Antigen-mediated T cell expansion regulated by parallel pathways of death

Irene L. Ch'en[a], Daniel R. Beisner[a], Alexei Degterev[b], Candace Lynch[c], Junying Yuan[d], Alexander Hoffmann[c], and Stephen M. Hedrick[a,1]

[a]Division of Biological Sciences and Department of Cellular and Molecular Medicine and [c]Department of Chemistry and Biochemistry, University of California, San Diego, CA 92093; [b]Department of Biochemistry, Tufts University Medical School, 136 Harrison Avenue, Stearns 703, Boston, MA 02111; and [d]Department of Cell Biology, Harvard Medical School, 240 Longwood Ave, Boston, MA 02115

Edited by Philippa Marrack, National Jewish Medical and Research Center, Denver, CO, and approved September 12, 2008 (received for review August 15, 2008)

T cells enigmatically require caspase-8, an inducer of apoptosis, for antigen-driven expansion and effective antiviral responses, and yet the pathways responsible for this effect have been elusive. A defect in caspase-8 expression does not affect progression through the cell cycle but causes an abnormally high rate of cell death that is distinct from apoptosis and does not involve a loss of NFκB activation. Instead, antigen or mitogen activated *Casp8*-deficient T cells exhibit an alternative type of cell death similar to programmed necrosis that depends on receptor interacting protein (Ripk1). The selective genetic ablation of caspase-8, NFκB, and Ripk1, reveals two forms of cell death that can regulate virus-specific T cell expansion.

apoptosis | necrosis | NFκB | Ripk1 | autophagy

The introduction of an infectious agent can provoke a small population of antigen-specific T cells to expand by as much as 10,000-fold within ≈8 days, and subsequently undergo an equally dramatic contraction (1–3). One concept to emerge is that lymphocytes are intrinsically programmed to cause their own death (4, 5). Whether they are quiescent naïve T cells (6, 7) or antigen-triggered, rapidly dividing T cells, the loss of survival signals causes a form of cell death characterized as apoptosis (8–11). The dynamics of infection-driven lymphocyte expansion are thus determined by a balance of signals that impact the coordinately regulated processes of quiescence, proliferation, and survival.

The death receptors (DR), such as Fas and TNFRI, mediate an "extrinsic" form of cell death, and yet their downstream mediators, Fadd and caspase-8, were found to be essential survival factors for antigen-mediated lymphocyte activation. T cells deficient for Fadd or caspase-8 do not accumulate normally in response to mitogenic or antigenic stimulation (12–18). Similarly, B cells deficient for Fadd or caspase-8 exhibit defects in Tlr3,4-mediated proliferation and mitogen-dependent antibody responses (19–21). To explain this effect, reports have supported a role for caspase-8 and Fadd in NFκB activation (20, 22–24), whereas other studies show their absence has no effect (18, 19, 21, 25).

Here, we considered an alternate hypothesis related to the observation that in the absence of caspase-8, DR signaling causes necrosis, also termed necroptosis (26–30). We tested the possibility that T cell antigen receptor (TCR)-mediated activation includes mechanisms to hold apoptosis in check, and in turn, the apoptosis circuitry itself prevents necroptosis. We report that T cells deficient for caspase-8, activated by mitogenic or antigenic stimuli, divide at a normal rate but die at a high rate such that their accumulation is greatly reduced. The mechanism of cell death does not take the form of apoptosis nor does it involve a deficiency in NFκB activation. Rather, *Casp8*-deficient T cells undergo Ripk1-dependent necroptosis. We demonstrate that either chemical inhibition of Ripk1 kinase activity or a genetic knockdown of Ripk1 completely rescues TCR-mediated, *Casp8*-deficient T cell proliferation. These results establish a second level of control in lymphocyte proliferation and cell death that is activated in the absence of death-inducing signaling complex (DISC) function.

Results

***Casp8* Deficiency Results in a Loss of T cell Accumulation Without a Defect in Cell Cycle Progression.** Mice with a conditional *Casp8* deletion (19) were crossed with *Cd4cre* mice to produce $Casp8^{f/f}$ and $Casp8^{f/f}$;*Cd4cre* offspring. Analyses by genomic PCR and Western blotting revealed that the vast majority of $Casp8^{f/f}$;*Cd4cre* T cells deleted both copies of *Casp8* and expressed no detectable caspase-8 protein [supporting information (SI) Fig. S1 *A* and *B* and *SI Text*] The mice were further analyzed for cell subsets in the thymus, lymph node, and spleen, and in agreement with the analysis of a similar mouse strain produced by Salmena *et al.* (18), there were no changes in the cell populations or in the number of activated/memory phenotype T cells (Fig. S2 and data not shown).

