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# Elucidating Sources and Roles of Granzymes A and B during Bacterial Infection and Sepsis

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## SUMMARY

**During bacterial sepsis, proinflammatory cytokines contribute to multiorgan failure and death in a process regulated in part by cytolytic cell granzymes. When challenged with a sublethal dose of the identified mouse pathogen *Brucella microti*, wild-type (WT) and granzyme A (gzmA)$^{-/-}$ mice eliminate the organism from liver and spleen in 2 or 3 weeks, whereas the bacteria persist in mice lacking perforin or granzyme B as well as in mice depleted of Tc cells. In comparison, after a fatal challenge, only gzmA$^{-/-}$ mice exhibit increased survival, which correlated with reduced proinflammatory cytokines. Depletion of natural killer (NK) cells protects WT mice from sepsis without influencing bacterial clearance and the transfer of WT, but not gzmA$^{-/-}$ NK, cells into gzmA$^{-/-}$ recipients restores the susceptibility to sepsis. Therefore, infection-related pathology, but not bacterial clearance, appears to require gzmA, suggesting the protease may be a therapeutic target for the prevention of bacterial sepsis without affecting immune control of the pathogen.**

## INTRODUCTION

Cytotoxic CD8$^+$ T (Tc) cells and natural killer (NK) cells use death ligands, granule exocytosis, and soluble proinflammatory cytokines to combat intracellular pathogens and cell transformation (Biron, 1994; Russell and Ley, 2002). Conventionally, granule exocytosis and death ligands exert this function by inducing target cell death; meanwhile, interferon γ (IFNγ) and tumor necrosis factor alpha (TNFα), among other cytokines, participate in the development of beneficial inflammatory responses by modulating the activity of macrophages and dendritic cells. However, if these pathways are not properly regulated, autoimmune and inflammatory disorders may develop.

Granule exocytosis may be described as a specialized form of intracellular protein delivery. Here, a pore-forming protein perforin (perf) aids the translocation of a family of serine proteases (granzymes [gzms]) into the cytosol of target cell (Voskoboinik et al., 2006). The gzms are ultimately responsible for target cell death by activating various mechanisms that execute the offending cell. Among the described proteinases (five in humans and ten in mice), gzmA and gzmB are the most abundant and thoroughly characterized (Bovenschen and Kummer, 2010; Chowdhury and Lieberman, 2008; Grossman et al., 2003). Whereas GzmB is clearly cytotoxic (Bleackley, 2005; Trapani and Sutton, 2003), the supposition that gzmA only induces cell death is controversial (Lieberman, 2010; Metkar et al., 2008). Gzms have been shown to inactivate viral particles, degrade extracellular matrix, and induce adherent cells to manufacture proinflammatory cytokines (Anthony et al., 2010a; Froelich et al., 2009; Hendel et al., 2010; Pardo et al., 2009; Romero and Andrade, 2008). Specifically, gzmA, gzmK, and gzmM in vitro may promote the release of proinflammatory cytokines from macrophages and gzmB may enhance the proinflammatory activity of interleukin 1α (IL1α) (Afonina et al., 2011). More compellingly, gzmA- or gzmM-deficient mice resist lipopolysaccharide (LPS)-induced septic shock (Anthony et al., 2010a; Metkar et al.,

2008), suggesting that gzmA and gzmM may contribute to the pathogenesis of bacterial sepsis.

Bacterial sepsis is presumed to be caused by uncontrolled production of proinflammatory cytokines (IL6, TNFα, and IL1β) secreted by macrophages (Angus and van der Poll, 2013). This outcome depends upon a process where macrophages are overactivated by recognition of bacterial pathogen-associated molecular patterns (PAMPs). They are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors in both plants and animals. Bacterial LPS, an endotoxin found on the bacterial cell membrane, is a prototypical PAMP (Van Amersfoort et al., 2003). Endotoxin is a potent mediator of gram-negative bacteria-induced sepsis, but other bacterial components also contribute to the inflammatory response (e.g., peptidoglycan; Van Amersfoort et al., 2003). Although macrophages are critical promoters of septic shock, other cell types likely contribute to this pathological outcome including Tc and NK cells (Sherwood et al., 2003). Several studies have shown a positive correlation between the severity of sepsis and the expression of gzms A and B in lymphocytes (Schaer et al., 2006; Zeerleder et al., 2005). Whether the contribution of perf and gzms to the pathology observed during sepsis is due to the induction of cell death or to proinflammatory effects is unknown (Hashimoto et al., 2000; Wesche-Soldato et al., 2007).

Unfortunately, the animal model of sepsis induced by LPS does not fully recapitulate the many facets of the septic shock response (Rittirsch et al., 2007). There is a need for animal models where the clinical signs of sepsis develop after exposure to endogenous bacterial pathogens. To test whether Tc/NK-cell-derived gzms control a replicating pathogen as well as paradoxically contribute to sepsis, we have used *Brucella microti*, a newly described mouse pathogen. *Brucella microti*, a member of the *Brucella genus* isolated from wild rodents, is an intracellular facultative pathogen that replicates primarily in macrophages and, in contrast to other *Brucella spp*, produces systemic bacteremia and fatal sepsis (Jiménez de Bagüés et al., 2010, 2011).

