Necroptosis of Intestinal Epithelial Cells Induces Type 3 Innate Lymphoid Cell-Dependent Lethal Ileitis

CFLARs Tg mice develop severe ileitis in utero

Intestinal epithelial cells die by apoptosis and necroptosis in CFLARs Tg mice

Blockade of necroptosis rescues lethality of CFLARs Tg mice

Necroptosis activates type 3 innate lymphoid cells, resulting in severe ileitis

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were activated and recruited to the small intestine along with upregulation of necroptosis and apoptosis, and rescued lethality of Tg mice. Deletion of CFLARs, essential genes of necroptosis, prevented both necroptosis and apoptosis, and rescued lethality of CFLARs Tg mice. Type 3 innate lymphoid cells (ILC3s) were activated and recruited to the small intestine along with upregulation of interleukin-22 (IL22) in CFLARs Tg mice. Deletion of ILC3s or IL22 rescued lethality of CFLARs Tg mice by preventing apoptosis, but not necroptosis of IECs. Together, necroptosis-dependent activation of ILC3s induces lethal ileitis in an IL-22-dependent manner.

INTRODUCTION

Apoptosis is the prototype of programmed cell death or regulated cell death and is executed by sequential activation of cysteine proteases, named caspases (Reedl and Salvesen, 2007; Yuan, 2006). Recent studies have reported another form of regulated cell death, which is also referred to as necroptosis (Christofferson and Yuan, 2010). Activation of death receptors induced by cognate death ligands including tumor necrosis factor (TNF), Fas, and TRAIL triggers the formation of death-inducing signaling complex, termed complex Iib, that is composed of Fas-associated protein with death domain (FADD), receptor-interacting protein kinase (RIPK)1, RIPK3, and caspase 8 (Pasparakis and Vandenabeele, 2015). Once caspase 8 is activated, it subsequently activates downstream caspases 3, 6, and 7, resulting in the execution of apoptosis. Activation of caspase 8 normally suppresses the execution of necroptosis by inactivating RIPK1 and CYLD (Chan et al., 2003; O’Donnell et al., 2011). In sharp contrast, in the presence of either caspase inhibitors, or deletion of Fadd or Caspase 8, the complex Iib evolves into the necrosome that is composed of RIPK1, RIPK3, and mixed lineage kinase domain-like (MLKL). Sequential phosphorylation of RIPK1, RIPK3, and MLKL results in oligomerization and subsequent plasma membrane translocation of MLKL, resulting in membrane permeabilization and necroptosis (Pasparakis and Vandenabeele, 2015). Necroptosis is induced by death ligands, polyinosinic:polycytidylic acid, and viral infection and is involved in various pathological conditions including drug-induced pancreatitis, ischemic reperfusion injury, and elimination of some types of viruses (Weinlich et al., 2017). Taken that germline deletion of Fadd and Caspase 8 results in embryonic lethality due to an increase in necroptosis (Kaiser et al., 2011; Oberst et al., 2011; Zhang et al., 2011), the FADD/caspase 8-dependent apoptotic pathway normally suppresses the necroptotic pathway during normal development. However, an interplay between apoptosis and necroptosis in vivo is not fully understood.

Cellular FLICE-inhibitory protein (cFLIP) is a catalytically inactive homolog of the initiator caspase, caspase 8, and blocks cell death induced by death ligands (Budd et al., 2006; Nakano et al., 2017). We and others have generated conditional Cflar-deficient mice and reported that cFLIP plays a crucial role in preventing cells from apoptosis and necroptosis (Dillon et al., 2012; Panayotova-Dimitrova et al., 2013; Piao et al., 2012, 2018; Schattenberg et al., 2011; Zhang and He, 2005). CFLAR gene encodes two proteins, designated as long form (cFLIPL) and short form (cFLIPs) due to alternative splicing. Intriguingly, recent studies have shown that cFLIPL blocks both apoptosis and necroptosis, whereas cFLIPs blocks apoptosis but promotes...
necroptosis (Feoktistova et al., 2011; Oberst et al., 2011). However, it is unclear whether the expression of cFLIPs promotes necroptosis in vivo, and the consequences of cFLIPs-dependent necroptosis are largely unknown.

