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(Article begins on next page)

Caspase-8 inactivation in T cells increases necroptosis and suppresses autoimmunity in *Bim*$^{-/-}$ mice

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Dysregulation of either the extrinsic or intrinsic apoptotic pathway can lead to various diseases including immune disorders and cancer. In addition to its role in the extrinsic apoptotic pathway, caspase-8 plays nonapoptotic functions and is essential for T cell homeostasis. The pro-apoptotic BH3-only Bcl-2 family member Bim is important for the intrinsic apoptotic pathway and its inactivation leads to autoimmunity that is further exacerbated by loss of function of the death receptor Fas. We report that inactivation of *caspase-8* in T cells of *Bim*$^{-/-}$ mice restrained their autoimmunity and extended their life span. We show that, similar to *caspase-8*$^{-/-}$ T cells, *Bim*$^{-/-}$ T cells that also lack caspase-8 displayed elevated levels of necroptosis and that inhibition of this cell death process fully rescued the survival and proliferation of these cells. Collectively, our data demonstrate that inactivation of caspase-8 suppresses the survival and proliferative capacity of *Bim*$^{-/-}$ T cells and restrains autoimmunity in *Bim*$^{-/-}$ mice.

Introduction

Apoptosis is essential for normal development and cellular homeostasis. It is a tightly controlled process that, in mammals and other vertebrates, can be activated through two distinct albeit ultimately converging pathways (Krammer et al., 2007). The extrinsic (also known as death receptor) apoptotic pathway is initiated by the interaction of death receptors with their ligands, as is the case for Fas (CD95) and its ligand FasL (CD95L). The binding of FasL to Fas results in the formation of the death-inducing signaling complex and the subsequent activation of caspase-8 (Strasser et al., 2009). FasL/Fas signaling is important for the deletion of peripheral autoreactive T cells. Similar to patients with the autoimmune lymphoproliferative syndrome (ALPS), which results from defective FasL/Fas signaling, mice with *Fas* mutations on MRL background display lymphadenopathy, splenomegaly, accumulation of double-negative T cells "T cell receptor (TCR) $\alpha\beta^+$ $B220^+CD4^-CD8^-$" and the production of autoantibodies (Cohen and Eisenberg, 1991; Nagata and Suda, 1995; Bidère et al., 2006). However, compared with MRL background, *Fas* mutant mice on C57BL/6 background display reduced lymphoproliferation and delayed onset of autoantibody production (Kelley and Roths, 1985). The intrinsic (also known as mitochondrial or Bcl-2–regulated) apoptotic pathway is controlled by the interplay of the pro- and anti-apoptotic members of the Bcl-2 protein family and can be triggered by developmental cues or a broad range of stimuli, including DNA damage, cytokine deprivation, or deregulated calcium flux (Youle and Strasser, 2008). Members of the Bcl-2 family include anti-apoptotic proteins, such as Bcl-2, Bcl-xL, Mcl-1, and A1, and pro-apoptotic proteins, such as Bax, Bak, and Bok, in addition to the BH3-only proteins Bim, Bik, Bid, Bad, Bmf, Hrk, Noxa, and Puma. Bcl-2 interacts with Bim and inhibits its pro-apoptotic functions (Youle and Strasser, 2008). Bim has

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Abbreviations used in this paper: ALPS, autoimmune lymphoproliferative syndrome; ANA, antinuclear autoantibody; ANOVA, analysis of variance; CFSE, carboxyfluorescein diacetate succinimidyl ester; Fadd, Fas-associated death domain; HE, hematoxylin and eosin; IL, interleukin; LN, lymph node; Nec-1, necrostatin-1; NF-κB, nuclear factor κB; TCR, T cell receptor; TEM, transmission electron microscopy; Treg, regulatory T cells; WT, wild type.

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emerged as a major player for mediating negative selection of autoreactive thymocytes (Bouillet et al., 2002) and deleting peripheral autoreactive T and B cells (Davey et al., 2002; Enders et al., 2003). *Bim*$^{-/-}$ mice develop progressive lymphadenopathy, splenomegaly, accumulate autoreactive lymphocytes, and autoantibodies; on a mixed C57BL/6 × 129SV background, they succumb to an autoimmune kidney disease resembling human systemic lupus erythematosus (Bouillet et al., 1999). On C57BL/6 background, *Bim*$^{-/-}$ mice do not develop the autoimmune kidney disease (Hughes et al., 2008; Weant et al., 2008)

Both the extrinsic and intrinsic apoptotic pathways have been proposed to be involved in the contraction phase of T cell immune responses and the elimination of autoreactive T cells, best demonstrated in studies of mice with dual germline inactivation of *Bim* and *Fas*. Compared with single mutant animals, mice doubly deficient for Fas and Bim develop accelerated lymphadenopathy, splenomegaly, and organ infiltration; accumulate effector memory T cells ($CD4^+CD44^+CD62L^-$); and exhibit higher levels of autoantibodies (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). These data demonstrate the cooperation of Fas and Bim in the shutdown of immune responses, maintaining peripheral tolerance, and preventing autoimmunity.

Caspase-8, an aspartate-specific cysteine protease, is essential for mediating apoptosis in response to activation of death receptors, such as Fas (Wilson et al., 2009). In addition to its role in apoptosis, caspase-8 also has important nonapoptotic functions, as it has been found to be critical for blood vessel formation during embryogenesis and mitogen- or antigen-induced proliferation of T and B cells (Chun et al., 2002; Salmena et al., 2003; Kang et al., 2004; Su et al., 2005; Lemmers et al., 2007). The importance of caspase-8 in the immune system is further demonstrated by the immunodeficiency associated with its homozygous mutation in the human ALPS-like syndrome (Chun et al., 2002). Patients with this syndrome display a combination of lymphadenopathy, splenomegaly, and impaired lymphocyte activation. Similar to these patients, mice with specific deletion of *caspase-8* in the T cell lineage (*tcasp8*$^{-/-}$) are also immunodeficient, exhibiting impaired T cell homeostasis characterized by T cell lymphopenia, defective proliferation of T cells after stimulation with mitogens or antigens, and impaired responses to viral infection (Salmena et al., 2003). *tcasp8*$^{-/-}$ mice also develop an age-dependent (ultimately fatal) lymphoproliferative disorder but show no autoantibody production or autoimmune kidney disease (Salmena and Hakem, 2005).

Through cleavage and consequent inhibition of the Receptor Interacting Protein Kinase 1 (RIPK1) and RIPK3, two serine/threonine kinases important for the death receptor–induced necroptosis, caspase-8 has been shown to suppress this programmed necrotic cell death (Holler et al., 2000; Lu et al., 2007; Rébé et al., 2007; Cho et al., 2009; He et al., 2009; Zhang et al., 2009; Vandenabeele et al., 2010). RIPK1 associates with death receptor–induced signaling complexes to modulate the switch between survival and death pathways (Holler et al., 2000). Under conditions that suppress the death receptor apoptotic pathway, RIPK1 plays a role in the alternative necroptotic cell death pathway (Degterev et al., 2008; Hitomi et al., 2008). In the death receptor–induced necroptosis, RIPK3 interacts with RIPK1 and has been shown to mediate its phosphorylation in vitro, although the physiological significance of this phosphorylation has not been determined (Cho et al., 2009; He et al., 2009). RIPK3 can modulate the switch between TNF-induced apoptosis and necrosis, and is required for RIPK1-mediated necrosis (Zhang et al., 2009). Pertinently, previous studies indicated that necrostatin-1 (Nec-1), a specific inhibitor of RIPK1 and necroptosis, rescues the proliferative defect of T cells lacking caspase-8 or Fas-associated death domain (Fadd; Bell et al., 2008; Degterev et al., 2008; Osborn et al., 2010). Recent studies demonstrated that Ripk3 deficiency rescues the embryonic lethality and defective T cell proliferation of *casp8*$^{-/-}$ mice (Ch'en et al., 2011; Kaiser et al., 2011; Oberst et al., 2011), further supporting the importance of caspase-8 in the regulation of necroptosis.

In the present study, we generated, on a mixed C57BL/6 × 129/J background, *Bim*$^{-/-}$ mice that lack caspase-8 only in their T cell lineage (*Lck-Cre*;*caspase-8*$^{fl/fl}$;*Bim*$^{-/-}$ mice are referred to here as *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice) and have investigated the interplay of caspase-8 and Bim in cell death, T cell homeostasis, lymphoproliferation, and autoimmune disease resembling human systemic lupus erythematosus. As expected, T cells from such double mutant mice showed resistance to apoptotic stimuli that trigger either the extrinsic or intrinsic apoptotic pathways. Remarkably, the *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice had reduced numbers of effector memory T cells compared with single mutant animals, and the impaired TCR-induced activation of T cells deficient for caspase-8 was not rescued by the concomitant loss of Bim. Elevated levels of necroptosis were observed in both *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells, and inhibition of necroptosis by Nec-1 was sufficient to fully rescue in vitro proliferation of these T cells. In contrast to *Fas*$^{-/-}$*Bim*$^{-/-}$ mice, *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice exhibited considerably less severe organ infiltration and kidney disease compared with single mutant mice, and serum levels of immunoglobulins and antinuclear autoantibodies (ANAs) were reduced in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice to levels comparable to their wild-type (WT) littermates. Remarkably, unlike the severely shortened lifespan of *Fas*$^{-/-}$*Bim*$^{-/-}$ mice (Hughes et al., 2008), deletion of *caspase-8* in the T cells of *Bim*$^{-/-}$ mice prolonged survival of Bim-deficient mice. Collectively, our results demonstrate the crucial role that caspase-8 plays in maintaining homeostasis of T cells, including autoreactive T cells that may escape thymic negative selection in the absence of *Bim*, and highlights the consequences of caspase-8 inactivation in inducing necroptosis of *Bim*$^{-/-}$ T cells and in suppressing autoimmunity caused by Bim deficiency.