In a previous analysis, there was found to be a defect in T cell proliferation as measured by the incorporation of ^{3}H-TdR (18). To determine the origin of this defect, T cells were labeled with CFSE, stimulated, and analyzed. For each condition, the same proportion of the culture was analyzed such that the area under each curve is representative of the accumulation of cells whereas the dilution of fluorescence indicates the number of cell divisions. Stimulation with plate-bound, anti-CD3 produced a mitogenic response in T cells from both $Casp8^{f/f}$ and $Casp8^{f/f}$;*Cd4cre* mice with a marked decrease in the accumulation of T cells from $Casp8^{f/f}$;*Cd4cre* mice (Fig. 1*A*). Costimulation through CD28 has been shown to further promote survival through its activation of PI3 kinase, Akt, $BclX_L$, and NFκB (31–33), and yet the further addition of anti-CD28 did not eliminate the reduced accumulation of $Casp8^{f/f}$;*Cd4cre* T cells. These effects were consistently more pronounced in CD8 T cells (Fig. 1*A Lower*). We note that the number of cell divisions and total accumulation of $Casp8^{f/f}$ and $Casp8^{+/+}$;*Cd4cre* T cells were equivalent (data not shown).

To analyze antigen-specific responses, $Casp8^{f/f}$;*Cd4cre* mice were crossed to TCR transgenic *AND* or *OT-I* mice (Fig. S2*D*). T cells were stimulated in culture with pigeon cytochrome *c* peptide or OVA peptide (OVAp), and these experiments mirrored the results using mitogenic stimulation. These data cannot be explained by a small population of T cells that failed to delete the *loxP*-flanked *Casp8* exon for two reasons. First, if the T cells with intact *Casp8* were selectively proliferating, there should emerge evidence of caspase-8 expression after 3 days of proliferation. This was shown

Author contributions: I.L.C. and S.M.H. designed research; I.L.C. and C.L. performed research; D.R.B., A.D., J.Y., and A.H. contributed new reagents/analytic tools; I.L.C. and A.H. analyzed data; and I.L.C. and S.M.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: shedrick@ucsd.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0808043105/DCSupplemental.

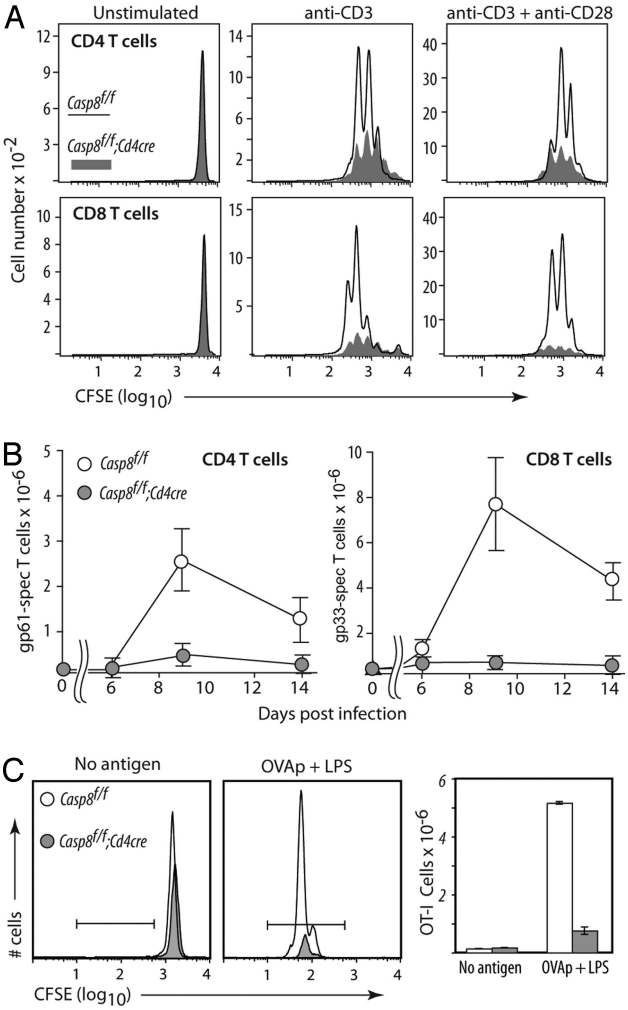

Fig. 1. Reduced accumulation of caspase-8-deficient T cells. (*A*) Purified T cells were labeled with CFSE and cultured for 3 days (representative of 10 experiments). (*B*) *Casp8*$^{f/f}$ and *Casp8*$^{f/f}$;*Cd4Cre* mice were infected with LCMV and splenic T cells were stained for intracellular IFNγ after restimulation in vitro (representative of 4 experiments). (*C*) *OT-I*;*Casp8*$^{f/f}$ and *OT-I*;*Casp8*$^{f/f}$;*Cd4Cre* T cells were adoptively transferred into congenic mice, immunized, and analyzed for live CD8$^+$Vα2$^+$CD45.2$^+$ T cells. Standard error was calculated for each condition (representative of 3 experiments).