Tissue homeostasis of the intestine is regulated by epithelial cells and various types of immune cells, including dendritic cells, macrophages, B and T cells, and innate lymphoid cells (Honda and Littman, 2016; Maloy and Powrie, 2011). Among them, Th17 cells and type 3 innate lymphoid cells (ILC3s) play a crucial role in preventing infection of the intestine from pathogenic bacteria (Ohnmacht, 2016; Vivier et al., 2018). The development of Th17 cells and ILC3s totally depends on the Rorc gene that encodes RAR-related orphan receptor gamma t (RORγt) protein. Under normal conditions, various stimuli such as colonization of commensal bacteria, food-derived metabolites, and cytokines activate macrophages or dendritic cells, resulting in the production of interleukin (IL)-23 and IL-1β (Manta et al., 2013; Mortha et al., 2014). IL-23 and IL-1β subsequently activate Th17 cells and ILC3s. IL-22 produced by activated ILC3s plays a dominant role in maintaining intestinal homeostasis and controls a set of genes showing antimicrobial activities, such as Regenerating islet-derived protein (Reg)3b and Reg3g (Eidenschenk et al., 2014; Parks et al., 2015). In sharp contrast, aberrantly activated ILC3s produce excessive amounts of IL-22, resulting in intestinal tissue injury under certain conditions including injection of anti-CD40 antibody, immaturity of acquired immunity, absence of regulatory T (Treg) cells, or transgenic expression of Il23 (Bauche et al., 2018; Buonocore et al., 2010; Chen et al., 2015). However, the mechanism underlying aberrant activation of ILC3s and ILC3-dependent tissue injury are not fully understood.

X chromosome inactivation is a process in which one of the two X chromosomes is randomly inactivated in female mammalian cells (Lyon, 1971). Hence integration of gene A onto one allele of two X chromosomes results in a mosaic pattern expression of gene A due to random inactivation of X chromosome. During generation of a promoter trap library, we obtained one ES line, designated B210, where a trap vector was integrated into the Diap2 locus on the X chromosome (Taniwaki et al., 2005). Using B210 ES line, we previously reported that mice harboring human SPINK1 gene in the Diap2 locus expressed human SPINK1 in a mosaic pattern (Sakata et al., 2016). This strategy might be useful to express cell death-promoting gene in mice by preventing potentially embryonic lethal phenotype.

To further understand the consequences of necroptosis and an interplay between apoptosis and necroptosis in vivo, we generated CFLARs Tg mice wherein the CFLARs gene was specifically integrated onto the X chromosome. Male and female CFLARs Tg mice were referred to as XcflpY and XcflpX mice, respectively. All XcflpY mice died in utero due to severe ileitis. Immunohistochemistry (IHC) with anti-phosphorylated RIPK3 (pRIPK3) antibody and transmission electron microscopy (TEM) revealed that a number of intestinal epithelial cells (IECs) died by necroptosis. Unexpectedly, large numbers of IECs died by apoptosis in the SI of CFLARs Tg mice. Surprisingly, deletion of Ripk3 or Mlkl rescued embryonic lethality of CFLARs Tg mice by preventing not only necroptosis but also apoptosis of IECs. Moreover, deletion of Rorc or Il22 prevented lethal ileitis in CFLARs Tg mice by preventing apoptosis, but not necroptosis of IECs. Together, necroptosis of IECs activated ILC3s, which further induced apoptosis of IECs in an IL-22-dependent manner.

