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Caspase-8 Acts as a Molecular Rheostat To Limit RIPK1- and MyD88-Mediated Dendritic Cell Activation

Carla M. Cuda,* Alexander V. Misharin,* Angelica K. Gierut,* Rana Saber,* G. Kenneth Haines, III,† Jack Hutcheson,‡ Stephen M. Hedrick,§ Chandra Mohan,‡ G. Scott Budinger,§ Christian Stehlik,* and Harris Perlman*

Caspase-8, an executioner enzyme in the death receptor pathway, was shown to initiate apoptosis and suppress necroptosis. In this study, we identify a novel, cell death–independent role for caspase-8 in dendritic cells (DCs): DC-specific expression of caspase-8 prevents the onset of systemic autoimmunity. Failure to express caspase-8 has no effect on the lifespan of DCs but instead leads to an enhanced intrinsic activation and, subsequently, more mature and autoreactive lymphocytes. Uncontrolled TLR activation in a RIPK1-dependent manner is responsible for the enhanced functionality of caspase-8–deficient DCs, because deletion of the TLR-signaling mediator, MyD88, ameliorates systemic autoimmunity induced by caspase-8 deficiency. Taken together, these data demonstrate that caspase-8 functions in a cell type–specific manner and acts uniquely in DCs to maintain tolerance.


Although caspase-8 is known to function in cell death, conditional deletion studies implicate caspase-8 in a number of cell death–independent activities, including cell motility (3), metastasis (4), suppression of inflammation (5, 6), and NF-κB activation (7). The current paradigm for these alternate roles for caspase-8 is that they are the consequences of unleashed necroptosis (8, 9). However, a number of recent studies point to the idea that caspase-8 may function in an entirely cell death–independent manner. TLR engagement can provoke RIPK signaling independent of DR activation, thereby leading to formation of a ripoptosome, a complex containing similar proteins involved in necroptosis, including caspase-8, RIPK1, cFLIP, and FADD (10). Additionally, ripoptosome and RIPK3 activity were shown to induce production of proinflammatory cytokine IL-1β in a caspase-8–dependent manner (11) independent of cell death. Activation of most TLRs requires the adaptor MyD88, which may lead to the phosphorylation and nuclear translocation of transcription factors IFN regulatory factors (IRFs), causing upregulation of proinflammatory gene expression (12). Previous studies showed that caspase-8 cleaves IRF3, targeting it for degradation and dampening NF-kB-dependent downstream gene induction (13). Taken together, these data suggest that heightened IRF3 transcriptional activity in the absence of caspase-8 may lead to hyperexpression of deleterious downstream IRF3-specific genes.

The vast majority of studies on the Fas-signaling pathway in the immune system and its role in apoptosis and necroptosis have focused on lymphocytes. Loss of Fas in lymphocytes has led to conflicting results (14–16), whereas deletion of caspase-8 yields lymphopenic mice as the result of a failure in proliferation and increased necroptosis (17). Although the phenotype of global and T cell–specific caspase-8 deletion is reversed by RIPK3 deficiency, which suggests that necroptosis is the underlying cause (18), a systemic autoimmunity develops that is similar to germline knockout of Fas (2, 17, 19). Because conditional deletion of Fas or caspase-8 in lymphocytes results in opposite phenotypes, and loss of Fas in dendritic cells (DCs) or overexpression of the general caspase inhibitor p35 in DCs induces a systemic autoimmune disease (14, 20), we investigated the role that caspase-8 plays in DC development and in maintaining tolerance.
Specific deletion of caspase-8 in DCs (CreCD11cCasp8fl/fl) is sufficient to induce a systemic autoimmune disease reminiscent of systemic lupus erythematosus (SLE) that is not a consequence of unleashed necroptosis, because this break in tolerance is neither ameliorated nor exacerbated by RIPK3 deletion. CreCD11cCasp8fl/fl DCs do not display a survival advantage, indicating that defective DC apoptosis is not the underlying cause of the observed inflammation. However, these DCs possess a heightened costimulatory capacity and an elevated response to TLR signaling that is abrogated by RIPK1 inhibition. Interestingly, IRF3 deletion in CreCD11cCasp8fl/fl mice exacerbates the observed break in tolerance. In contrast, concurrent deletion of caspase-8 and MyD88 in DCs (CreCD11cCasp8fl/fl, MyD88fl/fl) mice showed caspase-8 deletion (Supplemental Fig. 1). Transnetyx-sorted splenic conventional DC populations from CreCD11cCasp8fl/fl, MyD88fl/fl, and B6.CD45.1 mice (a gift from Mike Diamond), and B6.CD45.1 OT-II/RAG2−/− mice (The Jackson Laboratory) were bred to B6.CD45.1 OT-II/RAG2−/− mice (B6.CD45.1 OT-II/RAG2−/− CreCD11cCasp8fl/fl mice). Thus, these data demonstrate that caspase-8 in DCs maintains tolerance in a manner that is independent of cell death and IRF3 but requires dampening of RIPK1 and MyD88 signaling.

Materials and Methods

**Mice**

C57BL/6 (B6) mice homozygous for Ioxp-flanked caspase-8 allele (Casp8fl/fl) (21) were crossed with mice expressing Cre under control of the CD11c promoter (CreCD11c; The Jackson Laboratory, stock number 007567), generating CreCD11cCasp8fl/fl mice. PCR on FACS-sorted splenic conventional DC populations (B220−CD11c−CD8− and B220−CD11c+CD8+) from 6–10 wk-old mice showed deletion of caspase-8, but this was not the case for plasmacytoid DCs (CD11c+PDCA-1−B220−). Camperman, and macrophages (Supplemental Fig. 1). CreCD11cCasp8fl/fl bone marrow–derived DCs (BMDCs) showed caspase-8 deletion (Supplemental Fig. 1). OT-1/RAG2−/− and B6.CD45.1 mice were purchased from The Jackson Laboratory. RIPK3−/− mice (Genentech), IRF3−/− mice (a gift from Mike Diamond, Washington University, St. Louis, MO), IRF7−/− mice (a gift from Mike Diamond), and MyD88−/− mice (The Jackson Laboratory) were bred to CreCD11cCasp8fl/fl mice, generating IRF3−/−CreCD11cCasp8fl/fl, IRF3−/−/CreCD11cCasp8fl/fl, IRF7−/−/CreCD11cCasp8fl/fl, and MyD88−/−/CreCD11cCasp8fl/fl mice. Real-time PCR performed by Transnetyx on FACS-sorted splenic conventional DC populations from MyD88−/−/CreCD11cCasp8fl/fl mice showed caspase-8 and MyD88 deletion (Supplemental Fig. 1). Female mice were used in all studies. Proteinuria was assessed using Uristix (Siemens). Transnetyx performed genotyping. Experiments were approved by Northwestern University’s Institutional Animal Care and Use Committee.