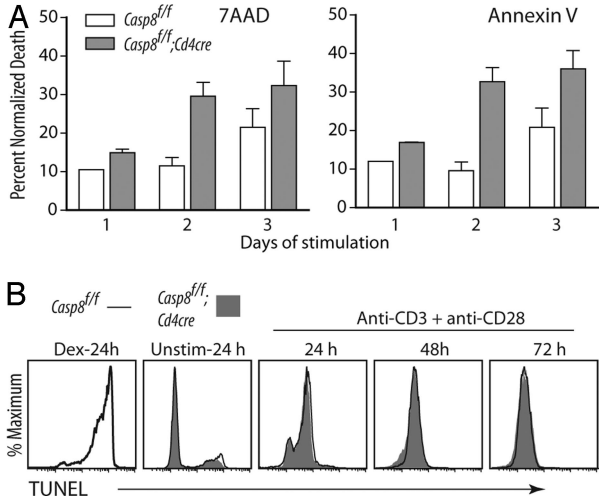

Fig. 2. Caspase-8 deficient T cell death is distinct from apoptosis. (*A*) CD8 T cells were stimulation with anti-CD3 and anti-CD28 and stained with Annexin V and 7AAD (representative of 4 experiments with each experiment normalized to the percentage of WT death at day 1). (*B*) Purified CD8 T cells were cultured and measured for TUNEL (representative of 9 experiments).

not to be true as T cells from *Casp8*$^{f/f}$;*Cd4cre* mice still lacked *Casp8* after 72 h of stimulation (Fig. S2*E*). Second, if the vast majority of T cells that deleted the gene failed to divide, there would be a large fraction of CFSE^{hi} undivided T cells after 3 days. This was also found not to be true (Fig. S2*D* and Fig. 1*A*).

To confirm that the defect occurs in an immune response, *Casp8*$^{f/f}$ and *Casp8*$^{f/f}$;*Cd4cre* mice were infected with lymphocytic choriomeningitis virus (LCMV) and analyzed at days 6, 9, and 14 after infection (Fig. 1*B*). As shown, there was a dramatic difference in the accumulation of both LCMV-specific CD4 and CD8 T cells with a more pronounced difference in CD8 T cells. These data are consistent with results showing differences in the percentage of blood T cells specific for LCMV and an absence of viral clearance (data not shown) (18). This was further demonstrated by the adoptive transfer of T cells from *OT-I* mice challenged with OVAp in the presence of LPS. As shown, *OT-I*;*Casp8*;*Cd4Cre* T cells were deficient in accumulation in response to antigen (Fig. 1*C*).

If activated T cells from *Casp8*$^{f/f}$ and *Casp8*$^{f/f}$;*Cd4cre* mice divide at the same rate, then there should be an equivalent percentage of T cells synthesizing DNA at any given point. T cells were pulsed with BrdU before harvesting and analyzed for BrdU incorporation and the amount of DNA as measured by 7AAD intercalation. As shown, the percentages of T cells synthesizing DNA and in S-G2 phase of the cell cycle were equivalent (Fig. S3*A*). As a second analysis, permeabilized T cells were stained with propidium iodide to detect the proportion of cells in each stage of the cell cycle, and we did not detect a difference in the number of cells in G1 (1n DNA), S, and G2-M phases (>1n DNA) (Fig. S3*B*). As a third analysis, we measured the proportion of adoptively transferred *OT-I* T cells that had incorporated BrdU after 2 days of stimulation with OVAp in vivo (Fig. S3*C*). Although the accumulation of T cells was defective as shown in Fig. 1*C*, the proportion of T cells that synthesized DNA was equivalent. Combined with the analysis of cell divisions by CFSE, these data establish that the reduction in accumulation of *Casp8*-deficient T cells is not the result of a defect in progression through the cell cycle (34, 35) but a decrease in survival associated with cell-cycle progression.

Effect of Caspase-8 on Survival. In principle, apoptotic cells lose membrane asymmetry such that phosphatidyl serine is exposed (Annexin V binding) before undergoing a loss of membrane integrity, whereas necrotic cells have been found to rapidly exhibit membrane permeability to dyes such as 7AAD (36, 37). The culture of disassociated T cells in the absence of overt stimulation resulted in a significant proportion of dead cells positive for both Annexin V and 7AAD (Fig. S3*D Upper*), and previous work showed that much of this cell death can be reversed with the overexpression of Bcl-2 or Bcl-X_L (38–40). The addition of anti-CD3 and anti-CD28 rescued T cell viability, but the level of cell death was greater in *Casp8*$^{f/f}$;*Cd4cre* T cells compared with WT (Fig. S3*D* and Fig. 2*A*). Similar results were found for CD4 T cells (data not shown).