## RESULTS

### Role of Cytolysis in the Control of *Brucella microti* Infection

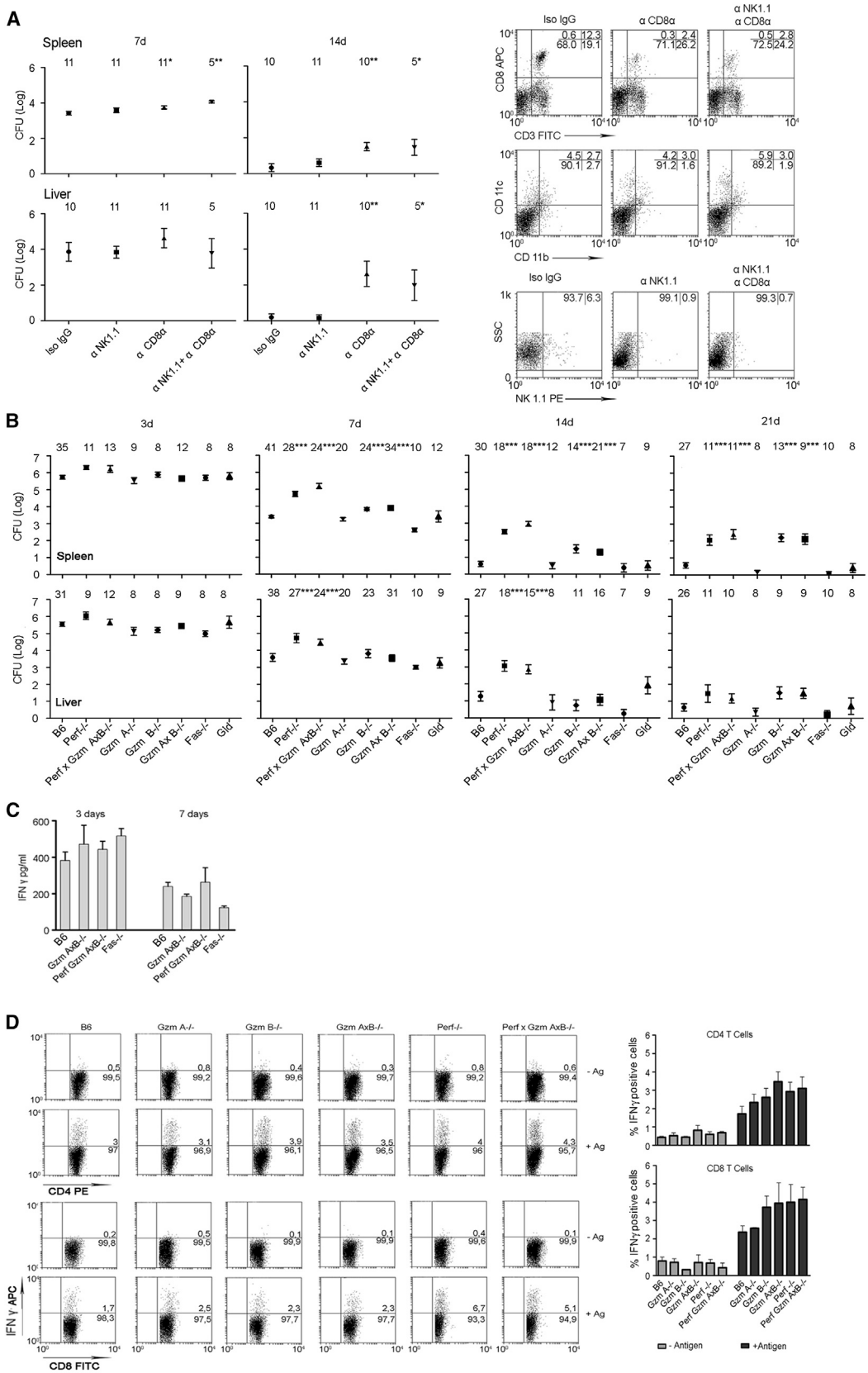
Overall, we asked whether alteration in the levels of cytolytic components might curtail inflammation during sepsis without affecting control of the bacterial infection and, thus, could constitute a selective therapeutic approach. In contrast to *Brucella spp*, which are nontoxic for mice, inoculating wild-type (WT) C57Bl6 (B6) mice with $10^6$ colony-forming units (cfu) of *Brucella microti* causes sepsis within 1 week (Alcaraz et al., 2010, Am. Coll. Vet. Pathol., conference; Jiménez de Bagüés et al., 2010). Thus, we applied a biological tool to study the control of bacterial sepsis in a mouse model. *Brucella spp* infect the host by varied routes including inhalation, inoculation through the conjunctival sac, ingestion of contaminated food, or through breaks in the skin. We chose intraperitoneal (i.p.) inoculation because this form of infection mimics entry through skin cuts (Turner et al., 2011).

To determine the contribution of $CD8^+$ T cells and NK cells in the control of infection, we performed in vivo immunodepletion experiments with anti-CD8α and anti-NK1.1 in mice inoculated with a sublethal dose ($10^5$ cfu) of bacteria. Only the depletion of $CD8^+$ T cells increased the splenic bacterial load at 7 and 14 days and in liver by 14 days (Figure 1A) whereas most WT animals had cleared the bacteria. The efficacy of the depletion protocol was verified by fluorescence-activated cell sorting (FACS) analysis of splenic NK, $CD8^+$ T cells, macrophages ($CD11b^+/CD11c^-$), and dendritic cells ($CD11c^+$; Figure 1A, right panel). Next, we examined the cytolytic pathways of Tc cells that might control infection in vivo by inoculating WT and various knockout (KO) mice with a sublethal cfu of *B. microti* and monitoring bacteria replication in spleen and liver. Compared to WT mice, bacterial clearance from the spleen (Figure 1B) was delayed in $perf^{-/-}$, $perfxgzmAxB^{-/-}$, $gzmB^{-/-}$, and $gzmAxB^{-/-}$ and was similar to $gzmA^{-/-}$, $Fas^{-/-}$, and gld mice. The time of maximum bacterial load (day 3) was similar among the animal groups. However, differences between WT and $perf^{-/-}$, $perfxgzmAxB^{-/-}$, $gzmB^{-/-}$, and $gzmAxB^{-/-}$ groups were noted at 7 days, the time when the WT animals began to clear infection. These differences then became more pronounced after 14 and 21 days. In the liver, the results were less dramatic; here, only $perf^{-/-}$ and $perfxgzmAxB^{-/-}$ mice showed higher cfu than WT mice. Finally, we found that the level of IFNγ in serum was similar for WT, $perfxgzmAxB^{-/-}$, $gzmAxB^{-/-}$, and $Fas^{-/-}$ mice after 3 and 7 days (Figure 1C), suggesting that defects in $perfxgzmAxB^{-/-}$ and $gzmAxB^{-/-}$ mice were not due to the failure of these animals to respond to *B. microti* inoculation. Further supporting this possibility, isolated $CD4^+$ and $CD8^+$ T cells from WT and KO mice produced similar levels of IFNγ after restimulation with *Brucella* antigens in vitro (Figure 1D).