Results

Loss of caspase-8 function does not affect the development of Bim-deficient T cells in the thymus

Although caspase-8 is dispensable for intrathymic T cell development (Salmena et al., 2003), Bim is essential for the deletion of autoreactive thymocytes (Bouillet et al., 2002). To investigate

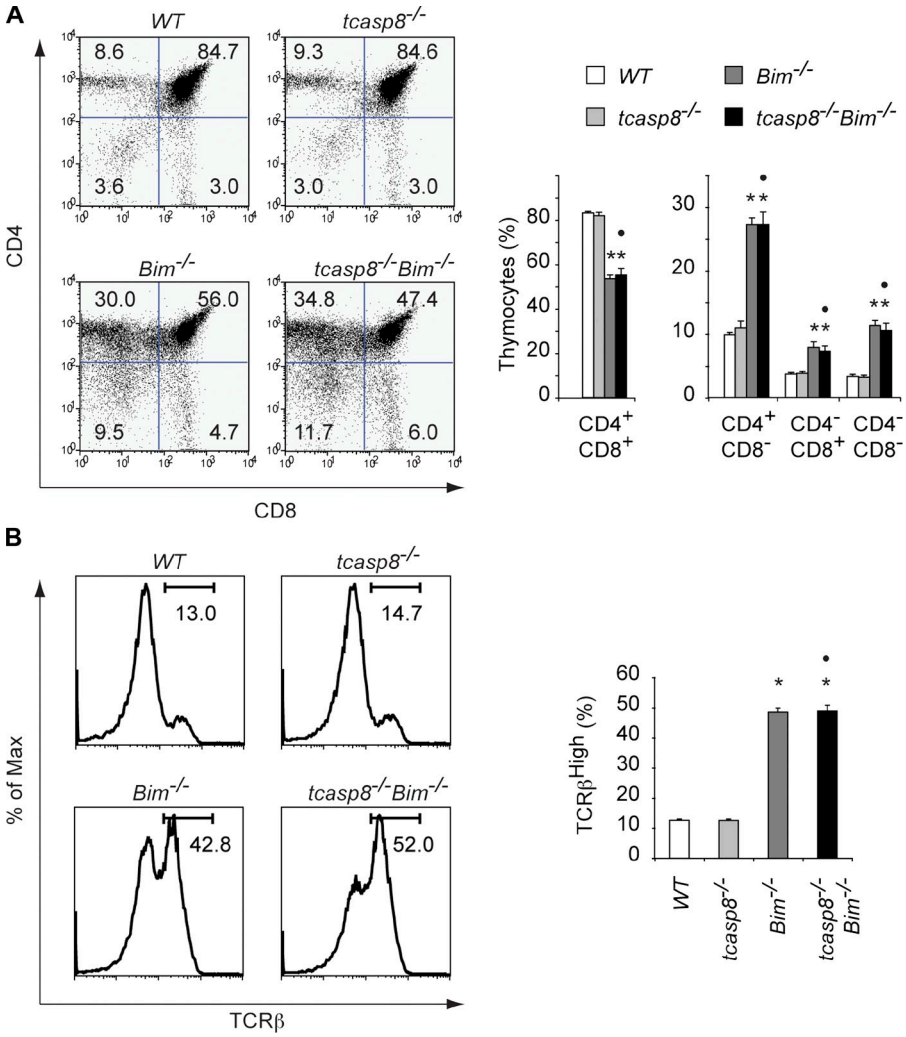

Figure 1. **Additional loss of caspase-8 does not rescue impaired intrathymic T cell development in Bim-deficient mice.** (A) Representative flow cytometric analysis of thymocytes from *tcasp8*$^{-/-}$*Bim*$^{-/-}$ and control (WT, *tcasp8*$^{-/-}$, and *Bim*$^{-/-}$) mice (left). Numbers in the quadrants indicate the percentages of the different thymocyte subpopulations. Histograms show the mean percentages of thymocyte subpopulations from 10 young mice of each genotype (right). (B) Representative expression levels of TCR-β on thymocytes of the mice indicated in A are shown (left). Histograms show the mean percentages of thymocytes with high TCR-β expression levels from 10 young mice for each genotype (right). Data represent the mean ± SEM (error bars). *, P < 0.05 as compared with WT; ●, P < 0.05 as compared with the *tcasp8*$^{-/-}$.

the effects of combined inactivation of *caspase-8* and *Bim*, we generated *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice that harbor homozygous germline mutations of *Bim* and deficiency of caspase-8 restricted to T cell lineage. All mice examined were on C57BL/6 × 129/J mixed genetic background, as on a mixed background, *Bim*$^{-/-}$ mice develop autoimmunity (Bouillet et al., 1999). We examined intrathymic T cell development in both young (6–8 wk old) and old (>6 mo) *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice and their controls. Total thymocyte numbers of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice were comparable to *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT mice at both ages (Fig. S1, A and B). FACS analysis demonstrated that there are no significant differences in the percentages (and numbers) of the four major thymocyte subpopulations (CD4$^-$CD8$^-$, CD4$^+$CD8$^+$, CD4$^+$CD8$^-$, and CD4$^-$CD8$^+$) between *tcasp8*$^{-/-}$ mice and their WT littermates at all ages, whereas, as reported (Bouillet et al., 1999), a significantly decreased proportion of the immature CD4$^+$CD8$^+$ and increased representation of CD4$^-$CD8$^-$, as well as the mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes, were observed in *Bim*$^{-/-}$ mice compared with WT controls (Figs. 1 A and S1 C). Similar to the *Bim*$^{-/-}$ mice, *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice contained reduced numbers of CD4$^+$CD8$^+$ thymocytes, whereas the numbers of CD4$^-$CD8$^-$ as well as the mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes were increased to a similar extent as in *Bim*$^{-/-}$ mice (Figs. 1 A and S1 C). Consistent with the increased representation of mature T cells in thymi from *Bim*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice, the proportions and numbers of thymocytes expressing high levels of TCR-β were increased in *Bim*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with *tcasp8*$^{-/-}$ and WT controls (Figs. 1 B and S1 D). These data indicate that caspase-8 deficiency does not further perturb intrathymic T cell development in Bim-deficient mice.

Combined loss of caspase-8 and Bim renders cells resistant to both the extrinsic and intrinsic apoptotic pathways

To investigate the effects of combined inactivation of caspase-8 and Bim on apoptosis, we examined the responses of *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT thymocytes as well as activated peripheral T cells to a range of cytotoxic stimuli that trigger either the extrinsic or intrinsic apoptotic pathway. Previous studies demonstrated the resistance of *tcasp8*$^{-/-}$ thymocytes and activated T cells to Fas-mediated killing, whereas *Bim*$^{-/-}$ thymocytes and activated T cells remain normally sensitive to this apoptotic stimulus (Bouillet et al., 1999; Salmena et al., 2003). Examination of apoptosis demonstrated that *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ thymocytes

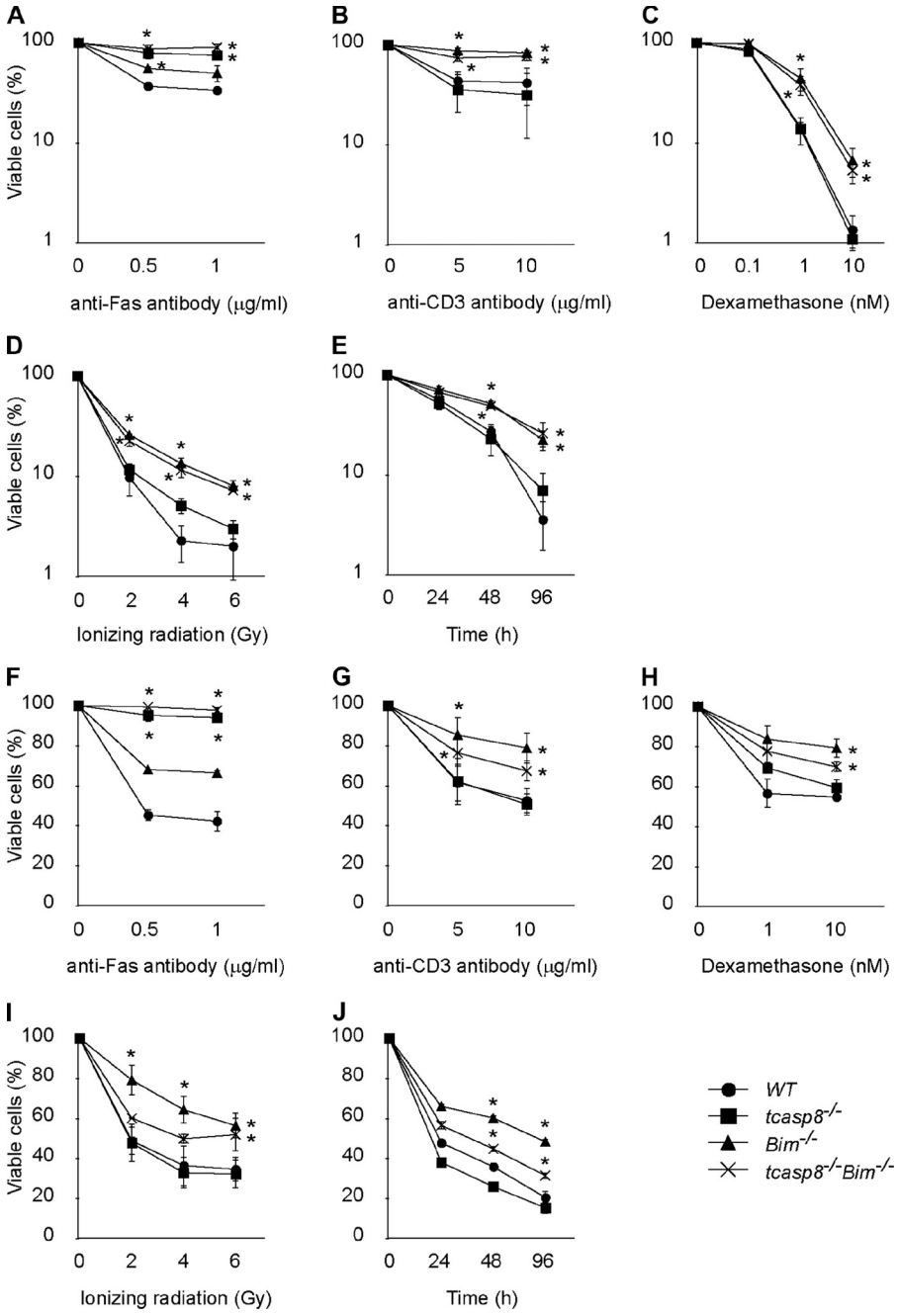

Figure 2. **Combined loss of caspase-8 and Bim causes additive but not synergistic resistance to apoptotic stimuli.** Apoptotic responses of thymocytes (A–E) and activated T cells (F–J) lacking caspase-8, Bim, or both are shown. Thymocytes or activated T cells from *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT littermates were challenged in culture with anti-Fas antibody (A and F), anti-CD3 antibody (B and G), dexamethasone (C and H), or ionizing radiation (D and I), and the extent of cell survival was assessed 24 h later. (E and J) Thymocyte and activated T cell survival in simple medium (termed death by neglect or growth factor deprivation–induced death) were also examined. Data represent the mean ± SEM (error bars) of 3–5 young mice for each genotype. Asterisks indicate significant differences compared with WT thymocytes or activated T cells ($P < 0.05$).

and activated T cells were highly resistant to Fas-mediated killing (Figs. 2, A and F). The effects of combined inactivation of *caspase-8* and *Bim* on thymocyte and activated T cell apoptosis in response to growth factor withdrawal, DNA damage, or treatment with dexamethasone were also assessed, as resistance to these apoptotic stimuli has been associated with loss of Bim (Bouillet et al., 1999) but not with loss of caspase-8 (Salmena et al., 2003). Bim is also critical for the deletion of autoreactive thymocytes and TCR/CD3 cross-linking–induced thymocyte apoptosis (Bouillet et al., 2002). Contrasting with *tcasp8*$^{-/-}$ thymocytes, *tcasp8*$^{-/-}$*Bim*$^{-/-}$ thymocytes exhibited similar resistance as *Bim*$^{-/-}$ thymocytes to anti-CD3 antibody treatment, dexamethasone, and ionizing radiation as well as growth factor deprivation (Fig. 2, B–E and G–J).