Apoptosis, which is defined morphologically, correlates closely with the presence of DNA fragmentation (4). As depicted in Fig. 2*B*, treatment of CD8 T cells with dexamethasone or culture in the absence of stimulation caused DNA fragmentation as determined by TUNEL, and this was reversed by mitogenic stimulation. These results were also visualized by an absence of DNA laddering in T cells stimulated with anti-CD3 and anti-CD28 (Fig. S3*E*). Finally, from a cell-cycle analysis, we noticed a lack of cells with subdiploid DNA (Fig. S3*B*). We conclude that T cells from *Casp8*-deficient mice can be stimulated to divide at a rate equivalent to WT cells but die at an abnormally high rate by a process that does not involve a

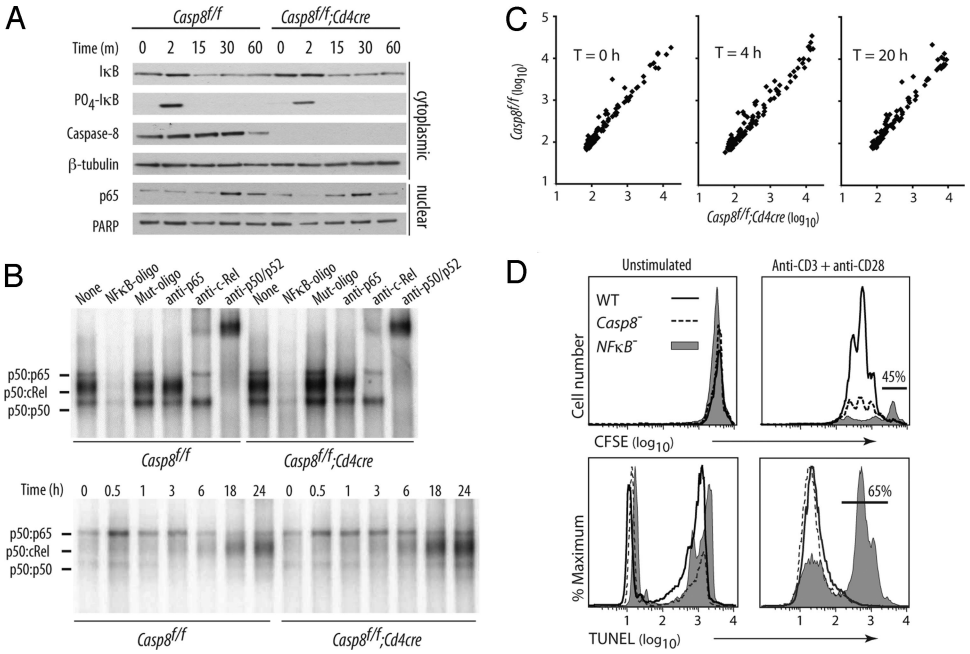

Fig. 3. Loss of caspase-8 in T cells does not affect NFκB signaling. (*A*) Cytoplasmic and nuclear lysates from anti-CD3 and anti-CD28 stimulated cells were resolved and immunoblotted with the antibodies listed above (representative of 5 experiments). (*B*) Nuclear lysates were assessed for NFκB nuclear DNA-binding activity by EMSA. Supershifts were performed on lysates from 24-h stimulated cells (above) (representative of 5 experiments). (*C*) Gene expression levels of NFκB targets were plotted between unstimulated and stimulated *Casp8*$^{f/f}$ and *Casp8*$^{f/f}$;*Cd4Cre* T cells. (*D*) CD8 T cells from *Casp8*$^{f/f}$, *Casp8*$^{f/f}$;*Cd4Cre*, and *NFκB*-deficient mice were stimulated for 3 days and analyzed for CFSE and TUNEL (representative of 3 experiments).

canonical hallmark of apoptosis. This is consistent with our results investigating the mechanism of cell death in activated T cells expressing a dominant-interfering Fadd death domain (17).

Role of NFκB in *Casp8*-Deficient T cells. To determine whether the phenotypic defects described in Fig. 1 can be ascribed to a loss of NFκB activation, we carried out a comprehensive analysis designed to assay the biochemical, transcriptional, and cell biological characteristics of NFκB activation. We first analyzed the degradation of IκB and the nuclear localization of the p65 RelA subunit of the NFκB complex. As shown, IκB was phosphorylated by 2 min and degraded by 15 min in both WT and *Casp8*-deficient T cells (Fig. 3*A*). Also, RelA (p65) was detected in the nucleus and peaked at 30 min subsequent to stimulation. These are characteristics of the canonical NFκB pathway (41, 42), but a direct technique to detect active NFκB is by EMSA (43). EMSA revealed 3 specific bands that could all be competed with unlabeled oligonucleotide but not an oligonucleotide with a mutation in the binding site (Fig. 3*B Upper*). Using specific antibodies, we identified each of the known dimeric complexes, and they were identical regardless of *Casp8* deletion. To determine the kinetics of NFκB activation, nuclear lysates were isolated from T cells stimulated from 0.5–24 h and analyzed (Fig. 3*B Lower*). No difference was observed comparing T cells from *Casp8*$^{f/f}$ and *Casp8*$^{f/f}$;*Cd4cre* mice.

NFκB components are subject to feedback control such that several components are themselves NFκB targets (44). As such, we followed the induction of 8 NFκB components and found no difference in levels of mRNA with time (Fig. S4*A*). In addition, we carried out a global analysis by using Agilent gene array technology. T cells were stimulated for 0, 4, or 20 h, and the RNA was isolated for analysis. Although expression levels of many genes changed quite dramatically as expected, there was no effect of *Casp8*-deletion at any of the stimulation time points (Fig. S4*B*). From these data, 107 known NFκB targets were plotted, and none were changed in *Casp8*-deficient T cells (Fig. 3*C*).