### Tc Cells Are Activated during *B. microti* Infection In Vivo whereas perf and gzmB Are Necessary to Inhibit *B. microti* Replication in Primary Macrophages

The in vivo experiments indicate that $CD8^+$ T cells and their cytolytic mediators, perf and gzmB, participate in the control of *B. microti* infection. Tc cells from mice infected with *B. microti* expressed gzmA, gzmB, and gzmK, indicating that these cells were activated during infection in vivo (Figure S1). Expression of the granzymes was accompanied by an increase in the ability of the ex vivo WT Tc cells to inhibit *B. microti* replication in bone-marrow-derived macrophages (BMDM) (Figure S2A). Tc cells from noninfected mice were unable to inhibit *B. microti* replication, indicating that the observed effects were antigen specific (Figure S2B). We then compared the ability of Tc cells from WT, $perfxgzmAxB^{-/-}$, $gzmA^{-/-}$, $gzmB^{-/-}$, $gzmAxB^{-/-}$, and gld mice to control *B. microti* replication in BMDMs. Bacterial growth was less reduced for Tc cells lacking perf or gzmB compared to WT or gzmA-deficient Tc cells (Figure 2A).

To directly test in our model that Tc control *B. microti* growth, we presumed that Tc cells containing perf and gzmB would reduce bacterial proliferation by eliminating infected BMDMs. Thus, in parallel experiments described for Figure 2A, we tested whether *B.-microti*-infected macrophages were killed by Tc cells containing perf and gzmB. Whereas *B. microti* was not toxic (Figure 2B; medium), ex vivo Tc cells isolated from WT and $gzmA^{-/-}$ *B.-microti*-infected mice induced a typical apoptotic phenotype characterized by phosphatidylserine translocation (annexin V staining) without membrane permeabilization (7AAD staining).

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The levels of apoptosis induced by WT and gzmA$^{-/-}$ Tc cells were significantly higher than observed for gzmB$^{-/-}$, gzmAxB$^{-/-}$, and perfxgzmAxB$^{-/-}$ Tc cells, indicating that perf and gzmB, but not gzmA, were primarily responsible for the destruction of BMDM infected with *B. microti*. Notably, Tc cell activity appears specific to *B. microti* because WT Tc cells from uninfected mice did not induce apoptosis (Figure 2B). The levels of apoptosis induced by Brucella-specific Tc cells against infected BMDMs was similar to lymphocytic choriomeningitis virus (LCMV)-specific Tc cells against noninfected, gp33-pulsed BMDMs, indicating a high level of Tc cell activation during *B. microti* infection.

Supporting a role for macrophage apoptosis in the control of bacterial replication, we found that the number of bacteria in macrophages treated with the protein kinase C inhibitor staurosporine was significantly lower than the untreated controls after 24 hr (Figure S3A). This outcome was observed for cells incubated with staurosporine for 24 hr, although apoptosis was evident after 7 hr (Figure S3C). Because *B. microti* starts to replicate in BMDM after 7 hr (data not shown), this finding suggests that apoptosis per se does not kill the bacteria but inhibits replication in the cells undergoing apoptosis. Supporting this possibility, if these macrophages (infected for 7 hr in vitro) acted as the source of *B. microti* for infection in vivo, a significantly lower level of infection was observed compared to viable infected BMDM (Figure S3B). Altogether, these experiments suggest that perf and gzmB indeed control *B. microti* replication by inducing apoptosis in cells that harbor the pathogen.

## GzmA Deficiency Enhances Mouse Survival after *Brucella microti* Infection and Reduces Disseminated Intravascular Coagulation and the Levels of Proinflammatory Cytokines In Vivo

GzmA does not appear necessary to eradicate *B. microti*. We therefore asked whether the protease might contribute to sepsis in mice challenged with a lethal dose of the organism (Alcaraz et al., 2010, Am. Coll. Vet. Pathol., conference; Jiménez de Bagüés et al., 2010) by assessing survival, bacteremia, disseminated intravascular coagulation (DIC), and the level of proinflammatory cytokines. WT, gzmA$^{-/-}$, gzmB$^{-/-}$, perf$^{-/-}$, and perfxgzmAxB$^{-/-}$ mice were inoculated with $10^6$ cfu of *B. microti*, and survival was monitored for 21 days. Approximately 60% of WT, gzmB$^{-/-}$, perf$^{-/-}$, and perfxgzmAxB$^{-/-}$ mice died during the first 10 days (Figure 3A). In contrast, gzmA-deficient animals were spared (20% died). Remarkably, if mice received a highly lethal dose ($10^7$ cfu), all WT animals died in 7 days, whereas 20% of gzmA$^{-/-}$ mice survived (data not shown). These data indicate that inflammation induced by gzmA contributes to septic response induced by *B. microti* infection and provides biologically relevant confirmation that gzmA deficiency protects mice from LPS-induced septic shock (Anthony et al., 2010b; Metkar et al., 2008). We cannot conclude if this effect depends on the presence of perf. Here, both perf$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice were just as susceptible as WT mice to sepsis whereas bacteremia was significantly higher in perfxgzmAxB$^{-/-}$ than gzmA$^{-/-}$ mice (Figure S4). Emphasizing that the lethal effect of *Brucella* was TLR-dependent, TLR4$^{-/-}$ mice survival was significantly greater than for WT animals (Figure 3).