Collectively, these data show that combined loss of caspase-8 and Bim provides thymocytes and activated T cells with additive but no additional protection from apoptotic stimuli. Consequently, defects in deletion of autoreactive T lymphoid cells in both the thymus and peripheral lymphoid cells existed in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice.

Consequences of combined loss of caspase-8 and Bim on peripheral T cell homeostasis

Caspase-8 and Bim are both important for maintaining peripheral T cell homeostasis (Bouillet et al., 1999; Salmena et al., 2003; Krammer et al., 2007). Examination of young mice revealed a similar extent of splenomegaly and lymphadenopathy in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ and *Bim*$^{-/-}$ mice, whereas *tcasp8*$^{-/-}$ mice

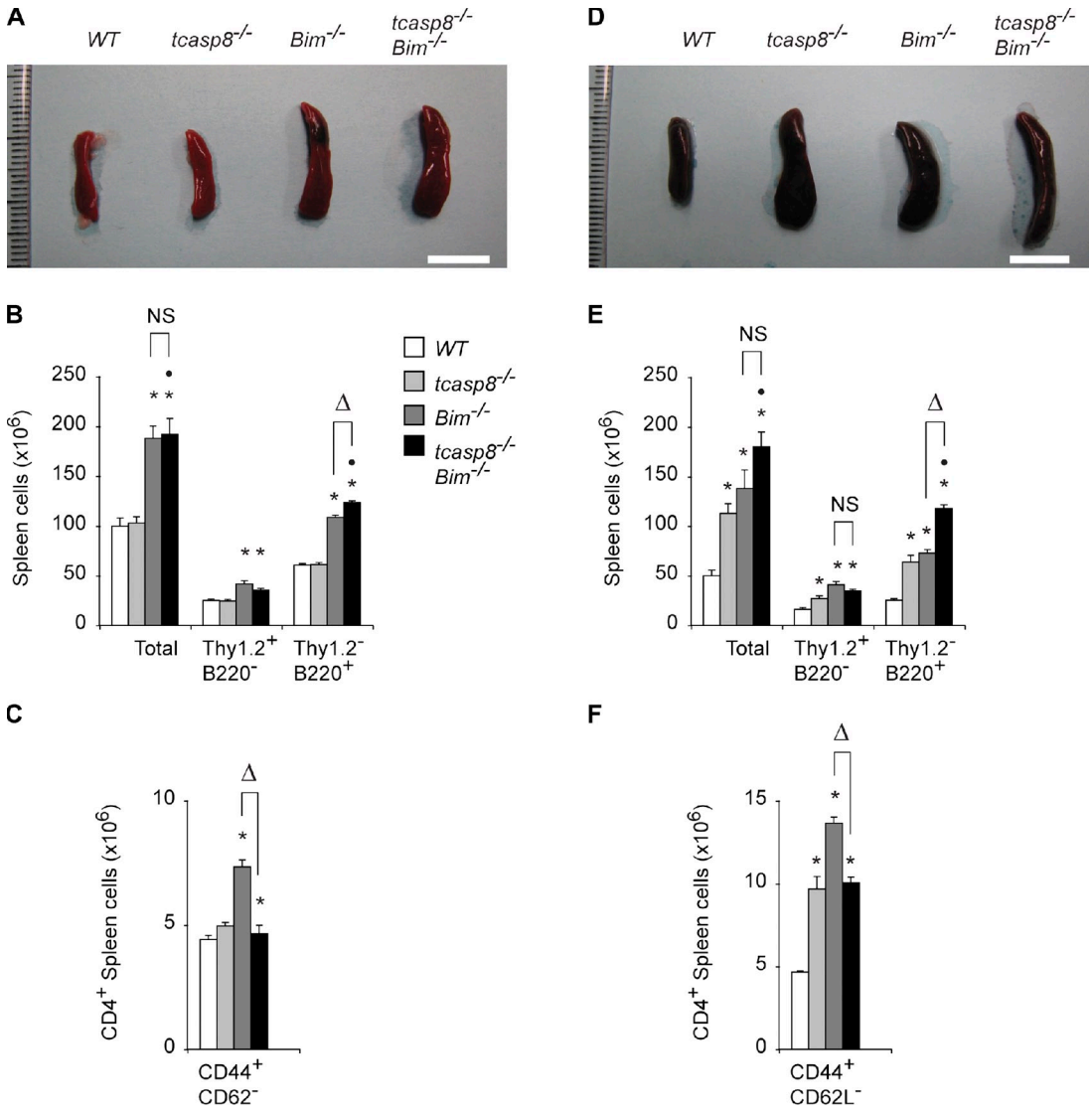

Figure 3. **Effect of T cell specific loss of caspase-8 on splenomegaly and lymphocyte hyperplasia in *Bim*$^{-/-}$ mice.** Representative spleens from *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT young (A) and old (D) mice. Total numbers of Thy1.2^{+}B220^{-} T cells and Thy1.2^{-}B220^{+} B cells in spleens from *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT young (B) and old (E) mice. Absolute numbers of effector memory T cells (CD4^{+}CD44^{+}CD62L^{-}) in spleens of *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT young (C) and old (F) mice. The values represent the mean ± SEM (error bars) of 10 mice for each genotype and age, and were compared by ANOVA testing. Young mice were 6–8 wk of age, and old mice were >6 mo of age. *, $P < 0.05$ compared with WT mice; ●, $P < 0.05$ compared with *tcasp8*$^{-/-}$ mice; Δ, $P < 0.05$ compared with *Bim*$^{-/-}$ mice. Bars, 1 cm.

had normal spleen and lymph node (LN) cellularity (Fig. 3, A and B; and Fig. S2, A and B). Splenomegaly of young *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice was mainly associated with accumulation of B cells, whereas their lymphadenopathy was associated with an increased number of both T and B cell populations (Figs. 3 B and S2 B).

With advanced age, splenomegaly was observed not only in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ and *Bim*$^{-/-}$ but also in *tcasp8*$^{-/-}$ mice (Figs. 3, D and E). T cell numbers were similarly elevated in spleens of old *Bim*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice, but splenic B cell numbers were significantly higher in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with *tcasp8*$^{-/-}$ or *Bim*$^{-/-}$ mice. Curiously, old *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice exhibited less lymphadenopathy (comparable to that of *Bim*$^{-/-}$ mice) than *tcasp8*$^{-/-}$ mice, and this was associated with a significant reduction in the numbers of T and B cells (Fig. S2, E and F).

Although the CD8^{+} T cell population was abnormally reduced in spleens and LNs of young *tcasp8*$^{-/-}$ mice, additional loss of Bim rescued this population and resulted in its accumulation in both spleen and LN (Fig. S2 D). The numbers of CD8^{+} T cells also increased in the spleens of old *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with *tcasp8*$^{-/-}$ littermates (Fig. S2 H). However, in accordance with their milder lymphadenopathy, old *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice contained significantly reduced numbers of CD4^{+} and CD8^{+} T cells compared with *tcasp8*$^{-/-}$ mice. Examination of the CD4^{+} effector memory (CD44^{+}CD62L^{-}) cells indicated that their numbers were reduced in spleens and LN of old *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with *Bim*$^{-/-}$ littermates (Fig. 3, C and F; and Fig. S2, C and G).

Collectively, these results indicate that T cell–specific inactivation of caspase-8 in old *Bim*$^{-/-}$ mice does not exacerbate