Finally, we sought to compare the phenotype of T cells from WT, *Casp8*-deficient, or *NFκB*-deficient mice. Purified T cells were stimulated and analyzed for cell division and apoptosis. Consistent with the experiments presented in Figs. 1 and 2, the stimulated WT and *Casp8*-deficient T cells divided 3–4 times and exhibited no DNA fragmentation. In contrast, T cells from *NFκB*-deficient mice were defective in cell cycle progression in that 45% of the cells did not divide even once (Fig. 3*D Upper*). In addition, 65% of the cells were positive for DNA fragmentation, measured by either TUNEL (Fig. 3*D Lower*) or DNA laddering (Fig. S3*E*, right 3 lanes). This pattern is in stark contrast to that of *Casp8*-deficient T cells that do not accumulate a population of undivided cells or exhibit TUNEL staining. From these experiments, we conclude that *Casp8*-deficient T cells exhibit reduced survival when stimulated to divide, but this defect cannot be readily explained by a defect in NFκB activation, cell division, or an increase in apoptosis.

T cells Lacking Caspase-8 Die by Ripk1-Dependent Necroptosis. Recently, TNF-induced necrosis was examined by screening for small molecule inhibitors (30). The first compound identified, necrostatin-1 (Nec-1), blocked all of the hallmarks of necrotic death with no detectable effect on apoptotic death caused by FasL and cycloheximide treatment. Nec-1 inhibited the loss of mitochondrial membrane potential and plasma membrane integrity found in TNF-treated Jurkat cells lacking either Fadd or caspase-8. No other signaling pathways were found to be perturbed at concentrations that completely blocked TNF-induced cell death.

To determine whether death in TCR-activated, *Casp8*-deficient T cells was mediated by a similar mechanism, T cells were cultured with and without Nec-1 or a derivative, 7-Cl-O-Nec-1 (Fig. 4). The increase in death observed in *Casp8*-deficient T cells was reduced to WT levels in the presence of necrostatin (Fig. 4 *Upper*). Moreover, the accumulation of *Casp8*-deficient T cells was completely rescued with no effect on WT T cells (Fig. 4 *Lower*). We also tested a series of Nec-1 derivatives and found a perfect correlation between their EC_{50} and ability to rescue TCR-mediated activation of *Casp8*-deficient T cells (45) (Fig. S5).

Notably, Jurkat T cells are susceptible to TNF-induced necrotic cell death in the absence of functional Fadd or caspase-8, and there is a requirement for RIPK1 (29). Recent experiments demonstrated that Necrostatin is a direct inhibitor of Ripk1, with no activity against the related Ripk2 or Ripk3, and acts by stabilizing its kinase-inactive conformation (45). Based on the rescue of T cell survival by Nec-1, our data suggest that Ripk1 is mediating cell death in *Casp8*-deficient T cells. To test this using primary mouse lymphocytes, T cells were stimulated with or without 7-Cl-O-Nec-1, and kinase activity was measured after immunoprecipitation with

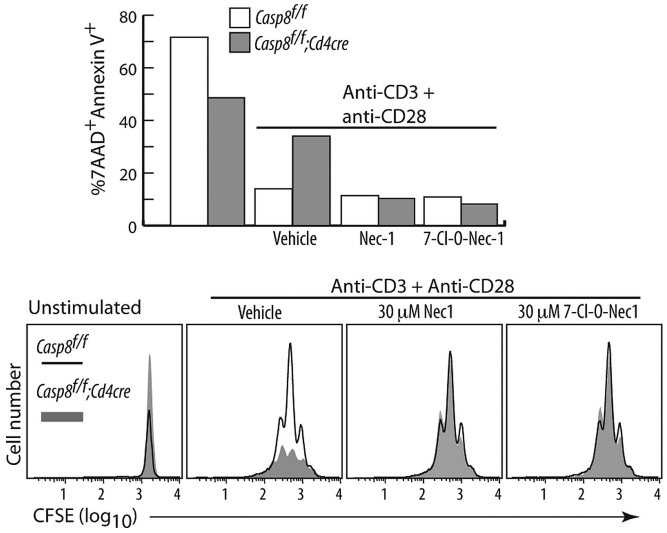

Fig. 4. Caspase-8-deficient death can be rescued by necrostatin-1. Purified T cells were cultured in media alone or stimulated for 3 days in the presence of vehicle (DMSO), necrostatin-1, or 7-Cl-O-Nec-1 (representative of 10 experiments).

anti-Ripk1. As shown in Fig. 5*A*, Ripk1 kinase activity was found in T cells after 48 h of stimulation, and the activity was blocked by the inclusion of 7-Cl-O-Nec-1 in culture. Given that 7-Cl-O-Nec-1 is so highly specific for Ripk1 activity (45), this result shows that the activity does not originate from an associated kinase but rather Ripk1 itself. The amount of Ripk1 immunoprecipitated from *Casp8*-deficient T cells was consistently decreased from that of WT, yet the specific activity from *Casp8*-deficient T cells was enhanced 4-fold (Fig. 5*B*). A second experiment using a different substrate is depicted in Fig. S6.