During sepsis, bacteria circulate throughout the vasculature, disseminating in multiple organs. Accordingly, *B. microti* was identified in blood, liver, spleen, brain, kidney, and lung 3 days after receiving a lethal dose inoculation (Figure 3B). Again, no difference was observed between WT and gzmA$^{-/-}$ mice.

DIC is a catastrophic complication of sepsis. Accordingly, the improved survival of gzmA-deficient mice was associated with a reduction in DIC (Figure 3C). After inoculation with $10^6$ cfu of *B. microti*, gzmA$^{-/-}$ mice had lower prothrombin times and higher platelet counts than WT mice. The level of serum alanine aminotransferase (ALT) was also lower in gzmA$^{-/-}$ mice, although the values did not reach statistical significance.

Finally, to verify that protection was partially due to reduced serum level of proinflammatory cytokines, we monitored the concentrations of IL1$\alpha$, IL1$\beta$, TNF$\alpha$, IL6, IFN$\gamma$, and MIP1$\alpha$ in WT and gzmA$^{-/-}$ mice during the 72 hr after lethal bacterial inoculation. Compared to levels observed in mice receiving a nonlethal dose ($10^5$ cfu), mice that received $10^6$ cfu had significantly higher level of cytokines (Table S1). When cytokine levels were measured in animals infected with $10^6$ cfu, the concentration of all cytokines except IFN$\gamma$ was significantly lower in gzmA$^{-/-}$ than in WT animals (Figure 3D).

## Mice Depleted of NK Cells Survive *B. microti* Infection, and the Transfer of WT NK Cells into gzmA$^{-/-}$ Mice Restores Susceptibility to Sepsis

We then determined the cell type that might be the source of gzmA during sepsis induced by *B. microti* infection. Because animals die after 3 days, we hypothesized that inflammation

**Figure 1. Role of CD8+ T and NK Cells and Their Cytolytic Mediators in the Control of *B. microti* Infection in Spleen and Liver**

(A) NK and/or CD8 T$^+$ cells in WT B6 mice were depleted using anti-NK1.1 and/or anti-CD8$\alpha$ monoclonal antibody (mAb). Subsequently, mice were infected i.p. with *B. microti* ($10^5$ cfu) and the number of cfu in liver and spleen at 7 and 14 days was quantified. Data are mean ± SEM of the indicated number of biological replicates performed in two independent experiments; *p < 0.05; **p < 0.01; ***p < 0.001, comparing each group with the control group (Iso IgG). In parallel (right panels), splenocytes were isolated from three animals of each group, stained with CD3/CD8, CD11b/CD11c, and NK1.1 mAbs and analyzed by flow cytometry. Numbers correspond to the percentage of cells in each quadrant.

(B) Mice were infected i.p. with *B. microti* ($10^5$ cfu), and the number of cfu in liver and spleen was determined 3, 7, 14, or 21 days later. Data are presented as mean ± SEM of the indicated number of biological replicates performed in four independent experiments; *p < 0.05; **p < 0.01; ***p < 0.001, comparing each group with the control group (B6).

(C) The level of IFN$\gamma$ was quantified in serum from indicated animals 3 and 7 days after infection. Data are presented as mean ± SEM from four biological replicates performed by duplicates.

(D) Ex vivo production of IFN$\gamma$ by CD4 and CD8 T cells was measured in splenocytes from mice 7 days after infection as indicated in materials and methods. Data in bar graphs on the right represent the mean ± SEM from two independent experiments combining duplicate samples. APC, antigen-presenting cell; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