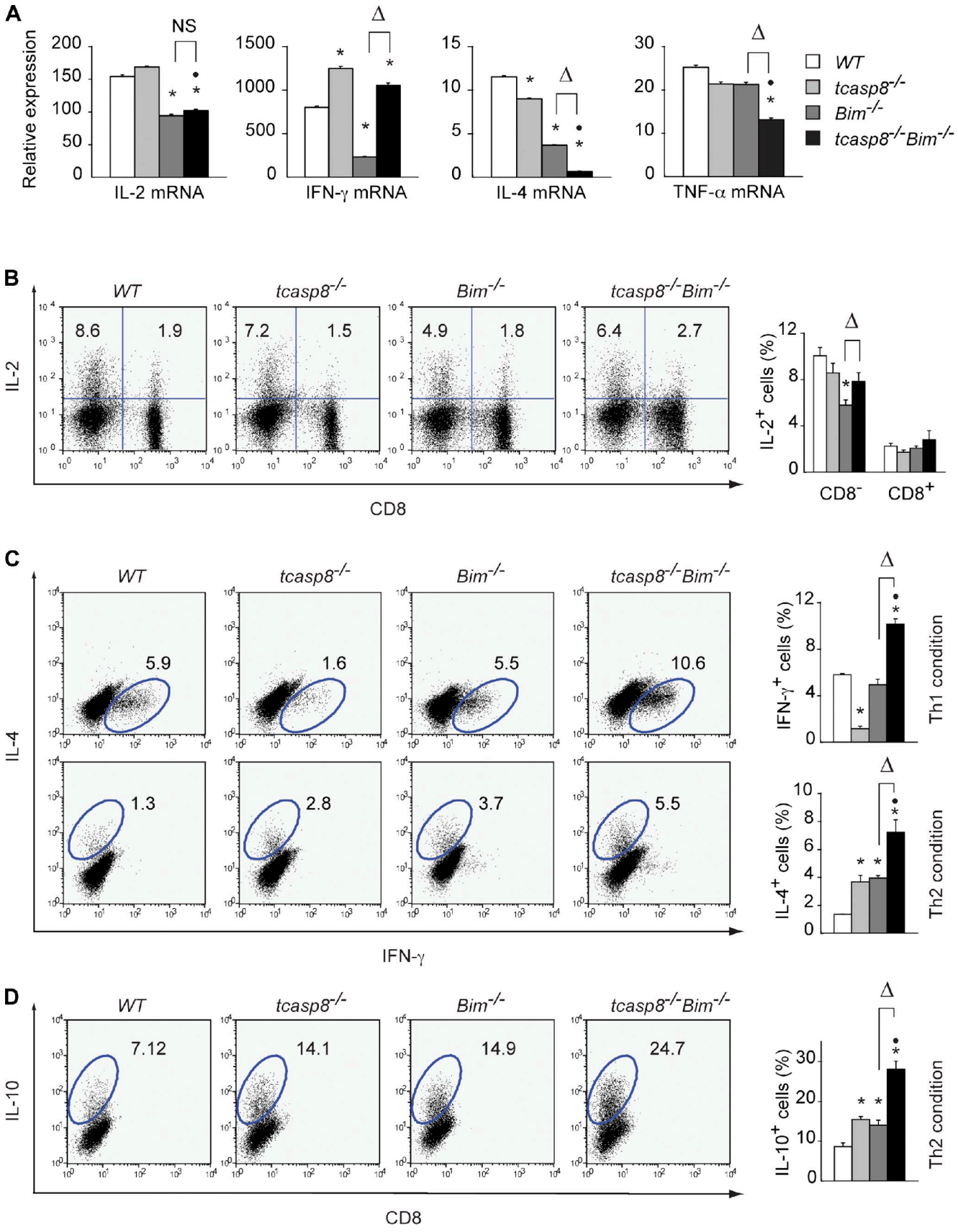

Figure 4. **Casapse-8 loss in *Bim*$^{-/-}$ T cells perturbs their pattern of cytokine production.** (A) Gene expression levels of *IL-2*, *IFN-γ*, *IL-4*, and *TNF* in *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT peripheral T cells stimulated with anti-CD3/anti-CD28 antibodies for 6 h. (B) Representative flow cytometric analysis of the intracellular levels of IL-2 in peripheral T cells stimulated with anti-CD3/anti-CD28 antibodies for 6 h. (C and D) Representative flow cytometric

their splenomegaly, but instead reduces their lymphadenopathy and their numbers of $CD4^+$ effector memory T cells in spleens and LN.

Inactivation of caspase-8 and Bim impairs T cell cytokine production

$CD4^+$ T cells play important roles in humoral and cellular immune responses, and are also involved in autoimmunity (Zhu et al., 2010). The most prominent subsets of $CD4^+$ T cells are T helper (Th) cells Th1, Th2, and Th17 and regulatory T cells (Treg), as defined by their patterns of cytokine production and functions (Zhu et al., 2010). Cytokines play important roles in the immune system and are critical for inflammation, hematopoietic cell growth, and homeostasis (O'Shea et al., 2002; Kunz and Ibrahim, 2009; O'Shea and Paul, 2010). T cell cytokine production patterns can affect lymphocyte homeostasis and the development of autoimmunity (Feldmann, 2008; O'Shea and Paul, 2010). As the effector memory T cells were abnormally increased in old *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, and *Bim*$^{-/-}$ mice compared with WT littermates, and as *tcasp8*$^{-/-}$ mice develop a lymphoproliferative disorder and *Bim*$^{-/-}$ mice develop systemic autoimmune disease (Bouillet et al., 1999; Salmena and Hakem, 2005), we examined the profile of cytokine production in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice and their littermate controls at the mRNA level and at the single cell level. First, we assessed the pattern of cytokine production in peripheral T cells and Treg ($CD4^+CD25^+FoxP3^+$). As previously described (Ludwinski et al., 2009), the expression levels of *interleukin 2* (*IL-2*), *IL-6*, *IFN-γ*, and *IL-4* genes were reduced in T cells from *Bim*$^{-/-}$ mice compared with WT littermates (Figs. 4 A and S3 A). The levels of *IL-2* mRNA and intracellular cytokines were comparable between *tcasp8*$^{-/-}$ and WT activated T cells (Fig. 4, A and B). In contrast, activated *tcasp8*$^{-/-}$ T cells displayed abnormally increased levels of *IFN-γ* and *IL-6* mRNA, whereas expression of *IL-4* mRNA was decreased compared with WT controls (Figs. 4 A and S3 A). When cytokine mRNA levels were examined in anti-CD3/anti-CD28 antibody-stimulated *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells, *IL-2*, *IL-6*, and *IL-17A* expression were found to be similar to those of *Bim*$^{-/-}$ T cells, whereas increased levels of *IFN-γ* and decreased levels of *IL-4* and *TNF* were observed compared with *Bim*$^{-/-}$ control T cells (Fig. 4, A and B; and Fig. S3 A). Examination of Treg in spleens and LN of young mice indicated no difference in their ratio in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with single mutant animals and WT littermates (Fig. S3 B).

We also examined the status of nuclear factor κB (NF-κB) activation in T cells from the four genotypes, as caspase-8 deficiency has been reported to lead to defective NF-κB nuclear translocation and impairment of its early signaling (Su et al., 2005). We analyzed degradation of IκBα and nuclear translocation of the p65/RelA subunit of the NF-κB complex in untreated as well as anti-CD3/anti-CD28 antibody–stimulated T cells. No differences were observed in the phosphorylation or degradation levels of IκBα between *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT T cells either left unstimulated or 15 and 60 min after stimulation (Fig. S3 C). As previously reported (Su et al., 2005), impaired early nuclear translocation of NF-κB p65/RelA was observed in *tcasp8*$^{-/-}$ T cells compared with WT controls. However, no differences in NF-κB p65/RelA nuclear translocation were observed between TCR stimulated *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *Bim*$^{-/-}$, or WT T cells.

Next, we focused on the pattern of Th1 and Th2 cytokine production in $CD4^+$ T cells because *IL-17A* mRNA levels and the proportion of peripheral Treg were similar in mutant mice. The production of the Th2 cytokines IL-4 and IL-10 was increased in *tcasp8*$^{-/-}$ and *Bim*$^{-/-}$ $CD4^+$ T cells compared with WT controls, whereas the expression of the Th1 cytokine IFN-γ was reduced in *tcasp8*$^{-/-}$ $CD4^+$ T cells but was normal in Bim-deficient $CD4^+$ T cells (Fig. 4, C and D; and Fig. S3 D). Remarkably, the productions of IFN-γ under Th1 culture conditions and the levels of IL-4 and IL-10 under Th2 culture conditions were increased in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ $CD4^+$ T cells compared with single mutant or WT T cells (Fig. 4, C and D). These results indicate that impaired T cell homeostasis caused by combined loss of caspase-8 and Bim is accompanied by deregulated cytokine production by T cells.

Bim inactivation does not rescue proliferative defects of caspase-8–deficient T cells

Loss of caspase-8 or its adaptor Fadd impairs proliferation of T cells in response to antigen or mitogen stimulation (Newton et al., 1998; Zhang et al., 1998; Salmena et al., 2003). Because loss of caspase-8 also increases the death of T cells (Salmena et al., 2003) and loss of Bim inhibits T cell apoptosis (Bouillet et al., 1999), we investigated whether loss of Bim could rescue the proliferative defects of *tcasp8*$^{-/-}$ T cells. In response to anti-CD3 antibody alone or together with anti-CD28 antibody or exogenous IL-2, purified *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells, similar to *tcasp8*$^{-/-}$ T cells, displayed decreased levels of $[^3H]$thymidine incorporation compared with *Bim*$^{-/-}$ and WT T cells (Fig. 5 A). As previously reported (Salmena et al., 2003), sub-G1 population increased in anti-CD3/anti-CD28–stimulated *tcasp8*$^{-/-}$ T cells compared with WT controls, which indicates increased cell death of T cells deficient for caspase-8 (Fig. S4 A). Sub-G1 population was also increased in anti-CD3/anti-CD28–stimulated *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells compared with WT and *Bim*$^{-/-}$ controls (Fig. S4 A). These results demonstrate that loss of Bim is not sufficient to rescue T cell proliferative defects associated with caspase-8 deficiency.

analysis of the intracellular levels of IFN-γ, IL-4, and IL-10 in $CD4^+$ T cells under Th1- or Th2-specific culture conditions. (C) The ovals indicate IFN-γ^+ (top) and IL-4^+ cells (bottom). (D) The ovals indicate IL-10^+ cells. *, $P < 0.05$ compared with WT control; ●, $P < 0.05$ compared with *tcasp8*$^{-/-}$ control; Δ, $P < 0.05$ compared with *Bim*$^{-/-}$ control. Data are derived from three independent experiments of young mice for each genotype. Histograms represent the mean ± SEM (error bars).