**Histopathologic studies**

Paraffin-embedded kidney sections (4 μm) were stained with periodic acid–Schiff (PAS), and a pathologist blinded to the study scored kidney sections using an Olympus BX41 microscope, as previously described (22). Frozen kidney sections (4 μm) were stained with anti-IgG–FITC (22). All images were photographed at ×400, ×200, or ×400 magnification using an Olympus BX41 microscope equipped with an Olympus DP20 camera.

**Flow cytometry**

Surface staining of cell suspensions and gating strategies were accomplished as previously described (23, 24). At least 100,000 events were acquired on a BD LSR II instrument. Data were analyzed with FlowJo software (TreeStar). Dead cells were excluded using Aqua Live/Dead staining (Invitrogen). For cell-sorting studies, splenocytes preincubated with Fc-block were stained with fluorochrome Abs (information available upon request). Spleenocyte populations sorted on a BD FACSaria II instrument at the University of Chicago’s Flow Core had an average purity of 97%.

**Bone marrow chimeras**

Bone marrow was aspiratively harvested from tibias, femurs, and humeri from 9-wk-old mice, erythrocytes were lysed (BD Pharm Lyse buffer), and cells were incubated with Fc-block, followed by incubation with PE-conjugated Abs against B220, CD4, CD8, CD11b, Ly6G, NK1.1, Siglec F, and CD16/32 (eBioscience). Cells were incubated with anti-PE MicroBeads (Miltenyi Biotec), and PE-labeled lineage cells were depleted (AutoMACS separator). Three-month-old B6.CD45.1 mice received a single 1000-cGy gamma radiation dose using a 137Cs-based Gammacell 40 irradiator (Nordion). After 12 h, 1.2 × 10⁶ lineage-depleted cells from Casp8fl/fl mice, Casp8fl/fl, and B6. CD45.1/2 mice (1:1 ratio), or CreCD11cCasp8fl/fl and B6.CD45.1/2 mice (1:1 ratio) were injected i.v. Presorted cells were stained with c-Kit (eBioscience) and Sca-1 (BioLegend) to analyze the LSK faction. Chimeric mice were maintained on autovclaved water plus antibiotics (Trimetoprim/sulfamethoxazole; Hi-Tech Pharmacal) for 4 wk after transfer and were phenotype 18 wk after transfer.

**In vivo assays**

For TLR ligand–injection studies, 3-mo-old mice were injected i.p. with LPS, imiquimod, or CpG (200 μg/20 g body weight; Invivogen) and analyzed 4 h later by flow cytometry. For oral antibiotic treatment, 3-wk-old mice were given autovclaved water with ampicillin (1 g/l), vancomycin (0.5 g/l), neomycin sulfate (1 g/l), metronidazole (1 g/l), and sucrose (10 g/l) twice a week for 8 wk, with no observable weight loss. For BrdU assays, mice were injected i.v. with 1 mg BrdU (BD Biosciences) for 3 d. On days 0, 1, and 3 postinjection, splenocyte and bone marrow suspensions were prepared as described above. After surface staining, cells were processed with BrdU staining kits (BD Biosciences), according to the manufacturer’s instructions. Fluorescence minus one controls were used to set gates for BrdU+ populations.

**In vitro assays**

For MLRs, splenocytes were incubated with anti-CD19 beads, and negative fractions were incubated with anti-CD11c MACs beads (Miltenyi Biotec) to purify APCs. Purified APCs were pulsed with 0.1 μg/ml OVA peptide (aa 323–339) for 60 min at 37˚C. OVA-specific splenic CD4+ T cells were isolated from B6.CD45.1/2 OT-1/RAG2−/− mice using a CD4+ T Cell Isolation Kit (Miltenyi Biotec), according to the manufacturer’s instructions. Purity of APCs and T cells was 90%. T cells were labeled with CFSE (500 nM for 12 min at 37˚C; Invitrogen). Pulsed APCs at various ratios were incubated with 2 × 10⁶ CFSE-labeled T cells, with or without 5 μg/ml CpG-B (ODN 1668), in triplicate in 96-well flat-bottom plates at 37˚C for 3 d. Cell clusters were dissociated with 7.5 mM EDTA for 15 min and stained with anti-CD4 (BD Biosciences), 7-Aminoactinomycin D (0.25 mg/ml; BD Biosciences) was used to exclude dead cells. A constant number of CaliBRITE beads (BD Biosciences) was added for acquisition of equal parts/culture. Live T cells were gated, and the number of divided cells showing less than maximal CFSE fluorescence intensity was determined. For cell death assays, bead-sorted CD4+ T cells were incubated with anti-CD3 (0.5 μg/ml) and anti-CD28 (1 μg/ml; both from BD Biosciences), with or without zVAD-FMK (20 μM; Promega) and necrostatin-1 (30 μM; Enzo Life Sciences), and stained with Annexin V (Invitrogen) and Aqua Live/Dead 72 h later, according to the manufacturer’s instructions.

BMDCs were generated as described (25). Briefly, bone marrow was resuspended in complete media with 50 μM 2-ME and cultured for 2 h. A total of 1 × 10⁶ nonadherent cells was plated in 24-well plates containing 1 ml complete media plus GM-CSF (10 ng/ml) and Flt3-L (50 ng/ml; both from PeproTech). Two thirds of the media was replaced on day 3. On days 5 and 7, nonadherent cells were transferred into six-well plates in media plus cytokines (2.5 × 10⁶ cells/well/ml) for 2 d, and BMDCs were used on day 9 at a concentration of 1.75 × 10⁶/ml. Supernatants and nuclear lysates from BMDCs that were stimulated for 6 h at 37˚C in 5% CO₂ with LPS (10 ng/ml; Sigma-Aldrich), CpG (5 μg/ml), and imiquimod (5 μg/ml) were evaluated for cytokine levels and transcription factor binding, respectively (see below). Total cell lysates from BMDCs that were stimulated for 30 or 60 min at 37˚C in 5% CO₂; with imiquimod (5 μg/ml) were evaluated for transcription factor expression levels. BMDCs also were stimulated for 6 h at 37˚C in 5% CO₂ with LPS (10 ng/ml), CpG (5 μg/ml), and imiquimod (5 μg/ml), with or without necrostatin-1 (30 μM), and/or zEtD-FMK (20 μM; BD Biosciences), and/or 1-methyl-b-tryptophan (30 μM; Sigma-Aldrich), and supernatants were evaluated for cytokine levels. ATP (5 μM; Sigma-Aldrich) was added for 30 min and then media were replaced for an additional hour to evaluate IL-1β levels. For cell death assays, 3 × 10³ BMDCs or total splenocytes were stimulated for 10 h at 37˚C in 5% CO₂ with SuperFasLigand (100 ng/ml; Enzo Life Sciences) or etoposide (10 μM; Alexis Biochemicals), with or without necrostatin-1 (30 μM), and then stained with Annexin V and Aqua Live/Dead.