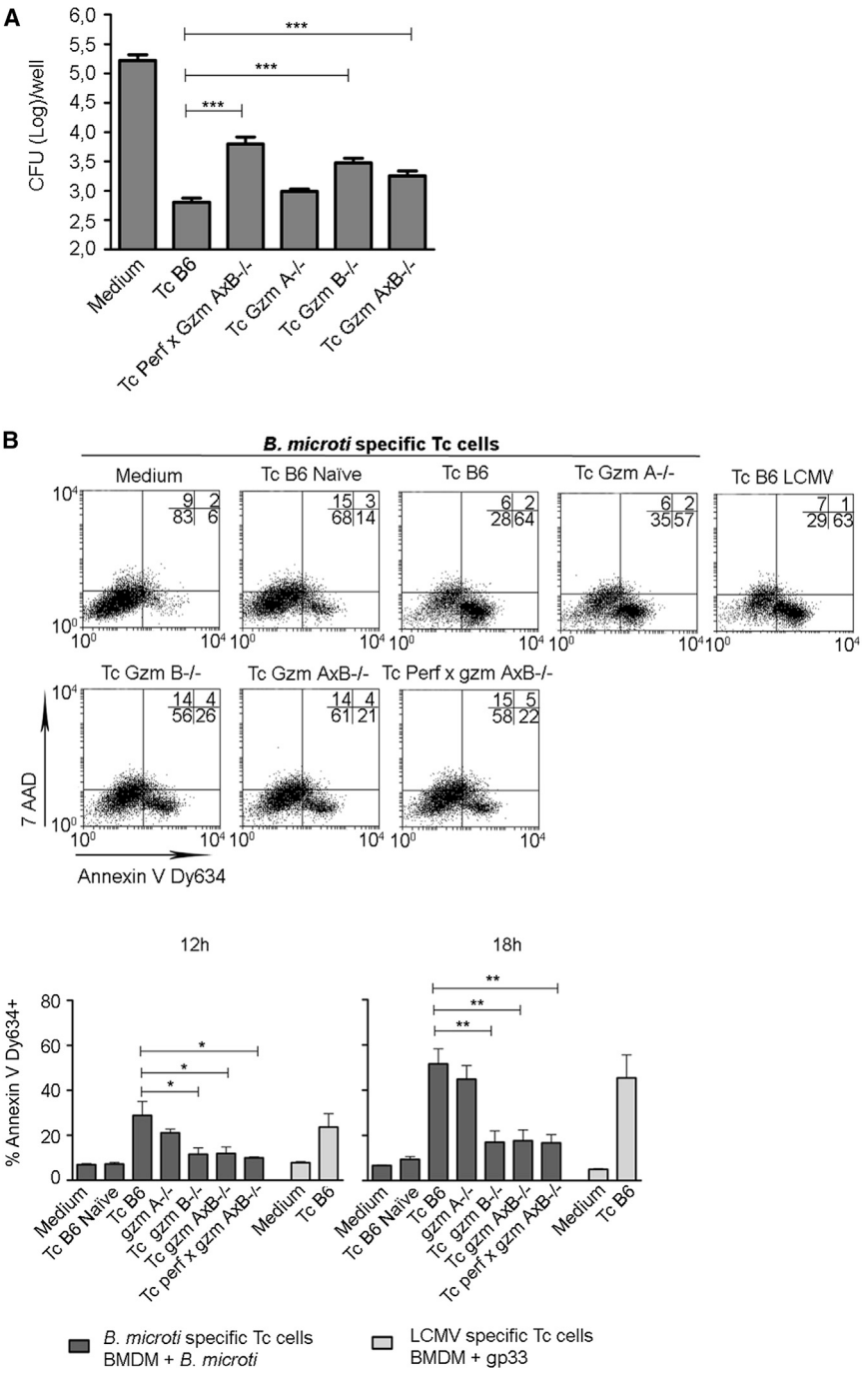


**Figure 2. Ex Vivo *B. microti*-Specific Tc Cells Use perf and gzmB to Control Bacterial Replication and Induce Apoptosis in BMDMs In Vitro**

B6, gzmA$^{-/-}$, gzmB$^{-/-}$, gzmAxB$^{-/-}$, and perfxgzmAxB$^{-/-}$ mice were infected with *B. microti* i.p. ($10^5$ cfu). CD8$^+$ cells were enriched from spleen using MACS 7 days afterward and incubated with BMDM from WT B6 mice infected with *B. microti*. After 24 hr, cells were lysed and the number of cfu was determined (A). In parallel, the percent apoptotic macrophages (annexin V/7AAD staining) was quantified (B). As a control, Tc cells from LCMV-infected B6 WT mice were incubated with BMDMs labeled with the immunodominant LCMV peptide gp33. A representative experiment is shown as dot plots. Numbers show the cell percentage in each quadrant. Data are presented as mean ± SEM from three independent experiments where samples were measured in triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, comparing each group with the group treated with B6 (WT) Tc cells.

induced by gzmA would be executed by NK cells from the innate arm of the immune system. We analyzed the survival of mice in which NK cells were depleted after inoculation with a lethal dose. After depletion of NK cells, 78% of mice survived bacterial sepsis compared to 27% of mice treated with control immunoglobulin G (IgG) (Figure 4A). However, NK cell depletion did not alter bacterial clearance in spleens of surviving mice (Figure 4B). In addition, NK cells isolated from mice infected with a lethal dose of *B. microti* expressed gzmA (Figure 4C).

To confirm that NK cells are indeed the source of gzmA during sepsis induced by *B. microti*, we performed transfer experiments in which magnetic-activated cell sorting (MACS)-enriched splenic NK cells from noninfected WT or gzmA$^{-/-}$ mice were isolated and transferred to gzmA$^{-/-}$ mice. Subsequently, mice were challenged with $10^6$ cfu *B. microti* and survival was monitored. Survival of gzmA-deficient mice was significantly higher than WT mice (Figure 4D). Notably, the survival of gzmA-deficient mice in which WT NK cells had been transferred was now similar to WT mice; meanwhile, transfer of gzmA-deficient NK cells did not alter the susceptibility to sepsis of gzmA$^{-/-}$ mice. These data strongly confirm that gzmA associated with NK cells are regulating the septic response during infection with the mouse bacterial pathogen *B. microti*.

## DISCUSSION

The genesis of septic shock remains poorly understood and has resisted treatment with a variety of therapeutics (Angus and van der Poll, 2013). Recent efforts have focused on alterations within the immune system to identify treatment options (Chiche et al., 2011). A major drawback in dissecting the innumerable steps that culminate in sepsis has been the relative lack of animal models to study this major health problem. We have employed a mouse gram-negative bacterial pathogen, *B. microti*, to analyze the capacity of Tc and NK cells to control bacterial infection as well as contribute to the development of sepsis. Our data show that WT