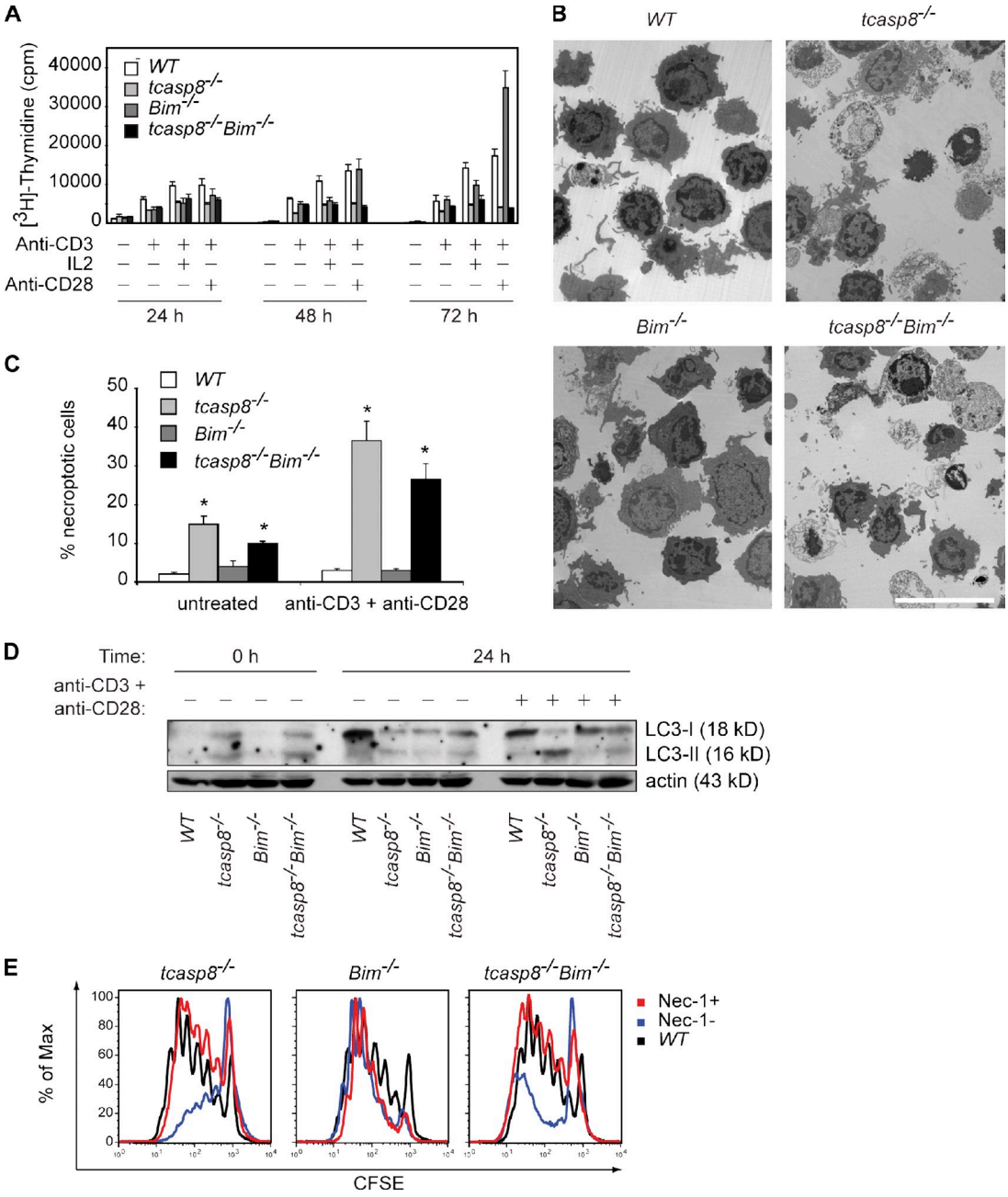

Figure 5. **Impaired proliferation and increased necroptosis in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells.** (A) [^{3}H]thymidine incorporation of purified T cells from mice of the indicated genotypes after 24, 48, or 72 h of stimulation with anti-CD3 antibody with or without costimulation with anti-CD28 antibody or IL-2. Representative data are shown from three independent experiments of young mice. (B) Representative TEM images of T cells activated for 24 h with anti-CD3/anti-CD28 antibody, showing increased frequency of necroptotic cells in cultures of anti-CD3/anti-CD28 antibody stimulated *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells. Bar, 10 µm. (C) The percentages of necroptotic primary and anti-CD3 antibody–activated T cells identified by electron microscopy. 125–209 untreated or activated T cells were scored for each genotype. *, $P < 0.05$ compared with WT and *Bim*$^{-/-}$ control mice. (D) Representative immunoblots of the levels of LC3-I and LC3-II in freshly isolated T cells (0 h) and T cells cultured for 24 h in the absence or presence of anti-CD3/anti-CD28 antibody. (E) FACS analysis of CFSE dilution profiles. Proliferation of CFSE-labeled T cells was examined after a 3-d activation with anti-CD3/anti-CD28 antibodies with or without 10 µM Nec1. Histograms represent the mean ± SEM (error bars).

Inhibition of necroptosis rescues proliferative defects of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells

In situations where apoptosis is inhibited (e.g., inactivation of Fadd or caspase-8), stimulation of death receptors, such as Fas, TNF-R, and TRAIL-R, triggers necroptosis, an alternative cell death process (Holler et al., 2000; Vandenabeele et al., 2010).

A genome-wide siRNA screen for genes that regulate necroptosis indentified 432 genes involved in this programmed necrotic death process (Degterev et al., 2008). Seven of the identified important genes for necroptosis have been previously known for their roles in apoptosis and include Bmf, a BH3-only Bcl-2 family member. The serine/threonine kinases RIPK1 and RIPK3 are critical for necroptosis, and their function is controlled

by caspase-8. Although activated caspase-8 triggers apoptosis by processing downstream effector caspases, such as caspases-3 and -7, and the BH3-only protein Bid, it also suppresses necroptosis by cleaving and inhibiting RIPK1 and RIPK3 (Vandenabeele et al., 2010).

To examine the type of cell death that takes place in T cells lacking caspase-8 alone or in combination with Bim, we used transmission EM (TEM). In contrast to primary T cells from WT or *Bim*$^{-/-}$ mice, *tcasp8*$^{-/-}$ T cells displayed increased numbers of necroptotic cells, as manifested by the swelling of the cell, mitochondria and cytoplasmic organelles, focal rupture of the plasma membrane, and moderate chromatin condensation (Figs. 5, B and C). Similar to *tcasp8*$^{-/-}$ T cells, the level of necroptosis was also significantly elevated in primary *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells (Fig. 5 C). Moreover, the frequency of *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells undergoing necroptosis was further increased after stimulation with anti-CD3/anti-CD28 antibodies. Consistent with our TEM data indicating no significantly increased apoptosis in anti-CD3/anti-CD28–stimulated *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells, the level of caspase activities was not increased in these T cells compared with WT controls (Figs. 5, B and C; and Fig. S4 B).

Autophagy is a catabolic process important for the lysosomes mediated degradation and turnover of long-lived proteins and organelles (Klionsky and Emr, 2000; Gozuacik and Kimchi, 2004). Because autophagy is induced in a number of necroptotic systems (Degterev et al., 2005), we investigated the levels of microtubule-associated protein 1 light chain 3 (LC3-II), a marker for autophagy, in peripheral T cells. Western blot analysis indicated increased LC3-II levels in untreated and stimulated T cells from *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with *Bim*$^{-/-}$ and *WT* controls (Fig. 5 D).

Next, we examined whether necroptosis of *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells accounts for their defective responses to mitogenic stimulation and increased cell death. Purified T cells from the four genotypes were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with anti-CD3/anti-CD28 antibodies in the presence or absence of the necroptosis inhibitor Nec-1 (Degterev et al., 2008). Although FACS analysis of CFSE dilution profiles confirmed the previously described (Degterev et al., 2008) proliferation defects of *tcasp8*$^{-/-}$ T cells, the addition of Nec-1 rescued mitogen-induced proliferation of *tcasp8*$^{-/-}$ T cells. Similarly, Nec-1 also rescued anti-CD3/anti-CD28 antibody–induced proliferation of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells to a level similar to WT and *Bim*$^{-/-}$ T cells (Fig. 5 E).

Because TNF-like cytokines are potent inducers of necroptosis, we examined the serum levels of TNF in the four genotypes. No detectable level of serum TNF was observed in the different strains of mice (Fig. S4 C). Expression levels of TNF-receptor 1 on peripheral T cells were also similar between mutants and WT controls (Fig. S4 D).

RIPK1 and RIPK3 interaction is important during TNF–receptor 1–induced necroptosis (Cho et al., 2009; Declercq et al., 2009; He et al., 2009; Zhang et al., 2009). As necroptosis is elevated in anti-CD3/CD28–stimulated T cells deficient for caspase-8, we investigated whether RIPK1 and RIPK3 interact in response to TCR stimulation of T cells from the four genotypes (Fig. S4 E). Immunoprecipitation of RIPK3 from anti-CD3/CD28–stimulated T cells from these mice failed to pull down RIPK1. These data are similar to the reported lack of interaction of RIPK1 and RIPK3 in anti-CD3/CD28 stimulated T cells deficient for Fadd (Osborn et al., 2010).

Collectively, these data indicate that the elevated necroptosis in *tcasp8*$^{-/-}$ T cells is not rescued by Bim inactivation. Furthermore, although defective proliferation of *tcasp8*$^{-/-}$ T cells was not rescued by loss of Bim, inhibition of necroptosis fully restored the proliferative defect of both *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells. Thus, necroptosis is the major cause for the impaired survival and proliferation of caspase-8–deficient T cells.

T cell–specific inactivation of caspase-8 diminishes autoimmunity in *Bim*$^{-/-}$ mice and increases their lifespan

Homozygous mutations of the human *CASPASE-8* gene cause the ALPS-like syndrome (Chun et al., 2002). Accordingly, *tcasp8*$^{-/-}$ mice develop an age-dependent lymphocyte infiltration of multiple organs, with lung infiltration being the likely cause of their premature death (Salmena and Hakem, 2005). In addition, *Bim*$^{-/-}$ mice on a C57BL/6 × 129SV genetic background develop progressive lymphadenopathy, splenomegaly, and fatal lupus-like autoimmune kidney disease (Bouillet et al., 1999).

To determine the effect of combined loss of both caspase-8 and Bim on the immunopathology associated with their individual inactivation, the levels of immunoglobulins, and ANA in sera from the old mice were examined using ELISA. In contrast to old *Bim*$^{-/-}$ mice, age-matched *tcasp8*$^{-/-}$*Bim*$^{-/-}$ littermates exhibited significantly decreased serum levels of ANA, and their overall levels of IgM, IgG1, IgG2a, and IgG3 were also reduced to the levels found in WT littermates (Figs. 6, A and B).

We also examined hematoxylin and eosin (HE)-stained sections of spleens, LN, lungs, livers, and kidneys from 13-mo-old *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice and their control littermates. As previously reported (Bouillet et al., 1999), kidneys from *Bim*$^{-/-}$ mice presented with abnormally increased glomerular size and mesangial area, loss of open capillary loops, and accumulation of immune complexes compared with age-matched *tcasp8*$^{-/-}$ and WT controls (Fig. 7 A). Remarkably, this pathology caused by Bim deficiency was substantially diminished by concomitant loss of caspase-8 (in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice, Fig. 7 A). Further histological examination of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice indicated that infiltration of T and B cells into nonlymphoid organs was significantly reduced compared with both *tcasp8*$^{-/-}$ and *Bim*$^{-/-}$ mice (Figs. 7 A and S5 A). In addition, in contrast to *Bim*$^{-/-}$ mice, aged *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice showed no glomerulonephritis, and their glomerular cell numbers were significantly reduced and indeed comparable to those of WT controls (Figs. 7 A and S5, A and B). Autoimmune glomerulonephritis in *Bim*$^{-/-}$ mice is accompanied by the accumulation of immune complexes in the kidneys (Bouillet et al., 1999).