**RESULTS**

**CFLARs Transgenic Mice Die Perinatally**

To circumvent embryonic lethality potentially induced by overexpression of cFLIPs in mice, we generated CFLARs Tg mice by utilizing X chromosome inactivation (Figure 1A). As we assumed that XcflpY mice might be embryonic lethal, we performed timed mating. XcflpY mice developed normally until embryonic day embryonic day (E) 16.5 but began to die at E17.5 to E18.5, and the rest of XcflpY mice died at birth (Figure 1B). Only 10% of XcflpX mice died perinatally, but the other XcflpX mice survived until adulthood. Although cFLIPs were expressed in various tissues (Figure 1C), an apparent histological abnormality was restricted to the small intestine (SI) (Figure S1A). The intestinal lumen was mostly occupied with villous structure in the SI of wild-type embryos, whereas the lumen was dilated and the length of villi was severely shortened in XcflpY mice, and to a lesser extent, in XcflpX mice at E18.5 (Figure 1D). IECs were detached from villi and accumulated in the lumen of the SI of XcflpY mice. Surprisingly, IECs in the lumen and a few IECs in villi were positive for cleaved caspase (CC) 3 staining, suggesting that these IECs died by apoptosis (Figure 1E). Consistently, CC3 was also detected in tissue extracts of the SI of CFLARs Tg mice at E18.5 (Figure 1F). We also found that large numbers of cells were positive for TUNEL staining in the lumen of the SI of XcflpY mice, and to a lesser extent, in XcflpX mice at E18.5 (Figure 1G).
Figure 1. CFLARs Tg Mice Die Perinatally
(A) Diagram of a vector for CFLARs Tg mice and genomic organization of the Diap2 locus of B210 cells. 
(B) XXY mice die perinatally. After timed mating, mice were sacrificed at the indicated days after coitus, and the 
genotypes of embryos were determined by PCR. Numbers in parentheses written in red characters indicate dead pups at 
the sacrifice. The genotypes of 2- to 4-week-old mice were determined by PCR.
(C) Tissue sections from mice of the indicated genotypes at E18.5 were stained with anti-cFLIP antibody (n = 3–5 mice per 
each genotype). The tyramid signal amplification (TSA) method was used to enhance cFLIPs-positive signals. Scale bars, 
50 μm. Notably, anti-cFLIP antibody recognized exogenously expressed human cFLIPs, but endogenous murine cFLIP at 
least under our experimental conditions.
(D) H&E-stained small intestinal sections of mice of the indicated genotypes at E18.5 (n = 10 mice per each genotype). 
Scale bars, 100 μm. (E, G, and H) Small intestinal tissue sections of mice of the indicated genotypes at E18.5 were stained with anti-cleaved 
caspase 3 (CC3) (E) or pRIPK3 (H) antibodies, or subjected to TUNEL staining (G) (n = 3–4 mice per each genotype). Scale 
bars, 100 μm. Red arrows indicate CC3+ IECs.
We next tested whether IECs of CFLARs Tg mice died by necroptosis. As phosphorylation of RIPK3 (pRIPK3) is a hallmark of cells dying by necroptosis, antibodies that recognize pRIPK3 have been used to detect necroptotic cells by IHC (Webster et al., 2018). We found that small numbers of pRIPK3-positive cells were detected in the SI and other tissues of CFLARs Tg mice (Figures 1H and S1B), suggesting these cells died by necroptosis. In contrast, apoptotic cells were not detected in tissues other than SI (Figure S1C). Together, these results suggest that IECs mainly died by apoptosis rather than necroptosis in CFLARs Tg mice at E18.5.

We established two lines of CFLARs Tg mice, designated C9 and C28, and verified the expression of cFLIPs in various tissues of adult XCFY mice by western blot (Figure S1D). We found that a few CC3-positive cells were still observed in the SI, but not in the colon of adult XCFY mice (Figures S1E–S1G). As the phenotypes of C9 and C28 mice were identical, we mainly analyzed a C28 line for further experiments.

To exclude the possibility that the phenotype of CFLARs Tg mice might come from inactivation of Diap2 gene, we also generated another Tg mice line, in which Cre-ERT2 was integrated into the same locus on the X chromosome, designated Cre-ERT2 Tg mice (Figure S2A). Cre-ERT2 Tg mice were born at the expected Mendelian ratios (Figure S2B) and did not show any abnormality of the SI and colon (Figures S2C–S2E). These results indicate that the phenotype of CFLARs Tg mice is not caused by inactivation of Diap2 gene, but the expression of CFLARs gene.

**A Few IECs Already Undergo Necroptosis in the SI of CFLARs Tg Mice at E17.5**

To investigate an interplay between apoptosis and necroptosis, we analyzed histology of IECs at an earlier time point E17.5. IECs of CFLARs Tg mice appeared to be normal at E17.5 compared with those at E18.5 (Figure 2A). Notably, pRIPK3-positive cells were detected in the SI of CFLARs Tg mice similarly to E18.5 (Figure 2B), whereas very few IECs were positive for CC3 staining (Figure 2C). TEM analysis revealed that some IECs exhibited a drastic decrease in electron densities of the cytoplasm with the dilatation of the endoplasmic reticulum and mitochondria in CFLARs Tg mice, suggesting that these IECs died by necroptosis (Figure 2D). In sharp contrast, a few IECs were detached from villi and exhibited chromatin condensation, a hallmark of apoptosis (Figure 2D). To quantify the relative populations of necrotic and apoptotic cells, we calculated TUNEL+ and CC3+ cells, respectively. TUNEL staining recognizes both necrotic and apoptotic cells. Numbers of TUNEL+ cells were higher than those of CC3 cells in the SI of CFLARs Tg mice (Figures 2C, 2E, and 2F), thus IECs already started to die by necroptosis, and to a lesser extent, by apoptosis at E17.5.