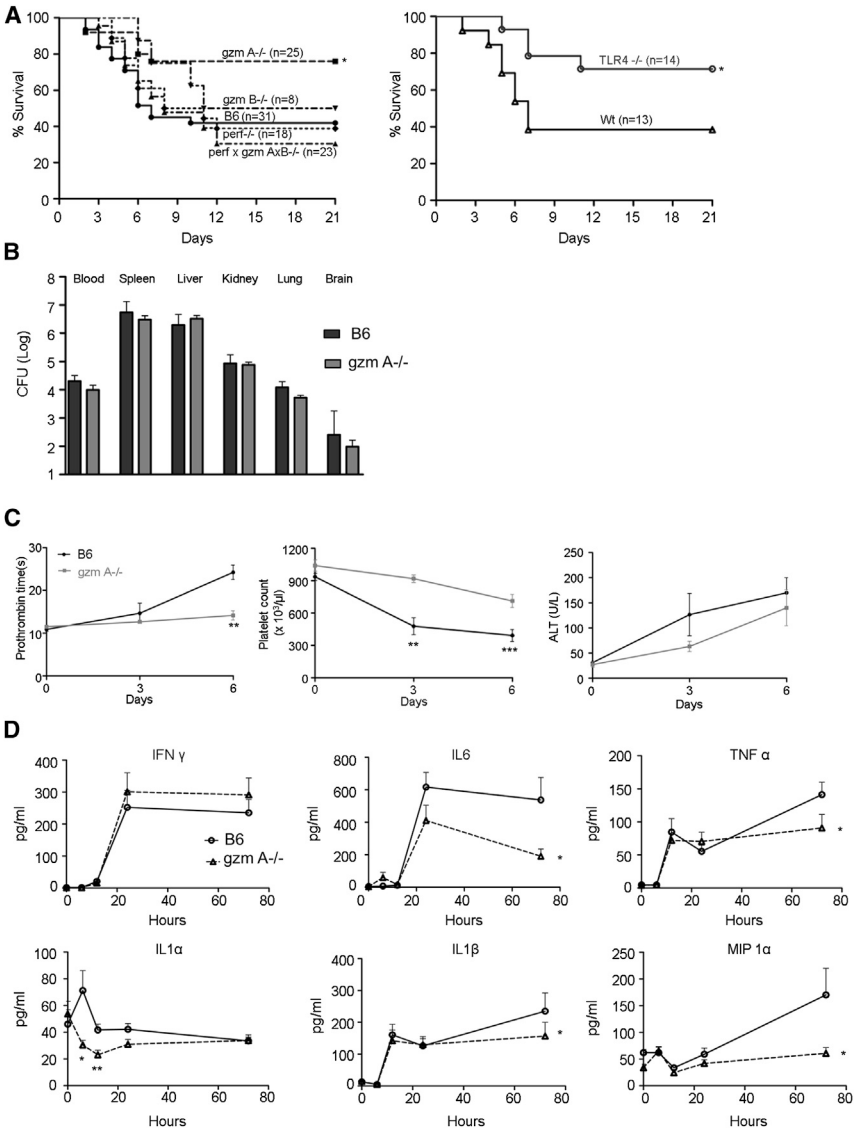


**Figure 3. GzmA-Deficient Mice Survive Sepsis Induced by *B. microti* Infection by a TLR4-Dependent Mechanism**

(A) B6 (WT), B10 (WT), perf$^{-/-}$, perfxgzmAxB$^{-/-}$, gzmA$^{-/-}$, gzmB$^{-/-}$, and TLR4$^{-/-}$ mice were infected i.p. with *B. microti* ($10^6$ cfu), and survival was monitored for 21 days. The data correspond to the indicated number of mice combined from two independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

(B) B6 (WT) and gzmA$^{-/-}$ mice were infected i.p. with $10^6$ cfu *B. microti*, and the number of cfu in blood, lung, brain, kidney, liver, and spleen was determined 3 days later.

(C and D) B6 (WT) and gzmA$^{-/-}$ mice were infected i.p. with $10^6$ cfu *B. microti*, and prothrombin time, platelet counts, and ALT (C) or cytokine (D) levels were measured in plasma or serum, respectively, at the indicated times postinfection as described in Experimental Procedures. Data are represented as mean ± SEM of four biological replicates performed by duplicates (C) or seven biological replicates performed by duplicates in two independent experiments (D). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

mice Tc cells participate in the control of *B. microti* infection in vivo and in vitro with the aid of perf and gzmB. However, other forms of immunity likely contribute to bacterial clearance because mice marginally reduce bacterial replication despite the absence of Tc cells or perf and gzmB. In marked contrast, gzmA contributes to the increased proinflammatory cytokine response during sepsis, a process that depends on the presence of NK cells.

What is the mechanism underlying the control of *B. microti* by perf and gzmB, and why would gzmA be dispensable? Unlike tumor models, understanding the immune etiopathogenesis of a pathogen that survives in macrophages is more complicated. For example, perf and gzmB certainly could kill an infected macrophage; however, *Brucella*-filled apoptotic bodies would be engulfed by adjacent macrophages allowing the bacteria to have a replicative advantage. Here, the inflammatory microenvironment would be predicted to enhance the capacity of bystander macrophage to kill these infected apoptotic bodies, a function served by gzmA. Inasmuch as gzmA induces cytokines that would augment the bactericidal activity of monocyte-macrophages, it is unclear why the protease does not aid clearance of the pathogen. Our data do not unequivocally discount a protective role for gzmA in the immune response against *B. microti* but suggest that other cell types that do not respond to gzmA may be bacterial reservoirs. Finally, the phagocytosis of infected apoptotic cells by bystander dendritic cells would enable cross priming of T cells enhancing the elimination of pathogens, a scenario that has been reported for *Salmonella* (Yrlid and Wick, 2000) and *Mycobacterium tuberculosis* (Schaible et al., 2003). However, these pathways have not been examined in the control of *Brucella spp*.

GzmA deficiency appears to dramatically protect mice from sepsis during *B. microti* infection. A similar safeguard occurs after NK cell depletion, suggesting that NK cells are the source of proinflammatory gzmA during bacterial sepsis. Confirming this possibility, the transfer of NK cells from WT mice into gzmA$^{-/-}$ mice enhances the susceptibility of mice to a septic response comparable to WT mice.