**Ab/cytokine measurements and transcription factor binding and expression quantification**

Anti-nuclear Abs, including anti-dsDNA, anti-ssDNA, or anti-chromatin, were measured as previously described (22). Total IgM and IgG isotypes

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and cytokine/chemokine expression were quantified using Luminex-based assays (Affymetrix). Transcription factor analysis was accomplished using Nuclear Extraction and Procarta TF Plex Kits, according to the manufacturer’s instructions (Affymetrix). Immunoblot analysis was performed as previously described (26), and the concentrations of the primary Abs were as follows: 1:1000 for rabbit anti-IRF3 Ab (Cell Signaling), 1 mg/ml for rabbit anti-IRF7 Ab (Abcam), and 1:500 for mouse anti-GAPDH Ab (US Biological).

Statistical analysis
GraphPad Prism 5.0 software was used for statistical analyses. Data are mean ± SD and were compared using the Mann–Whitney U test, unless otherwise noted.

Results
Mice with conditional deletion of caspase-8 in DCs develop a chronic systemic autoimmune disease
We examined the consequences of DC-specific deletion of caspase-8 (CreCD11cCasp8fl/fl). Loss of caspase-8 in DCs led to splenomegaly and lymphadenopathy in young (Supplemental Fig. 2A, 2B) and aged (Fig. 1A–C) mice. However, the observed splenomegaly in aged CreCD11cCasp8fl/fl mice was not attributed to the increased numbers of splenocytes (Fig. 1D) or CD45− cells or Ter119+ cells (Supplemental Fig. 2C, 2D). Additionally, there was a disruption of the splenic architecture in CreCD11cCasp8fl/fl mice compared

FIGURE 1. Mice with DC-specific deletion of caspase-8 exhibit systemic autoimmunity. (A–J) Eight-month-old female Casp8fl/fl (control) and CreCD11cCasp8fl/fl mice (n = 10) were evaluated for systemic autoimmune disease phenotypes. (A) Representative spleens and lymph nodes. (B) Splenomegaly. (C) Lymphadenopathy. (D) Number of splenocytes. (E) PAS-stained formalin-fixed kidney sections and anti-IgG–FITC–stained frozen kidney sections. (F) Kidney score. (G) Proteinuria. Serum levels of chromatin-, dsDNA-, and ssDNA-reactive IgG Abs (H), pathogenic IgG2a Abs (I), and cytokines and chemokines (J). Data are mean ± SD. *p < 0.05, **p < 0.005, ***p < 0.0005, Mann–Whitney U test. (K) Survival curve. **p < 0.005, log-rank Mantel–Cox test.
with control mice, as shown by the expansion of white pulp and reduction of red pulp (Supplemental Fig. 2E). There also was a slight increase in splenic collagen deposition in Cre\(^{CD11c}\)Cas\(^{p0/0}\) mice compared with control mice and no detectable liver pathology (Supplemental Fig. 2E). Cre\(^{CD11c}\)Cas\(^{p0/0}\) mice developed glomerulonephritis (Fig. 1E, 1F), IgG deposition in the kidney (Fig. 1E), and proteinuria compared with control mice (Fig. 1G). Cre\(^{CD11c}\)Cas\(^{p0/0}\) mice also exhibited spontaneous early mortality beginning at 7 mo of age; 50% of the mice died by 11 mo of age (Fig. 1K).

**Inflammation is independent of DC survival in DC-specific caspase-8–deficient mice**

Stimulation with FasL had a minimal effect on the survival of BMDCs (Fig. 2A) and splenic DCs (Fig. 2B), regardless of the presence of caspase-8, consistent with previous studies (27, 28). Splenic T cells from control and DC-specific caspase-8–deficient mice displayed similar levels of FasL-induced death (Fig. 2C) and etoposide-induced death (Fig. 2D). Consistent with recent studies (29), necrostatin-1, an inhibitor of RIPK1 kinase activity and

**FIGURE 2.** Inflammation related to DC-specific caspase-8 deficiency is independent of DC survival. Cas\(^{p0/0}\) (control) and Cre\(^{CD11c}\)Cas\(^{p0/0}\) BMDCs (n = 4) (A) and total splenocytes (n = 3) (B and C) were stimulated with SuperFasLigand (sFasL), with or without necrostatin-1 (Nec-1), for 10 h and stained with Annexin V and Aqua Live/Dead. Total splenocytes were gated into CD11c\(^{+}\) (B) and CD4/8\(^{+}\) (C) populations for analysis. (D) Additionally, control and Cre\(^{CD11c}\)Cas\(^{p0/0}\) BMDCs were stimulated with etoposide for 10 h and stained with Annexin V and Aqua Live/Dead. Data are the percentage of live divided by the unstimulated condition. (E and F) CD4\(^{+}\) T cells (n = 3), stimulated for 72 h with anti-CD3 and anti-CD28, with or without pan-caspase inhibitor zVAD-FMK (zVAD) and Nec-1, were stained with Annexin V and Aqua Live/Dead. Data are the percentage of live divided by the anti-CD3/28 condition. Control and Cre\(^{CD11c}\)Cas\(^{p0/0}\) mice (n = 4), injected with BrdU for 3 d (G and H), were evaluated for the percentage of splenic BrdU\(^{+}\) CD11c\(^{+}\)CD8\(^{+}\) (L) and CD11c\(^{+}\)CD8\(^{-}\) (M) conventional DCs. (I-M) Mice were reconstituted with equal portions of B6.CD45.1/2 (WT) and either control or Cre\(^{CD11c}\)Cas\(^{p0/0}\) bone marrow (n = 5). (I) Representation of experimental design. (J) Representative Lin\(^{+}\)Sca-1\(^{+}\)c-kit\(^{+}\) bone marrow cell percentages from 3-mo-old female control, Cre\(^{CD11c}\)Cas\(^{p0/0}\), and WT mice. Chimeric mice were evaluated 3 mo after transfer for numbers of conventional DCs (K), distribution of WT (45.1/2) and control or Cre\(^{CD11c}\)Cas\(^{p0/0}\) (45.2) derived conventional DCs (L), splenomegaly (M), and lymphadenopathy (N). Data are mean ± SD. *p < 0.05, **p < 0.005, ***p < 0.0005, Mann–Whitney U test.
necroptosis, had no effect on the viability of DCs (Fig. 2A, 2B, 2D), but it rescued activated T cells cultured with the pan-caspase inhibitor zVAD-FMK (Fig. 2E, 2F).