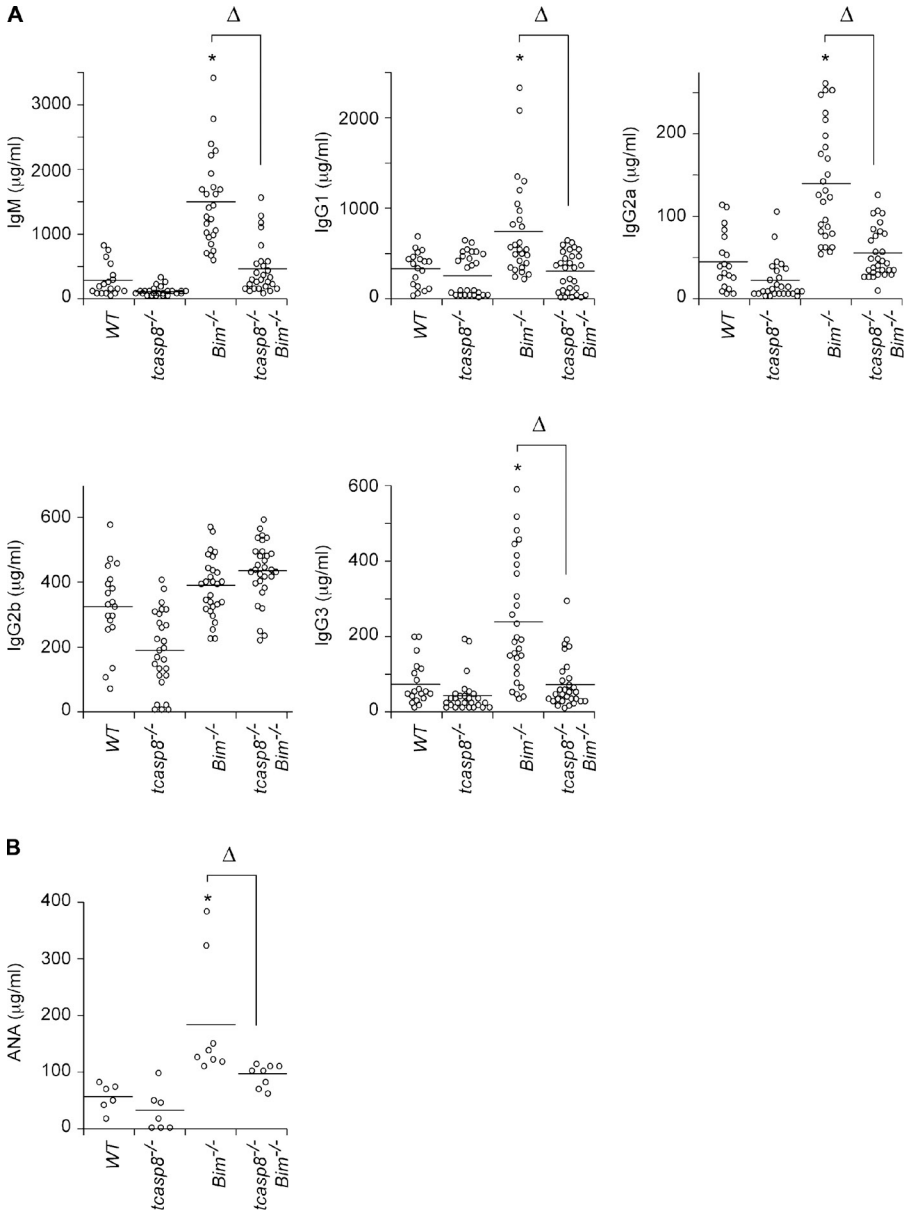

Figure 6. **Loss of caspase-8 in T cells suppresses the hypergammaglobulinemia caused by loss of Bim.** (A) Levels of IgM, IgG1, IgG2a, IgG2b, and IgG3 in sera of 6 mo or older WT, *Bim*$^{-/-}$, *tcasp8*$^{-/-}$, and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice (n = 18–30). (B) Levels of ANA in sera of 6 mo or older *tcasp8*$^{-/-}$*Bim*$^{-/-}$, *tcasp8*$^{-/-}$, *Bim*$^{-/-}$, and WT mice. Solid horizontal lines represent the mean for each genotype. *, $P < 0.05$ compared with *WT* mice; ●, $P < 0.05$ compared with *tcasp8*$^{-/-}$ mice; Δ, $P < 0.05$ compared with *Bim*$^{-/-}$ mice.

Anti-IgG staining of kidney sections indicated that the deposition of immune complexes in kidneys from *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice was markedly reduced compared with kidneys from aged *Bim*$^{-/-}$ mice (Fig. 7 A).

To examine the impact of *tcasp8* and *Bim* mutations, by themselves or in combination, on animal lifespan, we monitored cohorts of the four genotypes for 500 d. In accordance with their moderate organ infiltration by lymphocytes, absence of glomerulonephritis, relatively low serum levels of ANA and total immunoglobulins, the lifespan of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice was significantly extended compared with *tcasp8*$^{-/-}$ and *Bim*$^{-/-}$ mice and was indeed similar to that of WT littermates (Fig. 7 B). Collectively, these results demonstrate that specific loss of *caspase-8* in T cells is sufficient to restrain autoimmunity in *Bim*$^{-/-}$ mice. Despite the fact that Bim deficiency impairs negative selection of autoreactive thymocytes, the function and potency of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ peripheral T cells is likely restrained by their elevated level of necroptosis that results from loss of caspase-8 (Fig. 7 C). In addition, the infiltration of T and B cells into nonlymphoid organs that is apparent in both *Bim*$^{-/-}$ and *tcasp8*$^{-/-}$ mice was suppressed in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice. The restraining of these immune disorders in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice increased their lifespan.

Discussion

Deregulation of T cell homeostasis can lead to various immunopathologies, including immunodeficiency and autoimmunity (Theofilopoulos et al., 2001; Ohashi, 2002; Marleau and Sarvetnick, 2005). Similarly, impaired apoptosis contributes to the pathogenesis of these diseases and also tumorigenesis (Siegel, 2006; Bouillet and O'Reilly, 2009). Apoptosis is important for maintaining T cell homeostasis, and the intrinsic apoptotic pathway is crucial for the deletion of autoreactive

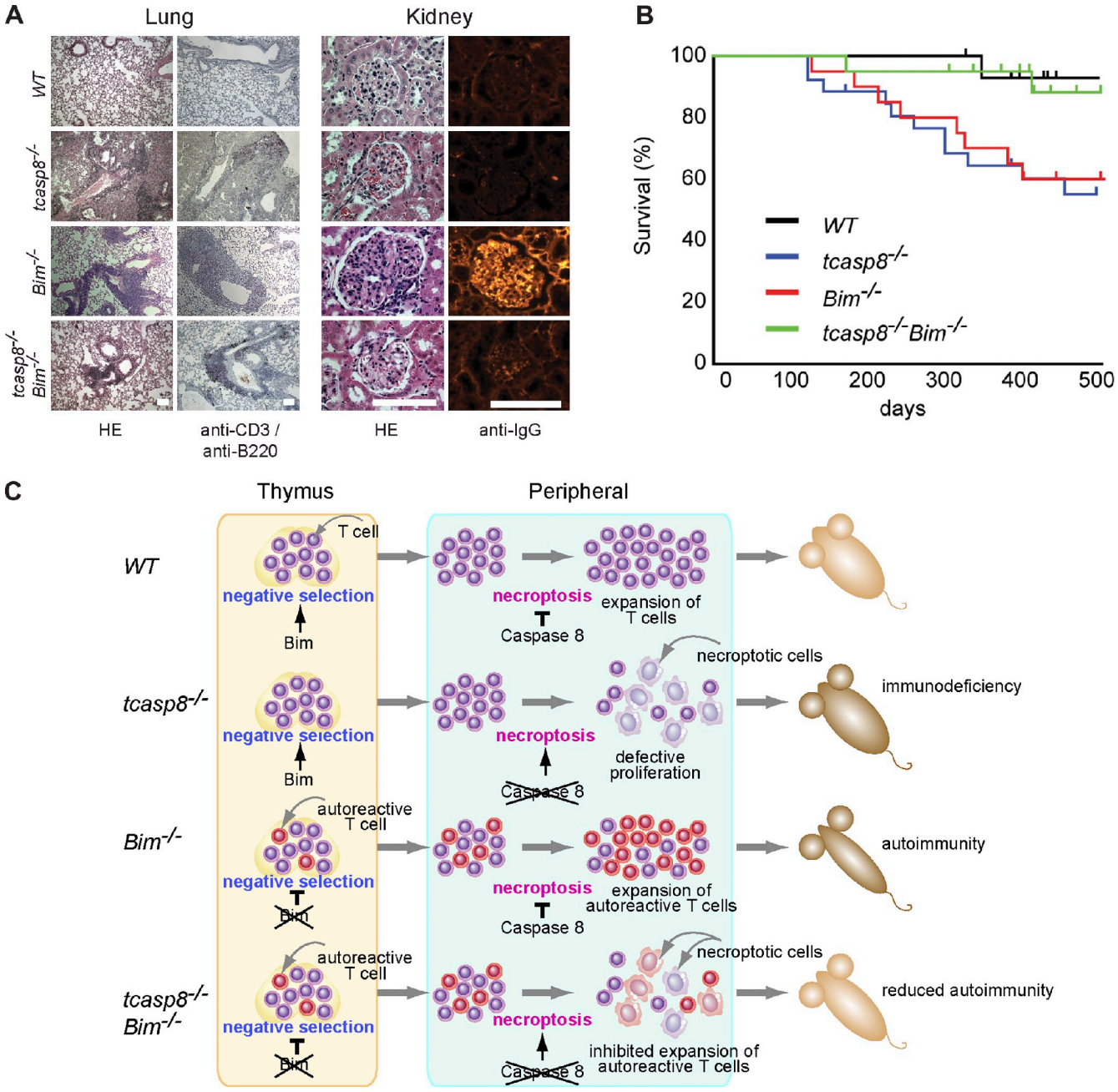

Figure 7. **T cell–specific loss of caspase-8 inhibits the development of autoimmune disease in *Bim*$^{-/-}$ mice and increases their lifespan.** (A) Representative immunohistochemical staining of lungs (left) and kidneys (right) from 13-mo-old mice of the indicated genotypes using HE, anti-CD3 antibody (red), and anti-B220 antibody (brown). Kidney sections were also stained with anti-IgG antibodies to assess the deposition of immune complexes (right). Bars, 100 µm. (B) Kaplan-Meier analysis representing the percent survival versus age in days of cohorts of WT (n = 15), *tcasp8*$^{-/-}$ (n = 25), *Bim*$^{-/-}$ (n = 20), and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ (n = 20) mice. The survival of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice was significantly extended compared with *tcasp8*$^{-/-}$ and *Bim*$^{-/-}$ mice ($P < 0.05$). (C) A model for the restrained autoimmunity of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with *Bim*$^{-/-}$ littermates. Autoreactive T cells that escape negative selection in *Bim*$^{-/-}$ mice expand and mediate autoimmunity. Caspase-8–deficient *Bim*$^{-/-}$ T cells that escape negative selection in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice display elevated levels of necroptosis and impaired proliferation, and these defects lead to restrained autoimmunity and prolonged survival of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice.

thymocytes that express TCR with high avidity for self-peptide major histocompatibility complexes (Sebzda et al., 1999; Bouillet and O'Reilly, 2009). The loss of caspase-8, a key component of the extrinsic apoptotic pathway, has no effect on T cell development in the thymus, including negative selection of autoreactive thymocytes, but does result in impaired homeostasis of peripheral T and B cells and abnormally reduced T cell activation; paradoxically, these defects ultimately lead to a lethal lymphoproliferative disorder (Salmena et al., 2003; Salmena and Hakem, 2005). Studies of *Bim*$^{-/-}$ mice indicated the essential role of Bim in the intrinsic apoptotic pathway and demonstrated its requirement for deletion of autoreactive thymocyte and for maintaining homeostasis of the lymphoid and myeloid compartments (Bouillet et al., 1999; Bouillet et al., 2002; Davey et al., 2002; Enders et al., 2003). Furthermore, Bim-deficient mice on a mixed C57BL/6 × 129SV genetic

background produce autoantibodies and develop lupus-like kidney disease.