The results reported here directly corroborate our recent observation that gzmA deficiency increases the resistance of mice to septic shock induced by LPS (Metkar et al., 2008), a finding confirmed by others (Anthony et al., 2010b). Nevertheless, these studies did not clarify whether the resistance of the gzmA KO mice was related to the amelioration of the cytokine storm induced by LPS because the serum levels of

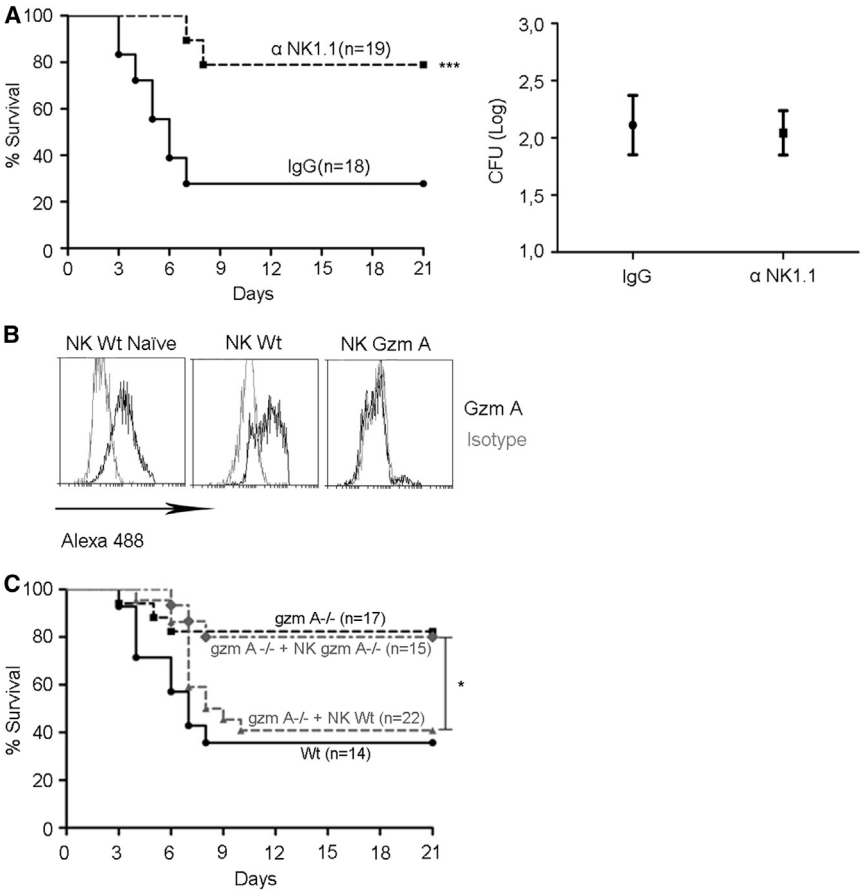


**Figure 4. NK Cell Depletion Protects Mice from Sepsis, and Transfer of WT NK Cells into gzmA-Deficient Mice Restores Susceptibility of gzmA$^{-/-}$ Mice to Sepsis**

(A and B) Control IgG-treated or NK-depleted B6 mice were infected i.p. with $10^6$ cfu of *B. microti*, and mouse survival was monitored during 21 days. (A) Survival curves corresponding to 20 biological replicates from two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001. (B) Surviving animals were sacrificed and the numbers of cfu in spleen were determined as described in the Experimental Procedures. Data are presented as mean ± SEM of cfu in spleen of each group; *p < 0.05; **p < 0.01; ***p < 0.001.

(C) B6 and gzmA$^{-/-}$ mice were mock treated (naive) or infected with $10^6$ cfu of *B. microti* i.p., and 3 days after infection, expression of gzmA was analyzed in MACS-enriched NK cells by flow cytometry.

(D) NK cells were enriched by MACS from WT or gzmA$^{-/-}$ mice and inoculated (1 × $10^6$ cells/mouse) i.p. in gzmA$^{-/-}$ mice. Subsequently, control or transferred mice were infected i.p. with $10^6$ cfu of *B. microti* and mouse survival was monitored during 21 days. *p < 0.05; **p < 0.01; ***p < 0.001. Data correspond to the indicated number of biological replicates from at least two independent experiments.

proinflammatory cytokines were not monitored. Until now, whether gzmA is indeed a physiological modulator of inflammation has been controversial. Furthermore, to maintain the homeostatic milieu, the immune system must balance the proinflammatory response necessary to control a bacterial infection without inducing immunopathology.

Recent evidence suggests that several gzms including gzmA, gzmB, and gzmK possess perf-independent extracellular functions that could influence hemostasis, vascular permeability, and other events that are considered hallmarks of sepsis (Hendel et al., 2010; Hiebert and Granville, 2012). Because perf$^{-/-}$ and perfxgzmAxB$^{-/-}$ mice are equally susceptible to sepsis as WT mice, we are unable to determine whether the participation of gzmA occurs intra- or extracellularly. In comparison, perf deficiency has been reported to protect mice from sepsis induced by LPS (Anthony et al., 2010b). Addressing this conflict, perf deficiency clearly increases the replication of *B. microti* (Figure S4). Thus, the beneficial "anti-inflammatory" effect in mice lacking both perf and gzmA is likely reduced by the higher bacterial load observed in these animals in comparison with animals that lack only gzmA.

Our data indicate that inflammation induced by gzmA in vivo plays a critical role in the development of sepsis associated with *B. microti* infection. Indeed, the absence of gzmA substantially protects mice from the lethal effects of *B. microti* where survival correlated inversely with serum levels of IL6, TNFα, IL1α, IL1β, and MIP1a. Importantly, IFNγ, the only cytokine that is present at similar levels in both WT and gzmA KO mice, is not produced by macrophages and thus is predicted to be unaffected by gzmA. Thus, the reduced level of cytokines in the absence of gzmA is likely due to the lack of protease-mediated induction of proinflammatory responses in macrophages, which are considered a major source of these cytokines during bacterial infections (Benoit et al., 2008). The reduction in the inflammatory response could also explain the amelioration of DIC parameters observed in gzmA-deficient mice, because it has been reported that inflammatory cytokines like IL1α/ILβ, IL6, or TNFα contribute to the activation of the coagulation cascade by enhancing the production of tissue factor and decreasing the expression of anticoagulation factors (van der Poll et al., 2000). Alternatively, it has been described that both gzmA and gzmK, which express similar cleavage specificity as gzmA, are able to activate the protease-activated receptors (PAR) in neurons and fibroblasts (Cooper et al., 2011; Suidan et al., 1994). These receptors are involved in platelet activation and thrombosis upon activation by thrombin, and accordingly, one could speculate that extracellular gzmA may directly promote thrombosis. However, it is not clear if gzmA-mediated PAR activation in platelets is sufficient to promote platelet aggregation (Suidan et al., 1996), and thus, this hypothesis requires further research.