Previous studies evaluating mice lacking Fas in DCs or over-expressing the p35 caspase inhibitor suggested a link between an autoimmune disease phenotype and deficiency in DC apoptosis (14, 20). Because caspase-8 is a downstream signaling component of Fas, and p35 inhibits all caspase activity, DC survival was examined using in vivo BrdU pulse chase assays and mixed chimeras. BrdU pulse chase assays showed no difference in DC turnover rates between CreCD11cCasp8fl/fl and control conventional DCs (Fig. 2G, 2H). Additionally, loss of caspase-8 in DCs did not result in enhanced survival, because splenic conventional DC numbers remained unchanged in mixed chimera mice (wild-type [WT]+CreCD11cCasp8fl/fl, Fig. 2I, 2J). Of note, the transferred LSK population (Lin−Sca-1+c-kit+) was similar in CreCD11cCasp8fl/fl, Casp8fl/fl, and WT mice (Fig. 2K). Further, there was no survival advantage attributed to the loss of caspase-8 in DCs, because there were no differences in the number of CreCD11cCasp8fl/fl and WT-derived splenic conventional DCs in mixed chimera mice (Fig. 2L), consistent with parallel DC turnover rates in BrdU pulse chase assays. In contrast, hallmarks of systemic autoimmunity, including splenomegaly (Fig. 2M) and lymphadenopathy (Fig. 2N), persisted in mixed chimera mice.

RIPK3 knockout fails to reverse the consequences of DC-specific caspase-8 deletion

Because RIPK3 knockout reverses the phenotype in global and T cell–specific caspase-8–deficient mice (18, 19), we crossed CreCD11cCasp8fl/fl mice to RIPK3−/− mice. Young RIPK3−/−CreCD11cCasp8fl/fl mice developed splenomegaly (Supplemental Fig. 2A), but lymphopenopathy was abated compared with CreCD11cCasp8fl/fl mice (Supplemental Fig. 2B). However, with age, RIPK3−/−CreCD11cCasp8fl/fl mice exhibited splenomegaly and lymphopenopathy at unchanged and exacerbated levels, respectively, compared with CreCD11cCasp8fl/fl mice (Fig. 3A–C). RIPK3−/−CreCD11cCasp8fl/fl mice also presented with glomerulonephritis (Fig. 3D, 3E) and IgG deposition in the kidney (Fig. 3D), although proteinuria was reduced compared with CreCD11cCasp8fl/fl mice (Fig. 3F). Further, although serum levels of chromatin-reactive, dsDNA-reactive, and ssDNA-reactive IgG Abs (Fig. 3G) were unaffected by the additional loss of RIPK3, pathogenic IgG2a Abs (Fig. 4I), pathogenic IgG2a Abs (Fig. 4J), and proinflammatory molecules, including IL-6, TNF-α, and sRANKL (Fig. 4K), were increased in RIPK3−/−CreCD11cCasp8fl/fl mice, compared with CreCD11cCasp8fl/fl mice.

Caspase-8–deficient DCs are hyperresponsive to TLR activation

Because of the chronic systemic inflammation observed in CreCD11cCasp8fl/fl mice regardless of the presence of RIPK3, we assessed the response to TLR agonists in the absence of caspase-8. Of note, expression of TLR2/4/7/9 was either unchanged or reduced in CreCD11cCasp8fl/fl splenic DC subsets compared with controls (Supplemental Fig. 3A). To determine the functional response of these TLRs in caspase-8–deficient DCs, BMDCs were treated with CpG, imiquimod, or LPS. CreCD11cCasp8fl/fl BMDCs produced higher levels of IL-12/23p40, IL-6, TNF-α, and IL-1β compared with control BMDCs in response to TLR7 (Fig. 5A), TLR9, and TLR4 activation (Supplemental Fig. 3B) without inducing cell death (Supplemental Fig. 3C). The loss of caspase-8 was sufficient to induce IL-1β release without the addition of ATP (Supplemental Fig. 3D). To expand upon these studies, the aforementioned TLR agonists were injected i.p. into mice. Although CpG and LPS had no effect on expression of activation markers in CreCD11cCasp8fl/fl mice (data not shown), imiquimod induced a large increase in CD86 expression and led to elevated MHC class II and CD40 expression on CreCD11cCasp8fl/fl CD11c+CDCD8− conventional DCs compared with controls (Fig. 5B, 5C).

Although DCs do not undergo necroptosis, we examined whether the necrosome has an effect on proinflammatory cytokine production. Necrostatin-1 inhibited secretion of IL-6, TNF-α, and IL-1β in all TLR agonist–treated caspase-8–deficient BMDC cultures, whereas it only reduced IL-12/23p40 secretion mediated by TLR9 activation (Fig. 5A, Supplementary Fig. 3B). Because necrostatin-1 was shown to block RIPK1, as well as IDO, the IDO2-specific inhibitor 1-methyl-o-tryptophan (1-MT) (31) was added to BMDC cultures as a control. 1-MT had no effect on cytokine secretion, with the exception of CpG-induced IL-12/23p40 production in BMDCs. The caspase-8–specific inhibitor zIETD-FMK was also added to BMDCs to address the requirement for caspase-8 enzymatic activity in the hypersecretion of cytokines and other proinflammatory molecules. Interestingly, specific blockade of caspase-8 activity did not mimic the deletion of caspase-8 in BMDCs (Fig. 5A, Supplementary Fig. 3B).

Caspase-8 suppresses MyD88 signaling

Over the past several years, gut microflora have been suggested to be a depot for TLR signaling (32). To reduce the potential for
endogenous TLR ligands from gut microflora to exacerbate SLE-like disease, young Cre\textsuperscript{CD11c}\textsuperscript{CD11b}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice were treated with oral antibiotics for 2 mo. Oral antibiotic treatment had no effect on disease development in Cre\textsuperscript{CD11c}\textsuperscript{CD11b}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice, because splenomegaly (Fig. 6A) and lymphadenopathy (Fig. 6B) were unchanged compared with untreated Cre\textsuperscript{CD11c}\textsuperscript{CD11b}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice.