Recent studies indicated that combined loss of Fas and Bim accelerates splenomegaly and lymphadenopathy, and promotes immune cell infiltration and autoimmune disease (Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008; Bouillet and O'Reilly, 2009). *Fas*$^{lpr/lpr}$*Bim*$^{-/-}$ mice develop a lupus-like syndrome with severe kidney pathologies and die prematurely, much earlier than either of the singly deficient mice (or WT mice). Fas-mediated apoptosis is caspase-8 dependent. However, caspase-8 is also essential for apoptosis mediated by other members of the TNF receptor family (Wilson et al., 2009). Because loss of caspase-8 renders lymphocytes resistant to more apoptotic stimuli than loss of Fas, one may have predicted to see greater defects in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice compared with *Fas*$^{lpr/lpr}$*Bim*$^{-/-}$ mice. However, in addition to its requirement for apoptosis, caspase-8 also has critical nonapoptotic functions, such as in early embryogenesis as well as in the activation of T cells, B cells, and macrophages (Chun et al., 2002; Salmena et al., 2003; Kang et al., 2004; Su et al., 2005; Lemmers et al., 2007; Maelfait and Beyaert, 2008).

Because loss of caspase-8 blocks Fas-mediated apoptosis and because Fas collaborates with Bim to suppress autoimmunity, we examined the consequences of loss of caspase-8 in T cells on intrathymic T cell development, T cell homeostasis, and autoimmunity of *Bim*$^{-/-}$ mice. Our data show that caspase-8 loss does not affect the developmental abnormalities of *Bim*$^{-/-}$ thymocytes (increased numbers of mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ cells and decreased numbers of CD4$^+$CD8$^+$ cells) in *Bim*$^{-/-}$ thymocytes. In addition, thymocytes lacking both caspase-8 and Bim, similar to *Bim*$^{-/-}$ thymocytes, were highly resistant to TCR/CD3 activation-induced apoptosis. These double mutant thymocytes were also resistant to all tested cytotoxic stimuli that trigger the extrinsic or the intrinsic apoptotic pathways; however, no synergistic resistance was observed in these cells compared with single mutants.

Although *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice of all ages displayed splenomegaly with abnormal accumulation of T and B cells, the lymphadenopathy observed in young *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice did not increase (and was even somewhat reduced) as they aged when compared with *tcasp8*$^{-/-}$ littermates. This remarkable amelioration of the lymphadenopathy in old *Bim*$^{-/-}$ mice by the additional loss of caspase-8 in their T cells completely contrasts with the massively enhanced lymphadenopathy of *Fas*$^{lpr/lpr}$*Bim*$^{-/-}$ mice (compared with *Bim*$^{-/-}$ mice; Hughes et al., 2008; Hutcheson et al., 2008; Weant et al., 2008). In addition, loss of caspase-8 in T cells of *Bim*$^{-/-}$ mice was sufficient to reduce T and B cell infiltration into nonlymphoid organs and suppressed hypergammaglobulinemia and ANA production associated with Bim deficiency. These data demonstrate the opposite effects of the loss of caspase-8 versus loss of Fas on autoimmunity caused by Bim deficiency.

Cytokines play critical roles in the pathogenesis of many diseases, including autoimmune pathologies (O'Shea et al., 2002). For example, IL-10 is critical to prevent inflammation and autoimmune colitis, and IFN-γ can either augment or suppress inflammation and autoimmunity, depending on the experimental setting (Hu and Ivashkiv, 2009; Saraiva and O'Garra, 2010). Cytokines are produced by different cell types, including CD4$^+$ T cells that play a central role in autoimmunity (O'Shea and Paul, 2010). Various functionally distinct subsets of CD4$^+$ T cells exist, including Th1, Th2, and Th17, and these subsets secrete distinct sets of cytokine (O'Shea and Paul, 2010). Inhibition of caspases in stimulated CD4$^+$ T cells increases their IL-4 production (Sehra et al., 2005), whereas inactivation of Bim in these cells reduces their production of IL-2, IL-4, IL-6, and IFN-γ (Ludwinski et al., 2009), possibly because cells that would normally have died instead survived but assumed a quiescent (nonactivated, low cytokine producing) state. Short-term mitogenic stimulation of CD4$^+$ T cells lacking both caspase-8 and Bim resulted in reduced levels of *IL-4* and *TNF* compared with single mutant or WT T cells, and the reduced IFN-γ production of *Bim*$^{-/-}$ CD4$^+$ T cells was rescued by the additional loss of caspase-8. In addition, in vitro re-stimulation of activated CD4$^+$ T cells demonstrated that in contrast to single mutant and WT control T cells, CD4$^+$ T cells from *tcasp8*$^{-/-}$ *Bim*$^{-/-}$ mice produced increased amounts of IFN-γ under Th1 culture conditions and increased levels of IL-4 and IL-10 under Th2 culture conditions. It is therefore possible that the cytokine profile changes in the absence of caspase-8 and Bim, including increased production of Th2 cytokine IL-4 and immunosuppressive cytokine IL-10, may contribute to suppressing autoimmunity in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice. These data support the notion that caspase-8 and Bim are required for balanced regulation of cytokine production by the CD4$^+$ T cell population.

Caspase-8 plays critical roles in the regulation of both apoptosis and necrosis: whereas caspase-8 activation promotes apoptosis, deficiency or inhibition of caspase-8 unleashes necroptosis as an alternative pathway to kill the cells. Because necroptosis was elevated in primary *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells and was further increased after TCR stimulation, it is possible that necroptosis contributes to the impaired homeostasis of peripheral T cells in *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice. This is supported by the full rescue of TCR-induced in vitro proliferation of *tcasp8*$^{-/-}$ and *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells in the presence of the necroptosis inhibitor Nec-1.

Our data are consistent with recent studies demonstrating important roles for necroptosis in the phenotypes of *casp8* mutant mice. Deficiency of Ripk3, a kinase important for necroptosis, was sufficient to rescue embryonic lethality and T cell proliferation of *casp8*$^{-/-}$ mutants (Ch'en et al., 2011; Kaiser et al., 2011; Oberst et al., 2011). Similarly, necroptosis is elevated in the absence of Fadd, and deficiency of Ripk1, another kinase important for necroptosis, was sufficient to rescue embryonic lethality and T cell proliferation of *Fadd*$^{-/-}$ mutants (Zhang et al., 2011). Therefore, it is likely that defective proliferation of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ T cells, because of their elevated necroptosis, may lead to the restrained autoimmunity of *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice.

Our data also indicate that loss of Bim diminishes the progressive lymphadenopathy caused by caspase-8 inactivation in T cells. We hypothesize that this rescue might be caused by increased survival of *casp8*$^{-/-}$ T cells lacking Bim as *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice display reduced lymphopenia early

in life compared with *tcasp8*$^{-/-}$ mice. This rescue of T cell survival could contribute to reduce homeostatic proliferation in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice and ultimately reduced lymphadenopathy and lymphoid infiltration into solid organs.

Collectively, our data demonstrate that inactivation of caspase-8 in T cells suppresses autoimmunity caused by Bim deficiency. Although autoimmunity induced as a consequence of loss of Bim is caused by the impaired apoptosis and the resulting escape of autoreactive T cells from deletion, inactivation of caspase-8 in *Bim*$^{-/-}$ T cells increases their spontaneous and activation-induced necroptosis, thereby likely leading to elimination of *Bim*$^{-/-}$ T cells, including autoreactive ones, and suppression of autoimmunity. A role for caspase-8 inhibition in suppressing autoimmunity associated with other genetic defects remains to be determined; nevertheless this study highlights the fact that germline mutations of *Fas* and caspase-8 loss in T cells have contrasting effects on autoimmunity associated with *Bim* deficiency and supports a role for necroptosis in the suppression of autoimmunity.

Materials and methods

Ethics statement

All experiments were performed in compliance with the guidelines of the Ontario Cancer Institute Animal Care Committee.

Mice

tcasp8$^{-/-}$ mice (Salmena et al., 2003) were crossed with *Bim*$^{-/-}$ mice (Bouillet et al., 1999) to generate *tcasp8*$^{-/-}$*Bim*$^{+/-}$ and *casp8*$^{fl/fl}$*Bim*$^{+/-}$ mice. *tcasp8*$^{-/-}$*Bim*$^{+/-}$ mice were crossed with *casp8*$^{fl/fl}$*Bim*$^{+/-}$ mice to generate *casp8*$^{fl/fl}$*Bim*$^{+/+}$ (WT), *tcasp8*$^{-/-}$*Bim*$^{+/+}$ (*tcasp8*$^{-/-}$), *tcap8*$^{+/+}$*Bim*$^{-/-}$ (*Bim*$^{-/-}$), and *tcap8*$^{-/-}$*Bim*$^{-/-}$ mice. Mice were genotyped by PCR using the following primers: Cre (5′-TCGCGATTATCTTCTATATCTTCAG-3′ and 5′-GCTCGACCAGTTTAGTTACCC-3′), caspase-8 (5′-CCAGGAAAAGATTTGTGTCTAGC-3′ and 5′-GGCCTTCCTGAGTACTGTCACCTGT-3′), WT *Bim* (5′-CTGTCTGATGGACTGTGATCA-3′ and 5′-CCTCCTTGTGTAAGTTTCGTT-3′), and mutant *Bim* (5′-CTCAGTCCATTCATCAACAG-3′ and 5′-CATTCTCGTAAGTCCGAGTCT-3′). All mice in this study were on a mixed C57BL/6 × 129/J genetic background. Mice referred to as young in this study were 6–8 wk of age, whereas mice >6 mo of age were considered to be old.

Cell culture

Thymocytes, splenocytes, and LN cells were cultured under 5% CO_2 at 37°C in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS (Wincent Inc.) plus 0.1% 2-ME (Invitrogen).