How does a protease from NK cells regulate macrophage function? Various studies have established that macrophage/NK cell

crosstalk occurs during bacterial infections (Hamerman et al., 2004; Nedvetzki et al., 2007), probably due to the interaction of NK-associated TLR ligands with macrophage-associated receptors. Notably, it has been reported that LPS stimulates the expression of the activating NKG2D ligand MicA on macrophages through a TLR-dependent mechanism (Eissmann et al., 2010). Ligation of NKG2D would stimulate the secretion of cytotoxic granule toward the interacting macrophage where the protease then modulates macrophage activity.

We have shown that two members (gzmB and gzmA) expressed by Tc and NK cells differentially contribute bacterial immunopathogenesis. By targeting gzmA, one may ameliorate bacteria-associated sepsis without compromising the ability of the immune system to control infection. This finding opens the possibility of treating inflammatory sepsis without causing immunosuppression.

## EXPERIMENTAL PROCEDURES

### Mouse Strains

Inbred C57BL/6 (B6) and mouse strains deficient for granzyme A (gzmA$^{-/-}$), granzyme B (gzmB$^{-/-}$), granzyme A and B (gzmAxB$^{-/-}$), perforin (perf$^{-/-}$), perforin and granzyme A and B (perfxgzmAxB$^{-/-}$), Fas (Fas$^{-/-}$), and gld (FasL mutant) bred on the B6 background and inbred C57BL/10 and a mouse strain with a spontaneous mutation for TLR4 were provided by Markus Simon and Marina Freudenberg (TLR4) from the Max-Planck-Institut für Immunbiologie, Freiburg and bred at the Centro de Investigación y Tecnología Agroalimentaria (CITA). Their genotypes were periodically analyzed as described (Pardo et al., 2008). Mice of 8–12 weeks of age were used in all experiments.

### Bacterial Strain and Determination of cfu

*B. microti* strain CCM4915 was grown to stationary phase in tryptic soy broth (Difco Laboratories), with shaking, at 37°C for 24 hr. The number of living bacteria in a sample was determined by counting the cfu after plating serial dilutions onto tryptic soy agar plates as described previously (Jiménez de Bagüés et al., 2011). In addition, the smooth phenotype of the strains was verified in all cases by crystal violet staining (Jiménez de Bagüés et al., 2010).

### Replication of *Brucella microti* In Vivo

A sublethal dose ($10^5$ cfu) of *B. microti* was injected intraperitoneally, and after different time points, the mice were euthanized by CO2 asphyxiation and blood, spleen, and liver samples were collected aseptically. The spleen and liver were weighed, homogenized, serially diluted, and plated onto tryptic soy agar for culture and counting of viable *Brucella* organisms after 24 to 48 hr of growth at 37°C.

### In Vivo Depletion of NK and CD8$^+$ T Cells

Depletion of NK and CD8$^+$ T cells was performed using the rat anti-NK1.1 monoclonal antibody (mAb) pk136 and an anti-mouse CD8α mAb (53-6.72), respectively, as indicated in Supplemental Experimental Procedures.

### Replication of *Brucella microti* in BMDM In Vitro

Macrophages were differentiated from mouse bone marrow during 6 days as described previously (Aporta et al., 2012) and in Supplemental Experimental Procedures. For replication experiments in vitro, 4 × $10^5$ macrophages/well were seeded in 24-well plates and infected with *B. microti* at a multiplicity of infection of 25:1 for 45 min at 37°C, 5% CO2. At this time point, in some experiments, staurosporine (500 nM) or MACS-enriched (positive selection; Miltenyi Biotec) splenic Tc or NK cells from WT or KO mice were added. Subsequently, medium was removed and cells were washed with PBS and further incubated with Dulbecco's modified Eagle's medium 10% fetal calf serum containing 30 μg/ml of gentamycin. After 24 hr, macrophages were lysed with Triton X-100 0.1%, and cfu number was determined.

### Flow Cytometry

Intracellular expression of granzymes and IFNγ were analyzed by FACS as previously described (Joeckel et al., 2011; see the Supplemental Experimental Procedures for more details).

#### *Cytotoxicity Assay*

Tc cells were isolated from mouse spleen using MACS-positive selection (Miltenyi Biotec) and used for cytotoxic analyses on target cells as previously described (Pardo et al., 2008; see the Supplemental Experimental Procedures for more details).

### Evaluation of Sepsis Induced by *Brucella microti* Infection

B6, KO, and NK-depleted mice were infected with $10^6$ cfu (lethal dose in B6 mice) of *B. microti*, and mouse survival was monitored during 21 days. In addition, some mice were sacrificed after 6, 12, 24, and 72 hr of infection, and IL1α, IL1β, IL6, IFNγ, TNFα, and MIP1α levels in serum (Multiplex Bead Array System; Luminex; Millipore) or prothrombin time, platelet counts, and ALT levels in plasma were analyzed.

### Statistical Analyses

Statistical analysis was performed using GraphPad Prism software. The difference between means of paired or unpaired samples was performed using the respective t test. Survival curves were compared using both the log rank test and the Gehan-Wilcoxon test. The results are given as the confidence interval (p) and are considered significant when $p < 0.05$. Biological replicates are considered as the number of individual mice.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.012.

## AUTHOR CONTRIBUTIONS

M.A.A. and M.P.J.d.B. performed and designed the experimental work, analyzed all data, and wrote the paper. N.A., S.M., and A.d.M. performed and designed experimental work. S.H.-S. provided essential reagents and experimental advice. A.A., M.M.S., and C.J.F. provided experimental advice and wrote the paper. J.P. conceived and designed the study, supervised the project, and wrote the paper.

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