Because of the heightened production of proinflammatory cytokines associated with caspase-8 deficiency, we sought to restrict TLR activation in our model by eliminating MyD88. Concurrent deletion of caspase-8 and MyD88 in DCs was sufficient to reduce splenomegaly and lymphadenopathy in aged Cre\textsuperscript{CD11c}\textsuperscript{CD11b}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice (Fig. 6C, 6D), but it did not affect splenocyte numbers (Fig. 6E).

Importantly, loss of MyD88 ameliorated kidney disease observed in Cre\textsuperscript{CD11c}\textsuperscript{CD11b}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice, as shown by reduced glomerulonephritis (Fig. 6F, 6G) and IgG deposition (Fig. 6F), although proteinuria levels (Fig. 6H) were unchanged. Further, although MyD88\textsuperscript{fl/fl}Cre\textsuperscript{CD11c}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice exhibited similar levels of chromatin- and dsDNA-reactive IgG Abs (Fig. 6I) and pathogenic IgG2a Abs (Fig. 6J), there was a trend toward reduced ssDNA-reactive IgG Abs (Fig. 6I) and significantly less proinflammatory serum cytokines/chemokines, including IL-22 and MCP-3 (Fig. 6K), compared with Cre\textsuperscript{CD11c}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice.

**DC-specific loss of caspase-8 intensifies DC and lymphocyte activation**

To determine the cellular mechanism responsible for the systemic autoimmunity that develops in Cre\textsuperscript{CD11c}\textsuperscript{Casp8\textsuperscript{fl/fl}} mice, multiparameter flow cytometry was used. Cre\textsuperscript{CD11c}\textsuperscript{Casp8\textsuperscript{fl/fl}} CD4\textsuperscript{+} T cells,
FIGURE 4. Deletion of IRF3 exacerbates the systemic inflammation in Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> mice. (A) Casp8<sup>fl/fl</sup> (control) and Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> BMDCs were stimulated with CpG, imiquimod, or LPS for 6 h, and isolated nuclear lysates were subjected to a multianalyte transcription factor bead-based assay. Data are represented as the fold change over unstimulated cells. (B) Control and Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> BMDCs were stimulated with imiquimod, and isolated total cellular lysates were subjected to immunoblot analysis for total IRF3. The blot was then stripped for total IRF7 and GAPDH expression, and the figures were cropped and pieced together. (C–K) Seven-month-old female Casp8<sup>fl/fl</sup> (control), Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, IRF3<sup>−/−</sup>Cas<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, and IRF7<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> mice (n ≥ 5) were evaluated for systemic autoimmune disease phenotypes. (C) Splenomegaly. (D) Lymphadenopathy. (E) Number of splenocytes. (F) PAS-stained formalin-fixed kidney sections and anti-IgG–FITC–stained frozen kidney sections. (G) Kidney score. (H) Proteinuria. Serum was evaluated for levels of chromatin-, dsDNA-, and ssDNA-reactive IgG Abs (I), pathogenic IgG2a Abs (J), and cytokines and chemokines (K). Data are mean ± SD. ∗p < 0.05, ∗∗p < 0.005, ∗∗∗p < 0.0005, control versus Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, Mann–Whitney U test. ∗p < 0.05, ∗∗p < 0.005, Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> versus IRF3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> or IRF7<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, Mann–Whitney U test.
but not CD8+ T cells, were increased in numbers (Fig. 7A, 7B). Further, both CD4+ and CD8+ T cell populations showed decreased naive (CD44+CD62L+) and increased effector (CD44+CD62L-) subsets compared with controls (Fig. 7C, Supplemental Fig. 4A, 4B). Elevated expression of PD-1 on CD8+ T cells and CD69 and PD-1 on CD8+ T cells in CreCD11cCasp8f/f mice further defined hyperactive T cell subsets (Supplemental Fig. 4C–F). Regulatory T cells (CD4+CD25Foxp3+) were present at greater numbers in CreCD11cCasp8f/f mice, and these cells expressed elevated PD-1 (Supplemental Fig. 4G, 4H). Although the loss of caspase-8 in DCs did not affect total B cell numbers (Fig. 7D), CreCD11cCasp8f/f mice displayed an increase in marginal zone (MZ) B cells. CreCD11cCasp8f/f total B cells expressed less IgD, indicating increased maturation and class-switching, and expressed more CD80, CD86, and PD-1 (Fig. 7E, Supplemental Fig. 4I–L), which correlates with the elevated Ab and autoantibody production observed in CreCD11cCasp8f/f mice.

Because T and B cell activation is associated with DC functionality, CreCD11cCasp8f/f DC populations were evaluated. CreCD11cCasp8f/f mice showed more splenic CD11c+CD8+ conventional DCs and less CD11c+CD8- conventional and plasmacytoid DCs compared with control mice (Fig. 7F). Further, both CreCD11cCasp8f/f CD11c+CD8+ conventional and plasmacytoid DCs were hyperactivated (Fig. 7G, Supplemental Fig. 4M), as indicated by increased expression of costimulatory molecules CD80 and CD86 and activation marker CD69, respectively. Similar to DC subsets, CreCD11cCasp8f/f mice exhibited increased numbers of Ly6Clo- and Ly6Chigh splenic monocytes/macrophages and neutrophils compared with control mice (Supplemental Fig. 4M). In addition, caspase-8-deficient CD11c+ cells incubated with OVA-peptide induced heightened OT-II-specific CD4+ T cell proliferation with and without TLR9 activation (Fig. 7H) as compared with control CD11c+ cells.

Knockout of RIPK3 or IRF3 not only exacerbated disease phenotypes, but also exaggerated the immune cell dysregulation found in CreCD11cCasp8f/f mice. Loss of RIPK3 increased the CD4+ (Fig. 7A) and regulatory (Supplemental Fig. 4G) T cell populations and heightened CD4+ and CD8+ T cell activation, as seen by increased skewing toward an effector phenotype (Figure 7C and Supplemental Fig. 4A, 4B) and elevated expression of CD69 on CD4+ T cells (Supplemental Fig. 4C) and PD-1 on CD4+, CD8+, and regulatory T cells (Supplemental Fig. 4D, 4F, 4H). RIPK3 deletion also modified B cells by reducing IgD levels (Fig. 7E, Supplemental Fig. 4I), indicating increased maturation, compared with caspase-8 deficiency alone. Systemic RIPK3 deletion increased DC numbers (Fig. 7F), and modified CD11c+CD8- conventional DC activation by decreasing CD80 expression (Fig. 7G) to control levels while increasing CD86 expression (Fig. 7G). Although loss of IRF3 did not affect T cell numbers overall (Fig. 7A, 7B), effector T cells were increased while naive T cells were reduced compared with CreCD11cCasp8f/f T cells (Fig. 7C, Supplemental Fig. 4A, 4B). Although IRF3 deletion increased neutrophil, eosinophil, and Ly6Clo- and Ly6Chigh splenic macrophage numbers (Supplemental Fig. 4N), DC numbers were unaffected (Fig. 7F). However, IRF3 deletion elevated CD11c+CD8- conventional DC CD86 expression (Fig. 7G).