To determine Ripk1 induction after lymphocyte activation, T cells were stimulated and Ripk1 was detected by direct immunoblotting (Fig. 5*C* and Fig. S6*C*). The data show that the amount of Ripk1 protein was highly induced from 6 to 48 h by mitogenic stimulation (46) but consistent with the immunoprecipitation data in Fig. 5*A*, the induction was diminished in the absence of caspase-8. Previous work demonstrated that Ripk1 can be a substrate for caspase-8 (47–49), and as such, *Casp8*-deficient T cells would be predicted to harbor an excess of Ripk1. As shown clearly in Fig. 5 and Fig. S6, cell death and the increase in Ripk1 activity cannot be simply explained by the absence of caspase-8-mediated Ripk1 proteolysis. To the contrary, in the absence of caspase-8, the total amount of Ripk1 is lower and the specific activity is higher (Fig. 5 and Fig. S6).

To show that Ripk1 mediates cell death in antigen-stimulated *Casp8*-deficient T cells, experiments were undertaken to effect a knockdown of Ripk1 in WT or *Casp8*-deficient T cells. T cells from *OT-I* mice were infected with retrovirus made from the MSCV-LMP vector in which a mi30-based miRNA is expressed from the viral LTR. After spin infection, the T cells were sorted for GFP fluorescence and examined for the level of expressed Ripk1 (Fig. 6*A*). Quantitation of Ripk1 protein by densitometry showed a diminution of ≈80–97% for 3 experiments. Equal numbers of sorted T cells were transferred into congenic recipients, the mice were immunized with OVAp and LPS, and analyzed 2 days later. As shown in Fig. 6*B*, the expansion of *OT-I*;*Casp8*$^{f/f}$;*Cd4Cre* T cells was 30% of the expansion of *OT-I*;*Casp8*$^{f/f}$ T cells, however, with the knockdown of *Ripk1*, WT and *Casp8*-deficient T cells expanded similarly. These results unequivocally demonstrate that *Casp8*-deficient T cells die on antigen-stimulation as a result of Ripk1-dependent cell death.

Discussion

Apoptosis is an integral part of defense against pathogenic agents of disease. As evidence, viruses often encode specific inhibitors of apoptosis, targeting many of the essential components leading to cell death, and in particular, a large number of viruses infecting vertebrates, nematodes, and arthropods have been found to inhibit caspases (50, 51). Because caspase-specific death by apoptosis is an ancient mechanism, most likely present in the common ancestor of nematodes, arthropods, and chordates, a possibility is that this immune-evasion strategy of viruses has selected an alternative pathway of cell death.

The studies presented in this report show that there are two parallel but interdependent pathways of cell death operative during T cell activation. Upon mitogenic or antigenic stimulation, studies have shown that NFκB is activated and promotes cell cycle progression and survival by inhibiting both intrinsic apoptosis through

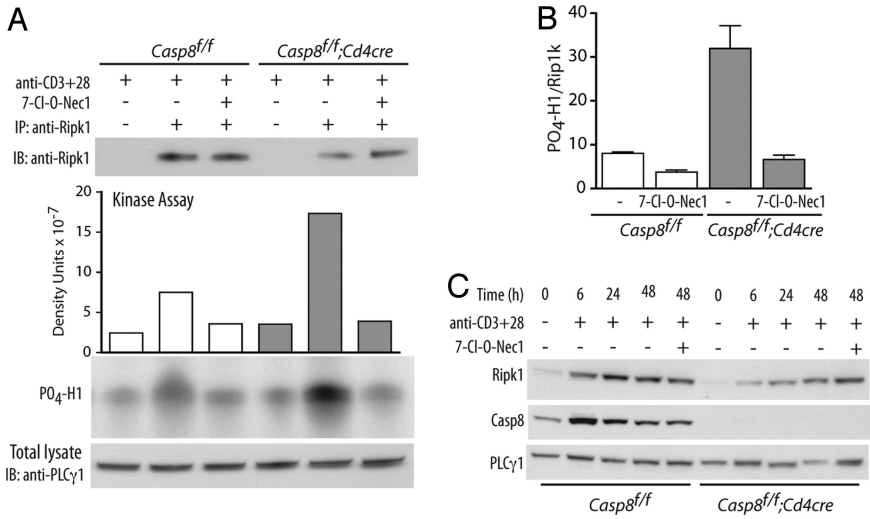

Fig. 5. Ripk1 activity is inducible and inhibited by necrostatin. (*A*) Purified T cells were stimulated 48 h in the presence of vehicle or 7-Cl-O-Nec-1. Kinase reactions were performed with Histone H1 as the substrate (representative of 4 experiments). (*B*) Specific activity was determined by plotting the ratio of Histone H1 phosphorylation to the amount of immunoprecipitated Ripk1. These data are compiled from 4 experiments. (*C*) Purified T cells were stimulated, resolved, and immunoblotted with the antibodies listed above (representative of 12 experiments).