**Deletion of Ripk3 Gene Partially Rescues Embryonic Lethality of CFLARs Tg Mice**

To investigate the causal relationship between necroptosis and apoptosis, we crossed CFLARs Tg mice with Ripk3−/− mice (Newton et al., 2004). Deletion of Ripk3 partially rescued embryonic lethality of XCFY mice and blocked the destruction of the villous structure in the SI of both XCFY and XCFX mice (Figures 3A and 3B). As expected, pRIPK3-positive IECs disappeared in the SI of XCFY;Ripk3−/− and XCFX;Ripk3−/− mice at E18.5 (Figure 3C). More importantly, CC3-positive IECs also disappeared in the SI of XCFY;Ripk3−/− and XCFX;Ripk3−/− mice at E18.5 (Figure 3D). TEM analysis confirmed that apoptosis and necroptosis of IECs disappeared in the SI of XCFY;Ripk3−/− mice (Figure S3A). Survived XCFY;Ripk3−/− mice appeared to be healthy (Figures S3B–S3D). cFLIPs protein was ubiquitously expressed in various tissues of surviving XCFY;Ripk3−/− mice, and their expression levels were higher than those of XCFX mice on a Ripk3−/− or Ripk3+/− background (Figure S3E). Necroptosis of IECs occurs in IEC-specific Fadd- or Caspase 8-deficient mice (Gunther et al., 2011; Welz et al., 2011), suggesting that necroptosis independently occurs in the absence of apoptosis. However, it is unclear whether necroptosis promotes or suppresses apoptosis in vivo. Taken that deletion of Ripk3 blocked necroptosis and apoptosis, necroptosis might promote apoptosis of IECs of CFLARs Tg mice in vivo.

Consistent with these results, TNF and zVAD-fmk (TNF/zVAD)-induced necroptosis were enhanced in murine embryonic fibroblasts (MEFs) derived from XCFY;Ripk3+/− mice compared with Ripk3+/− mice, and TNF/zVAD-induced necroptosis was abolished in MEFs from XCFY mice on a Ripk3-deficient background (Figure 3E).
Figure 2. A Few IECs Already Undergo Necroptosis in the SI of CFLARΔs Tg Mice at E17.5

(A–C and E) Small intestinal sections of mice of the indicated genotypes at E17.5 were stained with H&E (A), anti-pRIPK3 (B), or anti-CC3 (C) antibodies, or subjected to TUNEL staining (E). Red arrowheads and arrows indicate CC3+ (C) and TUNEL+ cells (E), respectively. Results are representative of four independent experiments (n = 4 mice per each genotype). Scale bars, 100 μm.

(D) Tissue sections described as in (A) were analyzed by TEM (n = 4 mice per each genotype). Red arrows and arrowheads indicate cells showing necroptotic and apoptotic morphology, respectively. Scale bars, 5 μm.

(F) Numbers of CC3-positive and TUNEL-positive cells were counted in randomly selected fields and are expressed as numbers of positive cells per field. Results are mean ± SEM (n = 4–7 mice per genotype). Statistical significance was determined by two-tailed unpaired Student’s t test. **p < 0.01; ns, not significant.
Although RIPK3 is essential for necroptosis, several studies have shown that RIPK3 promotes apoptosis and also inflammation under certain conditions (Mandal et al., 2014; Newton et al., 2014). Taken that MLKL solely promotes necroptosis (Murphy et al., 2013; Wu et al., 2013), we crossed CFLARs Tg mice with Mlkl/C0 mice (Dannappel et al., 2014). Consistent with deletion of Ripk3, deletion of Mlkl partially rescued the lethal phenotype of XCFY mice and blocked the disruption of the villous structure of the SI of XCFY mice (Figures 4 A and 4B). Moreover, CC3-positive, but not pRIPK3-positive, IECs disappeared in the SI of XCFY;Mlkl/C0 mice (Figures 4 C and 4D). These results suggest that MLKL-dependent necroptosis subsequently triggers apoptosis of IECs in vivo.