Although knockout of IRF7 did not affect disease phenotypes in found in CreCD11cCasp8f/f mice, IRF7 deletion not only decreased numbers of CD4+ and CD8+ (Fig. 7A, 7B) T cells, and reduced expression of PD-1 on CD4+ and CD8+ and regulatory T cells (Supplemental Fig. 4D, 4F, 4H), but also reduced CD11c+CD8- conventional DC CD80 expression (Fig. 7G) in CreCD11cCasp8f/f mice. However, these alterations at the cellular level were insufficient to reduce disease activity.

DC-specific deletion of MyD88 not only partially reversed the SLE-like disease phenotype in CreCD11cCasp8f/f mice, but loss of this TLR signaling mediator also altered the observed immune cell dysregulation found in these mice. Although loss of MyD88 did not affect T cell numbers (Fig. 7A, 7B), CD8+ T cells expressed less CD69 (Supplemental Fig. 4E) and regulatory T cells...
FIGURE 6. Caspase-8 suppresses MyD88 signaling. (A and B) Three-week-old Casp8<sup>fl/fl</sup> (control) and Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> (n = 4) mice were treated with oral antibiotics for 8 wk and evaluated for splenomegaly (A) and lymphadenopathy (B). (C–K) Eight-month-old female control, Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, and MyD88<sup>fl/fl</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> mice (n ≥ 4) were evaluated for systemic autoimmune disease phenotypes. (C) Splenomegaly. (D) Lymphadenopathy. (E) Number of splenocytes. (F) PAS-stained formalin-fixed kidney sections and anti-IgG-FITC-stained frozen kidney sections. (G) Kidney score. (H) Proteinuria. Serum was evaluated for levels of chromatin-, dsDNA-, and ssDNA-reactive IgG Abs (I), pathogenic IgG2a Abs (J), and cytokines and chemokines (K). Data are mean ± SD. *p < 0.05, **p < 0.005, ***p < 0.0005, control versus Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, Mann–Whitney U test. &p < 0.05, ##p < 0.005, Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> versus MyD88<sup>fl/fl</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, Mann–Whitney U test.
FIGURE 7. Caspase-8-deficient CD11c−CD8+ conventional DCs express increased activation markers and confer a hyperactive phenotype on lymphocytes. (A–G) Splenocytes from 6–8-mo-old female Casp8fl/fl (control), CreCD11c-Casp8fl/fl, RIPK3fl/fl CreCD11c-Casp8fl/fl, IRF3fl/fl CreCD11c-Casp8fl/fl, IRF7fl/fl CreCD11c-Casp8fl/fl, and MyD88fl/fl CreCD11c-Casp8fl/fl mice (n ≥ 4) were analyzed by flow cytometry. CD4+ (A) and CD8+ (B) T cell numbers. (C) Representative naive (CD44−CD62L+) and activated (CD44+CD62L−) T cell percentages of total CD4+ and CD8+ populations. (D) Total B cell (B220+) numbers and subsets: follicular (FO; CD19+CD21/35+CD23+), MZ (CD19+CD21/35+CD23low), transitional 2 (T2; B220+AA4.1+CD23+), transitional 1 (T1; B220+AA4.1+CD23−), and plasmablasts (PB; CD19+B220lowCD138+CD21/35−CD23−). (E) B cell IgD, CD80, CD86, and PD-1 expression. (F) Conventional (CD11c−CD8+ and CD11c+CD8−) and plasmacytoid (CD11cintPDCA-1+B220+) DC numbers. (G) CD11c−CD8+ conventional DC CD80 and CD86 expression. (H) Bead-separated CD11c+ cells pulsed with OVA were cocultured with OT-II/RAG−/− CD4+ T cells. Data are mean ± SD. *p < 0.05, **p < 0.005, ***p < 0.0005, control versus CreCD11c-Casp8fl/fl, Mann–Whitney U test. #p < 0.05, ##p < 0.005, ###p < 0.0005, control versus CreCD11c-Casp8fl/fl versus experimental knockouts, Mann–Whitney U test.
expressed less PD-1 (Supplemental Fig. 4H) compared with CreCD11cCasp8fl/fl T cells. MyD88 deletion also altered the B cell pool by augmenting the MZ, transitional 2, and transitional 1 subsets, reducing plasmablasts (Fig. 7D), and decreasing CD86 expression on total B cells compared with caspase-8 deficiency alone (Fig. 7E, Supplemental Fig. 4K). DC-specific loss of MyD88 led to increased numbers of CD11c^+ CD8^+ conventional DCs compared with CreCD11cCasp8fl/fl mice (Fig. 7F) and reduced CD11c^+ CD8^- conventional DC CD86 expression (Fig. 7G) that to control DCs. Taken together, these results suggest that caspase-8 dampens MyD88 signaling in DCs; when caspase-8 is no longer present, unchecked signaling through this TLR mediator occurs, leading to the onset of systemic autoimmunity.

Discussion

Previous studies linked increased DC survival to the development of autoimmune disease. In this study, we show that CreCD11cCasp8fl/fl mice develop splenomegaly, lymphadenopathy, autoantibodies, glomerulonephritis, immune complex deposition in the kidney, exacerbated proteinuria levels, heightened amounts of serum proinflammatory cytokines, and early mortality. In contrast to the other experimental models of apoptotic regulators in DCs, loss of caspase-8 in DCs does not affect their survival. There is no difference to the other experimental models of apoptotic regulators in DCs, loss of caspase-8 in DCs leads to their hyperactivation that requires a vastly different manner in DCs compared with Fas (14) or pan-caspase inhibitors (20).

Recently, a number of studies implicated caspase-8 in the regulation of the inflammasome, in particular the Nlrp3 inflammasome, independently of cell death in a number of cell types (29, 33, 34). Our findings are consistent with one such study (29), which showed that deletion of caspase-8 in DCs resulted in splenomegaly and lymphadenopathy through an apoptosis- and necroptosis-independent mechanism. Both studies show that caspase-8-deficient BMDCs secrete IL-1β without the requirement for secondary ATP stimulation in response to TLR4 activation and that this is abrogated by the addition of necrostatin-1. However, our findings expand on the role of caspase-8 in DCs, because we show a novel necrostatin-1-specific inhibitory effect on proinflammatory cytokine secretion in response to TLR7/9 activation. In addition, we show that these changes are associated with the development of SLE-like disease, which results in early spontaneous mortality. Further, we show that the loss of caspase-8 in DCs leads to their hyperactivation that requires RIPK1 activity, and culminates in development of disease by a MyD88-dependent IRF3-dependent mechanism. Differences between the studies may stem from differing cell-specific caspase-8 deletion constructs and/or the chosen transgenic CD11c-Cre line.