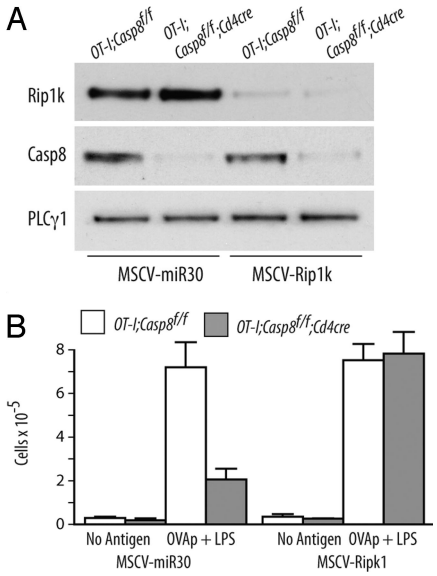

Fig. 6. Ripk1 knockdown rescues a caspase-8 deficiency. (*A*) Ripk1 deletion of day 7 sorted *OT-I* T cells (purity 98%). Average for mi30 transduction was 11,640 arbitrary density units; average for Ripk1-mi30 was 359. (*B*) Splenocytes were gated on live $GFP^+CD8^+V\alpha2^+CD45.2^+$ T cells and plotted with the standard error for 4 mice each (representative of 3 experiments).

the induction of genes such as Bcl-X_L and extrinsic apoptosis through the induction of c-Flip (5, 44, 52–54). The present experiments demonstrate that T cell activation also results in the induction of Ripk1 with the potential to mediate necroptotic cell death. This pathway is held in check by caspase-8, but in its absence, Ripk1 mediates necroptosis (Fig. S7). Whereas the currently accepted paradigm is that caspase-8 is required for NFκB activation, here, we establish that the enigmatic requirement for caspase-8 in *survival* of antigen-activated T cells is to prevent Ripk1-mediated necroptosis. Infection with LCMV, absent caspase-8, invokes a high level of cell death substantially diminishing cell accumulation, and perhaps this mimics a T cell-tropic virus infection that includes a caspase-inhibitory virulence factor. The proposition is that the antigen-induced activation of T cells includes a circuitry that requires an intact DISC to avoid a nonapoptotic death. Because some viruses require T cell proliferation for replication, this, in turn, serves as a sensor to prevent T cell expansion in the absence of a functional caspase-dependent apoptotic pathway. As such, this pathway could be considered a resistance factor that detects, not viruses themselves, but the effects of their anticaspase virulence factors (Fig. S7). This scheme also includes our work showing that B cell proliferation mediated by Tlr3,4, which are the two Tlrs that stimulate through the adapter Trif, also require caspase-8 for expansion. Because Trif, but not other adapters, interacts with Ripk1, we propose that Tlr3,4 activation engages Ripk1, and without caspase-8, B cells die via the necroptosis pathway.

In this report, we elucidate and clarify the effects of a caspase-8 loss-of-function in three aspects of activated T cell physiology: cell cycle progression, NFκB activation, and cell death. Based on the distribution of cell divisions using CFSE, the rate of BrdU incorporation or cell cycle analysis based on DNA content, we show that *Casp8*-deficient T cells undergo cell division at a rate indistinguishable from WT T cells—both in culture and in vivo. Although these results contradict the interpretation of some earlier experiments, especially related to the effects of Fadd and Flip deletion, there can be no doubt that caspase-8 is not required for cell cycle progression (34, 55, 56).

In studying the role of caspase-8 in T cell activation, nothing has been more confounding than the topic of NFκB activation. As discussed in the introduction, one hypothesis was that caspase-8 and Fadd are required for NFκB activation. In the report of a CASP8-deficient family, deficient T cell activation was correlated with a deficit in NFκB p65 nuclear localization (16, 22). The initial study of *Casp8*-deficient mouse T cells found no defect in NFκB activation (18), whereas reanalysis of these mice revealed a defect (22). In the present study, we investigated the TCR-mediated activation of NFκB by analyzing: RelA (p65) nuclear localization; IκB phosphorylation and degradation; EMSA, identifying p50, cRel, and p65 dimers; and NFκB target gene expression. In no instance, looking at many repeat experiments, have we found evidence for an NFκB defect in *Casp8*-deficient T cells. We also show that a loss of NFκB activity has dramatic effects on T cell proliferation and survival. *NFκB*-deficient T cells fail to proliferate, and a large proportion of these T cells fragments their DNA which is indicative of apoptosis—a phenotype completely distinct from anti-CD3-stimulated *Casp8*-deficient T cells. We assert that proliferation defects associated with a *Casp8* deficiency do not result from the absence of NFκB activation or the lack of NFκB-targeted gene expression.

