tg CD8 T cells was analyzed by gating on Thy1.1⁺/Va2⁺ CD8 T cells three days after injection (day 14). Data are representative of two independent experiments.

Figure 4.

Aberrant antigen-specific T cell contraction in perforin/TNF-deficient mice is a result of the prolonged infection. WT and DKO mice were infected with *actA*-deficient LM-OVA and left untreated or treated with ampicillin from day 4 to day 7 p.i. Total number of OVA-specific CD8 T cells (A) and LLO-specific CD4 T cells (C) in the spleen as detected by ICS for IFN- γ . Data (mean $\pm$ S.D.) are from 3 mice per genotype per time point. Percent survival (mean $\pm$ S.D.) of OVA-specific CD8 T cells (B) and LLO-specific CD4 T cells (D) on day 42 p.i. relative to day 7 of the respective genotype. All data are representative of two independent experiments. Data were analyzed by unpaired two-tailed t Test.

Figure 5.

Antigen-specific T cells exhibit sustained proliferation through the contraction phase in the absence of perforin and TNF. WT and DKO mice were infected with *act4*-deficient LM-OVA and pulsed with BrdU from day 4–7, 8–11, 11–14, 14–17, or 34–41. BrdU incorporation was analyzed in OVA-specific CD8 T cells (A–C) and LLO-specific CD4 T cells (D–F) on the final day of the indicated pulse. (A and D) Representative histograms are shown. Open histograms are BrdU staining and filled histograms are isotype controls. Percent of OVA-specific CD8 T cells (B) or LLO-specific CD4 T cells (E) that were BrdU positive following the indicated BrdU pulse. (C and F) are the same as (B and E) respectively) only mice were treated with ampicillin day 4–7 p.i. Data (mean $\pm$ S.D.) in B–C

and E–F are from 3 mice per group per timepoint and are representative of two independent experiments. Data were analyzed by unpaired two-tailed t Test comparing DKO group to WT group at the respective time point.

Figure 6.

Antigen-specific T cells in DKO mice exhibit an effector-like phenotype. WT and DKO mice were infected with *act4*-deficient LM-OVA. (A) Representative histograms showing the expression of CD62L, CD127, and IL-2 on OVA-specific CD8 T cells. Open histograms are the indicated marker and filled histograms are isotype controls. Number within each histogram is the fraction of OVA-specific CD8 T cells that are marker positive. Data (mean $\pm$ S.E.M.) are from 6 mice per time point per genotype from two experiments on OVA-specific CD8 T cells (B) and LLO-specific CD4 T cells (C). (D) Representative histograms showing the expression of PD-1 and LAG-3 on OVA-specific CD8 T cells. Open histograms are the indicated marker and filled histograms are isotype controls. Number within each histogram is the fraction of OVA-specific CD8 T cells that are marker positive. (E) Data (mean $\pm$ S.D.) are from 3 mice per time point per genotype on OVA-specific CD8 T cells. Data were analyzed by unpaired two-tailed t Test comparing DKO group to WT group at the respective time points.

Figure 7.

Perforin mediates contraction of OVA-specific CD8 T cells and LLO-specific CD4 T cells during a prolonged *actA*-deficient LM-OVA infection. Total number of OVA-specific CD8 T cells (A) and LLO-specific CD4 T cells (C) in the spleen as detected by ICS for IFN- γ . Data (mean $\pm$ S.E.M.) are from three experiments with 8–9 mice per timepoint per genotype. Percent survival (mean $\pm$ S.E.M.) of OVA-specific CD8 T cells (B) and LLO-specific CD4 T cells (D) on day 41 p.i. relative to day 7 of the respective genotype. (E) LM-OVA burden in the liver on day 3 p.i. (F–I) Mice were pulsed with BrdU from day 11–14 and the percent of OVA-specific CD8 T cells (F–G) and LLO-specific CD4 T cells (H–I) that incorporated BrdU on day 14 p.i. are shown. Data (mean $\pm$ S.D.) are from 3–6 mice per genotype and representative of two independent experiments. (G and I) Same as (F and H) only mice were treated with ampicillin day 4–7 p.i. (J) OVA_{257–264} antigen-display was detected by injection of CFSE labeled OT-I TCR Tg CD8 T cells. CFSE dilution in OT-I TCR Tg CD8 T cells as a measure of proliferation was analyzed by gating on Thy1.1+/Va2+ CD8 T cells

three days after injection. Representative histograms (3 mice/genotype/condition) are shown. Data were analyzed by One-way ANOVA followed by Tukey's post test analysis.

Figure 8.

CD4 T cells possess the cytolytic machinery necessary for regulating T cell contraction during prolonged *L. monocytogenes* infections. Mice were infected with 5×10^6 actA-deficient LM-OVA. (A) Relative expression of perforin RNA in cells from a spleen of an uninfected mouse, naïve CD4⁺/CD11a^{lo}/CD49d^{lo} T cells or *Listeria*-specific CD4⁺/CD11a^{hi}/CD49d^{hi} T cells [37] (Butler N, et al., *Nature Immunology*, In Press) sort purified five days after infection. Data are representative of two separate experiments. Granzyme B (GrB) expression in OVA-specific CD8 T cells (B) and LLO-specific CD4 T cells (C) following stimulation with OVA_{257–264} and LLO_{190–201}, respectively, five days post LM-OVA infection. Representative histograms show GrB expression (open histogram) and

isotype control (shaded histogram). Number within each histogram is the fraction of OVA-specific CD8 T cells or LLO-specific CD4 T cells that are GrB positive. Bar graph data (mean $\pm$ S.E.M.) are from 6 mice per time point from two experiments.