TLR7 and TLR9, which are intracellular receptors known to be activated by nucleic acids, have been linked to both human and murine models of SLE (35). TLR engagement induces RIPK signaling independent of DR activation, thereby leading to formation of a ripoptosome (10). Blocking RIPK1 kinase activity dampens TLR4/7/9-induced secretion of proinflammatory cytokines in caspase-8-deficient DCs without affecting cell survival. Thus, RIPK1 appears to function in a cell-specific manner that is vastly different based on the studies using T cell caspase-8-deficient mice (17). However, deletion of RIPK3 in CreCD11cCasp8fl/fl mice is unable to reverse the observed phenotypic outcome, which is contrary to T cell caspase-8-deficient mice and similar to a recently published study (18, 29). Thus, our data substantiate a new function for caspase-8, namely that it suppresses the inflammatory DC phenotype independent of activating apoptosis or inhibiting necroptosis but requires components of the ripoptosome.

Immunization studies revealed that T cell differentiation by DCs requires TLR activation (36). Teichmann et al. (37) found that deletion of DCs in a murine model of SLE ameliorates disease by limiting T cell expansion and subsequent kidney damage. Although most TLRs signal through MyD88, TLR3 requires the mediator TRIF, and TLR4 signals through both MyD88 and TRIF (38). Although DC-specific deletion of MyD88 in MRL.Fas^lpr mice reduces lymphoproliferation and controls dermatitis, nephritis development persisted (39). However, we were able to suppress lymphoproliferative and end-organ disease by cell-specific deletion of MyD88 in CreCD11cCasp8fl/fl mice. Lyn is a Src family tyrosine kinase that phosphorylates caspase-8 and blocks its downstream activity in neutrophils (40). Although DC-specific deletion of Lyn mimics the systemic autoimmunity induced by deletion of caspase-8, these activities are independent of one another. However, similar to CreCD11cCasp8fl/fl mice, DC-specific MyD88 deletion suppressed the autoimmunity induced in mice by DC-specific Lyn deficiency (41). These data indicate that both Lyn and caspase-8 limit MyD88-dependent TLR signaling in DCs. In contrast to CreCD11cCasp8fl/fl mice, DC-specific deletion of MyD88 is unable to abrogate systemic inflammation caused by DC-specific FADD deficiency (42). Further, administration of broad-spectrum antibiotics suppresses systemic inflammation in DC-specific FADD-deficient mice (42) but has no effect in CreCD11cCasp8fl/fl mice. These results suggest that, although caspase-8 and FADD together are intimately involved in cell death, caspase-8 mediates its suppressive action in DCs, in part, via a MyD88-dependent mechanism, whereas FADD may function to block MyD88-independent TLR signaling through TRIF. Our in vitro results suggest that the enzymatic activity of caspase-8 is dispensable for its suppressive activity, indicating that caspase-8 may act as a scaffolding protein that, in this case, may sequester MyD88. Future studies are required to define the exact interaction. Additionally, because we did not observe a complete abrogation of disease with MyD88 deletion, it is possible that caspase-8 also may dampen TRIF-dependent TLR signaling. Therefore, future studies are required to determine whether deletion of TRIF can ameliorate the systemic autoimmunity in CreCD11cCasp8fl/fl mice.

A majority of SLE patients present an IFN signature, namely constitutive production of type I IFNs (IFN-α and IFN-β) and increased expression of type I IFN–regulated genes (12). This IFN signature is also detected in DCs from a murine model of SLE (43) and in mice lacking caspase-8 in DCs. Further, caspase-8-deficient BMDCs showed sustained DNA binding of IRF and IRF signaling response elements following extended TLR4/TLR7/TLR9 stimulation. These studies suggest that the loss of caspase-8 may increase the transcriptional activity of IRFs. Because previous studies showed that caspase-8 processes IRF3 for degradation (13), it would follow that IRF3 is elevated in the absence of caspase-8. Indeed, expression of IRF3 is increased in caspase-8-deficient BMDCs. Thus, we hypothesized that the presence of elevated levels of IRF3 in caspase-8-deficient DCs and the increased potential for deleterious transcriptional products may be the root cause of the autoimmunity observed in CreCD11cCasp8fl/fl mice. However, to our surprise, deficiency in IRF3 exacerbates the lymphoproliferative disease in CreCD11cCasp8fl/fl mice. These data indicate that IRF3 may be crucial in providing a compensatory mechanism to dampen inflammation induced by loss of caspase-8. Because recent evidence suggests that IRF3 acts not only as a transcription factor, but also as an apoptotic mediator through interaction with Bax via its newly discovered BH3 domain (44),
a failure to undergo apoptosis may be a potential explanation for the enhanced lymphoproliferative disease in Cre^{CD11c-Casp8}^{−/−} mice. Although deletion of either IRF3 or IRF7 is unable to correct the inflammation associated with DC-specific caspase-8 deficiency, it is possible that other IRFs may be involved. For instance, IRF5 interacts with MyD88 downstream of TLR signaling and is phosphorylated and activated after TLR engagement (45). Further, polymorphisms in the IRF5 gene in SLE patients result in their constitutive expression, thereby upregulating type I IFN and proinflammatory cytokine production (46).

Both conventional and plasmacytoid DCs from patients with SLE were shown to possess abnormal phenotypes (47, 48). CD11c^+CD8^+ conventional DCs from SLE patients display a more activated and mature phenotype, including enhanced MHC class II function or expression is reduced in DCs of SLE patients. Future studies will be required to ascertain whether caspase-8 plays a role for caspase-8 in DCs in the pathogenesis of murine SLE-like disease; the number of DCs is increased, as is their secretion of interferons and dendritic cells. Thus, our data document a critical role for caspase-8-mediated cleavage inhibits IFN-3 protein by facilitating its proteasome-mediated degradation. Histopathol 28: 63–64.