What aspect of lymphocyte physiology is affected by the loss of caspase-8 making stimulated, dividing T cells sensitive to cell death? We found that the process is related to necroptosis, characterized as the TNF- or Fas-ligand-mediated cell death associated with the absence of Fadd or caspase-8. An inhibitor of necroptosis, necrostatin, completely rescued the accumulation of *Casp8*-deficient T cells in culture. Based on the observation that necrostatins block Ripk1 kinase activity (45), we analyzed the activation of Ripk1 to find that it is strongly induced by TCR-mediated activation over a period of 48 h, and its activity is substantially enhanced by the absence of caspase-8. These data suggest that Ripk1-mediated phosphorylation is required for the death of *Casp8*-deficient activated T cells, and this demonstrates a function for the kinase activity of Ripk1. Finally, a knockdown of Ripk1 was sufficient to completely rescue the antigen-induced proliferation of *Casp8*-deficient T cells in vivo.

T cells placed in culture without otherwise being stimulated undergo apoptosis at a high rate, and this "death by dissociation" is apoptotic and Bax/Bak dependent (57). As we show here, apoptosis can be almost completely rescued by anti-CD3 and anti-CD28 activation, and this reversal is NFκB-dependent because T cells from *NFκB*-deficient mice were not rescued by activation. At the same time, T cell activation also induces Ripk1, but in the presence of caspase-8, there is no induction of necroptosis (Fig. S7). Previous work showed that caspase-8 can proteolytically inactivate Ripk1 thus creating a dominant interfering form of Ripk1 with respect to NFκB activation (47, 48). Although we have not detected an increase in the levels of Ripk1 in the absence of caspase-8, we find a reproducible decrease associated with increased activity. Apparently, the presence of caspase-8 inactivates Ripk1 without obviously causing its degradation in T cells. As shown for TNFRI signaling, TRADD, FADD, caspase-8, and Ripk1 can form a complex with c-Flip that is inactive (58). We propose a similar complex is formed upon TCR-mediated, T cell activation, possibly including Atg5 (59), and in the absence of caspase-8, Ripk1 is perhaps uncomplexed and active but less stable. An association of Atg5 implies the possibility of autophagy as an additional pathway to cell death.

In WT T cells, the function of Ripk1 is difficult to discern. It does blunt the proapoptotic activity of TNF by inducing NFκB activation, and perhaps, there are situations in which T cell expansion in vivo occurs in the presence of high amounts of TNF. We propose that its selective value has been to act as a sensor for the absence of caspase-8. We further propose that the absence of caspase-8 signals the presence of viral infection, and in such cells, Ripk1 promotes death via a pathway completely independent of caspase-mediated apoptosis.

Materials and Methods

Mice. *Casp8$^{f/f}$* conditional mice (19) were backcrossed 12 generations to C57BL/6J mice and crossed to *Cd4Cre$^{+/-}$* (Taconic), *OT-I*, and *AND* TCR transgenic mice. *NFκB$^{-/-}$* mice (*cRel$^{-/-}$p50$^{-/-}$p65$^{+/-}$*) have been described (60). Mice were analyzed between 6–12 weeks of age.

T cells. T cells from spleen and lymph nodes were isolated by magnetic separation with biotinylated-B220, -MHCII, -CD11b, and -DX5 (eBioscience) and streptavidin microbeads (Miltenyi Biotech) and assessed for proliferation as described (17). Where indicated, 30 μM necrostatin-1 (Axxora LLC) or 7-Cl-O-necrostatin-1 was used. Cultured cells were resuspended in an equal volume of FACS buffer and collected for a constant length of time on the flow cytometer.

Adoptive Transfer. Donor cells (1.5×10^6) from *OT-I*;*Casp8$^{f/f}$* and *OT-I*;*Casp8$^{f/f}$*;*Cd4Cre* mice were transferred into congenically marked CD45.1 recipients. Mice were immunized with OVAp (257–264, SIINFEKL) and LPS (Sigma–Aldrich) and analyzed 2 days later.

Kinase Assay. Lysates were precleared with Protein G Sepharose (Sigma–Aldrich), quantified, and immunoprecipitated with anti-RIP or IgG2a (BD Biosciences). Substrates used were myelin basic protein (MBP) (Upstate) and Histone H1 (Calbiochem). Kinase activity was quantified with ImageQuant (Molecular Dynamics).

In Vitro Knockdown. A shRNA retroviral construct containing a target sequence for Ripk1 (AAGAGAAAGTTTACCAAATGCT) was created by using the MSCV-LMP (Murine Stem Cell Virus-LTR miR30-PIG) vector (Open Biosystems). Retroviral supernatants were prepared as described (61). Purified *OT-I* T cells were stimulated for 24 h and infections were conducted as described (61). Cells were sorted for GFP expression by FACSAria and adoptively transferred into congenic recipients as described for Adoptive Transfer.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grant AI037988 (to S.M.H.) and by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Disease Training Grant T32DK007233 (to I.L.C.)..

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