Thymocyte apoptosis

Thymocytes (2×10^5 in 1 ml of culture medium) were treated with the following apoptotic stimuli: anti-Fas antibody (0.5 or 1 µg/ml; BD), dexamethasone (0.1, 1, or 10 nM; Sigma-Aldrich), γ-irradiation (2, 4, or 6 Gy), or plate-bound anti-CD3 antibody (5 or 10 µg/ml; 2C11 clone; BD) in combination with 1 µg/ml anti-CD28 antibody (clone 37.51; BD). Extent apoptosis and cell death were measured 24 h after treatment using the Annexin/propidium iodide Apoptosis Detection kit (R&D Systems) or staining with 7-amino-actinomycin D (7AAD; Sigma-Aldrich) followed by flow cytometric analysis.

T cell activation and cell death

T cells derived from spleen or LN were activated overnight in tissue culture plates coated with 5 µg/ml anti-CD3 antibody, and for 3 d in medium containing 50 U/ml IL-2. Viable, activated T cells were isolated using Lympholyte-M (Cedarlane) and washed, and 2×10^5 cells were replated in 24-well dishes in 1 ml of medium. Cells were treated with the apoptotic stimuli as thymocytes (see Materials and methods). 24 h after treatment, cell viability was determined using Annexin/propidium iodide or 7AAD staining.

FACS analysis

Single-cell suspensions prepared from the spleen, thymus, or LN (cervical, inguinal, and axillary) were stained with antibodies at 4°C in PBS with 1% FBS. The following antibodies were used: anti-CD4, anti-CD8, anti–TCR-β, anti-Thy1.2, anti-B220, anti-CD25 (IL-2Rα chain), anti-FoxP3, anti-CD44, and anti-CD62L, all conjugated to allophycocyanin, R-PE, FITC, or perCP (BD or eBioscience). Lymphocytes were analyzed by flow cytometry (FACSCalibur; BD) with CellQuest software (BD) or FlowJo analysis software (Tree Star).

T cell cytokine production

Flow cytometric determination of the levels of IL-2, IFN-γ, and IL-4 in the cytoplasm of activated peripheral T cells was performed using Cytofix/Cytoperm Plus (BD) according to the manufacturer's instructions. To measure the levels of IL-2 production, purified T cells were stimulated with tissue culture plate coated anti-CD3/anti-CD28 antibodies for 6 h. To assess the cytokine production of helper T cells, naive CD4$^+$ T cells were purified using the mouse naive CD4$^+$ T cell isolation kit (R&D Systems). For Th1 and Th2 differentiation, purified naive CD4$^+$ T cells (5×10^6/ml) from spleen and LN were stimulated with 1 µg/ml anti-CD3 antibody plus 10 µg/ml anti–IL-4 antibody (11B11) for Th1 differentiation, or with 10 µg/ml anti-IFN-γ antibody (XMG1.2) for Th2 differentiation. After overnight culture, Th1 cultures received 50 U/ml recombinant murine IL-2 (Invitrogen), whereas Th2 cultures received 50 U/ml murine IL-2 plus 500 U/ml murine IL-4 (eBioscience) as described previously (Hao et al., 2008). After 3–5 d of culture, Th1 and Th2 cells were restimulated with anti-CD3/anti-CD28 antibodies for 6 h in the presence of Golgi-Stop (BD). The proportions of Th1 cells secreting IFN-γ and Th2 cells secreting IL-4 and IL-10 were determined by intracellular anti-cytokine antibody staining followed by flow cytometric analysis. Antibodies and cytokines were purchased from eBioscience.

Analyses of caspase activities in vivo

Caspase activities were assessed using CaspACE FITC-VAD-FMK In Situ Marker (Promega) according to manufacturer's instructions. In brief, purified T cells stimulated with anti-CD3 and anti-CD28 antibodies for 24 h were treated with CaspACE FITC-VAD-FMK In Situ Marker for 20 min and analyzed by FACS.

Analyses of T cell proliferation

T cells were enriched from spleen and LNs by depletion of B cells using anti-B220 antibody-conjugated magnetic beads (Invitrogen). T cell purity was generally >95% as determined by FACS analysis. For analysis of proliferation, purified T cells (1×10^5) were placed into round-bottom 96-well plates in RPMI-1640 media containing 10% FCS and 0.1% β-mercaptoethanol, and were stimulated in triplicate with precoated anti-CD3 antibody (5 µg/ml) with or without plate-coated anti-CD28 antibody (1 µg/ml) or IL-2 (100 U/ml). T cells were pulsed for the last 18 h of culture with 1 µCi [^{3}H]thymidine (GE Healthcare) per well and harvested at 48 and 72 h after start of culture. Purified T cells were also labeled with CFSE and cultured with anti-CD3/anti-CD28 for 3 d with or without 10 µM Nec-1.

RNA extraction, cDNA synthesis, and real-time PCR analysis

To assess the gene expression levels in peripheral T cells, purified T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 6 h. Total RNA was isolated from activated purified T cells using TRIZOL reagent (Invitrogen) according to the manufacture's instructions. 1 µg of total RNA was reverse transcribed by Superscript II (Invitrogen) in the presence of oligo(dT) primers according to the manufacturer's instructions. Real-time PCR was performed using the 7900HT Fast Real Time PCR System, and Power SYBR Green PCR Master Mix (both from Applied Biosystems). Gene expression levels were normalized to β-actin expression levels.

Primers for real-time PCR

The following primers were used: *IL-2* (5′-CCTGAGCAGGATGGAGAATTACA-3′ and 5′-TCCAGAACATGCCGCAGAG-3′), *IL-4* (5′-ACAGGAGAAGGGACGCCAT-3′ and 5′-GAAGCCCTACAGACGAGCTCA-3′), *IL-6* (5′-TAGTCCTTCCTACCCCAATTTCC-3′ and 5′-TTGGTCCTTAGCCACTCCTTC-3′), *IFN-γ* (5′-ATGAACGCTACACACTGCATC-3′ and 5′-CCATCCTTTTGCCAGTTCCTC-3′), *TNF* (5′-CCCTCACACTCAGATCATCTTCT-3′ and 5′-GCTACGACGTGGGCTACAG-3′), *β-actin* (5′-TATTGGCAACGAGCGGTTC-3′ and 5′-CCATACCCAAGAAGGAAGGCT-3′).

Enzyme-linked immunosorbent assay

Levels of antinuclear autoantibodies and immunoglobulins in sera of mice were quantified using the ANA mouse ELISA kit (Alpha Diagnostic International) and SBA clonotyping system/HRP and mouse immunoglobulin

isotype panel (SouthernBiotech), respectively. Levels of serum TNF were quantified using the mouse TNF ELISA Ready-SET-Go kit (eBioscience).

Western blot and immunoprecipitation analysis

Total protein extracts from cells were prepared using modified RIPA buffer (2 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.025% SDS, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets [Roche]). For cytoplasmic protein extraction, cells were resuspended by lysis buffer (10 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM KCL, 1 mM DTT, 0.1 mM Na_3No_4, and protease inhibitor cocktail tablets) and incubated for 15 min on ice. After incubation, 25 µl of 10% NP-40 was added and cells were incubated for 5 min on ice. Cells were centrifuged for 30 s at 13,000 rpm, and supernatant was collected for cytoplasmic fraction. Nuclear extraction buffer (25 mM Hepes, pH 7.5, 500 mM NaCl, 1 mM DTT, 10 mM NaF, 10% glycerol, 0.2% NP-40, and 5 mM $MgCl_2$) was added to the pellet. The pellet was centrifuged for 5 min at 13,000 rpm, and the supernatant was collected for the nuclear fraction. For immunoprecipitation experiments, protein extracts from cells were prepared using 150 mM NaCl-Tris buffer (150 mM NaCl, 50 Mm Tris-HCl, pH 7.5, 0.5% Triton X-100, and protease inhibitor cocktail tablets). Proteins were separated on 10% homemade gels. The following antibodies were used in 5% powdered milk (Carnation; Nestle) or 5% bovine serum albumin (Sigma-Aldrich) in TBS-T: anti-phospho-IκBα (Ser32) antibody (Cell Signaling Technology), anti-IκBα antibody (Cell Signaling Technology), anti–NF-κBp65 antibody (Santa Cruz Biotechnology, Inc.), anti-HDAC1 antibody (Cell Signaling Technology), anti-RIPK1 antibody (BD), anti-RIPK3 antibody (Imgenex Corp.), anti-LC3 antibody (Santa Cruz Biotechnology, Inc.), anti-actin antibody (Santa Cruz Biotechnology, Inc.), and anti–caspase-8 antibody (home made). HRP–Protein A (GenScript) was also used.

Immunohistochemistry

Organs fixed in buffered formalin were processed for paraffin-embedded sectioning at 5 µm, and stained with hematoxylin plus eosin (Thermo Fisher Scientific). For immunohistochemistry, paraffin-embedded sections were incubated with anti-B220 or anti-CD3 antibodies. Anti–rat Ig antibodies conjugated to horseradish peroxidase (Dako) and anti–hamster Ig antibodies conjugated to alkaline phosphatase (BD) revealed B220 and CD3 labeling, respectively. Single antibody immunohistochemistry staining was revealed with horseradish peroxidase. Immunoreactivity was revealed by incubation in diamine benzidine and p-nitrophenylphosphate. Cy3-conjugated anti–mouse IgG antibody was used to examine the presence of immune complex deposits in kidney and other tissue sections. Images were captured with a 10× or a 20× HCX lens on a microscope (DM4000B; Leica) equipped with digital camera (DC 300RF; Leica). Images were acquired using Leica Image Manager software. All digital images were imported into Photoshop (Adobe) and adjusted for gain, contrast, and gamma settings.

TEM

Purified T cells were either untreated or activated for 24 h in the presence of anti-CD3 antibody. Cells were pelleted, fixed in cold 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, and postfixed in 1% aqueous osmium tetroxide for 1 h. Samples were rinsed and dehydrated through a series of graded ethanol solutions before embedding in epoxy resin.

Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance of experimental data (P-values, values < 0.05) was determined using the analysis of variance (ANOVA) test. A log-rank test was used to analyze the mortality of mouse cohorts.

Online supplemental material

Fig. S1 shows thymic T cell populations in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ and control mice. Fig. S2 shows the effect of caspase-8 loss in T cells on lymphadenopathy caused by loss of Bim. Fig. S3 shows cytokine production levels and the ratio of Treg in periphery in *tcasp8*$^{-/-}$*Bim*$^{-/-}$ mice. Fig. S4 shows cell cycle analysis, caspase activities, and necroptosis complex in peripheral T cells treated with anti-CD3 and anti-CD28 antibody. Fig. S5 shows that loss of caspase-8 in T cells suppresses lymphocyte infiltration and autoimmune kidney disease of Bim-deficient mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201103053/DC1.

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