Supplemental Figure 1. PCR for genotype validation. (A) Splenocyte populations from Cre\textsuperscript{CD11c}Casp8\textsuperscript{fl/fl} mice were sorted as: B cells (CD19\textsuperscript{+}), CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, NK cells (NK1.1\textsuperscript{+}), red pulp macrophages (CD11b\textsuperscript{+}F4/80\textsuperscript{+}), neutrophils (CD11b\textsuperscript{+}Ly6G\textsuperscript{+}), monocytes/macrophages (CD11b\textsuperscript{+}CD11c\textsuperscript{low/negative}SSC\textsuperscript{low}F4/80\textsuperscript{low}) further subdivided into Ly6C\textsuperscript{−} and Ly6C\textsuperscript{+}, pDC (mPDCA-1\textsuperscript{−}B220\textsuperscript{+}CD11c\textsuperscript{intermediate}) and conventional DC (B220\textsuperscript{−}CD11c\textsuperscript{+}CD8\textsuperscript{+} and B220\textsuperscript{−}CD11c\textsuperscript{+}CD8\textsuperscript{−}), and subjected to PCR for Casp8\textsuperscript{floxed} and Casp8\textsuperscript{deleted} alleles. (B) BMDCs generated from Cre\textsuperscript{CD11c}Casp8\textsuperscript{fl/fl} mice were subjected to PCR for Casp8\textsuperscript{floxed} and Casp8\textsuperscript{deleted} alleles. (C-D) Splenocyte populations from MyD88\textsuperscript{fl/fl}Cre\textsuperscript{CD11c}Casp8\textsuperscript{fl/fl} mice were sorted as in (A) and subjected to real time PCR for (C) caspase-8 and (D) MyD88 deletion.
Supplemental Figure 2. Phenotypes of young and aged mice. (A-B) 2-3-month-old female Casp8<sup>fl/fl</sup> (control), Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, RIPK3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, IRF3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> and IRF7<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> mice (n≥4) were evaluated for (A) spleen and (B) cervical lymph node weights. (C-E) 6-8-month-old control and Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> mice (n≥7) were evaluated for numbers of (C) CD4<sup>+</sup> and (D) Ter119<sup>+</sup> splenocytes. (E) Representative formalin-fixed spleen sections (4 μm) stained with hematoxylin and eosin (H&E) and trichrome and formalin-fixed liver sections (4 μm) stained with H&E. (F-H) Splenocytes from 3- and 6-month-old control, Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, RIPK3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> and B6.lpr mice (n≥5) were analyzed by flow cytometry. (F) Representative CD4<sup>−</sup>CD8<sup>−</sup>CD3<sup>+</sup>B220<sup>+</sup> T-cell percentages of total splenocytes at 3 and 6 months. Numbers of CD4<sup>−</sup>CD8<sup>−</sup>CD3<sup>+</sup>B220<sup>+</sup> double negative T-cells at (G) 3 and (H) 6 months. (I) Splenocyte populations were sorted as: B-cells (CD19<sup>+</sup>), CD4<sup>+</sup>, CD8<sup>+</sup> and DN T-cells (CD4<sup>−</sup>CD8<sup>−</sup>CD3<sup>+</sup>B220<sup>+</sup>) and subjected to real time PCR for caspase-8 deletion. Data are represented as mean ± SD and compared by Mann Whitney test. * denotes comparison between control and Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, # denotes comparison between Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> and RIPK3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, B6.lpr, IRF3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> or IRF7<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>. *, #:p<0.05; **, ##:p<0.005; ###:p<0.0005.
Supplemental Figure 3. TLR ligation does not induce cell death. (A) Splenocytes from 6-8 month-old Casp8fl/fl (control) and CreCD11cCasp8fl/fl (n=5) were analyzed by flow cytometry for expression of TLRs. (B) GM-CSF + Flt3-L-treated BMDCs from control and CreCD11cCasp8fl/fl mice were stimulated with CpG or LPS ± Nec-1 and/or zIETD-FMK (zIETD), a caspase-8 inhibitor and/or 1-Methyl-D-tryptophan (1-MT) for 6 hours ± ATP (5mM) and supernatants evaluated for IL-12/IL-23p40, IL-6, TNFα, and IL-1β. (C) Control and CreCD11cCasp8fl/fl BMDCs were stimulated with imiquimod (5 μg/mL) for 6 hours and cells were stained with Annexin-V and Aqua live/dead. Representative percentages of Annexin-V- and LIVE/DEAD-stained BMDCs. (D) GM-CSF + Flt3-L-treated BMDCs from control and CreCD11cCasp8fl/fl mice were stimulated with CpG, LPS or imiquimod ± Nec-1 and/or zIETD-FMK (zIETD) and/or 1-Methyl-D-tryptophan (1-MT) for 6 hours ± ATP (5mM) and supernatants evaluated for IL-1β. Data are represented as mean ± SD and compared by Mann Whitney test.
Supplemental Figure 4. Alterations in splenic populations. (A-L) Splenocytes from 6-8-month-old Casp8<sup>fl/fl</sup> (control), Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, RIPK3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, IRF3<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> and IRF7<sup>−/−</sup>Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> mice (n≥4) were analyzed by flow cytometry. Naive (CD44<sup>−</sup>CD62L<sup>+</sup>), memory (CD44<sup>+</sup>CD62L<sup>+</sup>) and effector (CD44<sup>+</sup>CD62L<sup>−</sup>) (A) CD4<sup>+</sup>T-cell and (B) CD8<sup>+</sup>T-cell numbers. CD4<sup>+</sup> (C) CD69 and (D) PD-1 expression levels and CD8<sup>+</sup>T-cell (E) CD69 and (F) PD-1 expression levels. Regulatory T-cell (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) (G) numbers and (H) PD-1 expression level. Total B-cell (I) IgD, (J) CD80, (K) CD86 and (L) PD-1 expression levels. (M) plasmacytoid DC (CD11c<sup>int</sup>PDCA-1<sup>+</sup>B220<sup>+</sup>) CD69 expression. (N) Neutrophil (CD11b<sup>+</sup>F4/80<sup>−</sup)Ly6G<sup>+</sup>), Ly6<sup>Chigh</sup> macrophage (CD11b<sup>+</sup>F4/80<sup>+</sup>), Ly6<sup>Clow</sup> macrophage (CD11b<sup>+</sup>F4/80<sup>+</sup>), red pulp macrophage (CD11b<sup>+</sup>F4/80<sup>−</sup>), NK (NK1.1<sup>+</sup>) and eosinophil (SiglecF<sup>+</sup>) cell numbers. Data are represented as mean ± SD and compared by Mann Whitney test. * denotes comparison between control and Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup>, # denotes comparison between Cre<sup>CD11c</sup>Casp8<sup>fl/fl</sup> and experimental knockouts. *, #:p<0.05; **, ##:p<0.005; ###:p<0.0005.