RIPK1 kinase activity is required for formation of the complex IIb that is composed of FADD, caspase 8, RIPK1, and RIPK3 and induces apoptosis or necroptosis in a context-dependent manner (Feoktistova et al., 2011; Tenev et al., 2011). Crossing of CFLARs Tg mice with mice expressing a kinase-inactive mutant of Ripk1 (Ripk1DN/DN) (Polykratis et al., 2014) more efficiently rescued lethal phenotype of CFLARs Tg mice.
Tg mice compared with Mlkl−/− mice by preventing both necroptosis and apoptosis (Figures 4E–4H). Unexpectedly, Tnfrsf1a deficiency (Pfeffer et al., 1993) could not rescue lethal phenotype of XCFY mice (Figure 4I), suggesting that the cFLIPs promote the RIPK3-MLKL-dependent necroptotic pathway in IECs of CFLARs Tg mice in a TNFR1-independent manner.
The Expressions of Reg3β and Reg3γ Are Elevated in the SI of CFLARs Tg Mice

To further investigate the consequences of cFLIPs-dependent cell death in vivo, we performed genome-wide transcriptome analysis of the SI of wild-type and CFLARs Tg mice at E18.5. We found that genes associated with cytokine responses, inflammatory responses, innate immune responses, and host defense responses were enriched in the SI of CFLARs Tg mice compared with wild-type mice (Figures S4A–S4C, Table S1). We focused on Regenerating gene (Reg3)β and Reg3γ. Reg3β and Reg3γ are produced by IECs and act as antimicrobial proteins (Vaishnava et al., 2011). Notably, the expressions of Reg3β and Reg3γ are tightly regulated at the developmental stages: both Reg3β and Reg3γ are not expressed in the SI of wild-type fetus, but their expression is gradually increased in the SI after birth possibly due to colonization with commensal bacteria (Matsumoto et al., 2012). We confirmed that the expression of Reg3β and Reg3γ was undetectable in the SI of wild-type mice, but elevated in the SI of CFLARs Tg mice at E18.5 by qPCR, IHC, and western blot (Figures S5A–S5C; Table S2).

We next investigated the mechanism underlying the elevation of Reg3β and Reg3γ in the SI of adult mice. The expression of Reg3β and Reg3γ in the SI of adult mice was abolished in Il22−/− (Zheng et al., 2008) or Rorc-gfp/gfp (Eberl et al., 2004), but not in Rag2−/− (Hao and Rajewsky, 2001) mice (Figure S5D). However, Rorc is essential for the development of ILC3s and T H17 cells, and the expression of Il22 was abolished in the SI of Rorc-gfp/gfp, but not in Rag2−/− mice (Figure S5E). These results suggest that IL-22 produced by RORγt+ ILC3s, but not T H17 cells, is essential for the expression of Reg3β and Reg3γ in the SI of adult mice. Thus we surmised that RORγt+ ILC3s might be activated in response to cell death of IECs of CFLARs Tg mice at the prenatal stage.

ILC3s Accumulate in the SI of CFLARs Tg Mice

Although ILC3s play a crucial role in maintaining tissue homeostasis of the intestine (Ouyang and Valdez, 2008; Parks et al., 2015), activation of ILC3s promotes intestinal tissue injury under certain conditions (Bauche et al., 2018; Eken et al., 2014). ILC3s are aberrantly activated in the SI of mice lacking CD4+ T cells or young mice wherein acquired immunity is not fully maturated (Mao et al., 2018). As expected, the expression of Foxp3, a hallmark of Treg cells, was very low in the SI of either wild-type or CFLARs Tg mice at E18.5 compared with that of adult mice (Figure S6A). To test whether ILC3s contribute to the development of ileitis at preterm mice, we first crossed Rorc-gfp reporter mice with CFLARs Tg mice. As the expression of the green fluorescent protein (GFP) is under the control of endogenous promoter of Rorc in Rorc-gfp reporter mice, RORγt+ cells that contain both T H17 cells and ILC3s are recognized as GFP+ cells by flow cytometry or IHC. We found that large numbers of CD11b+Ly-6G+ neutrophils infiltrated in the SI of CFLARs Tg mice at E18.5 (Figures 5A and 5B). CCR6+ ILC3s are recruited to the SI via its ligand CCL20 that is produced by IECs (Esplugues et al., 2011). To further investigate the consequences of cFLIPs-dependent cell death in vivo, we performed genome-wide transcriptome analysis of the SI of wild-type and CFLARs Tg mice at E18.5 (Figure S6D), suggesting an intimate cross talk between the expression of Reg3β and Reg3γ in the SI of wild-type fetus, but their expression is gradually increased in the SI after birth possibly due to colonization with commensal bacteria (Matsumoto et al., 2012). We confirmed that the expression of Reg3β and Reg3γ was undetectable in the SI of wild-type mice, but elevated in the SI of CFLARs Tg mice at E18.5 by qPCR, IHC, and western blot (Figures S5A–S5C; Table S2).

To determine whether apoptotic cells were responsible for accumulation of RORγt+ cells, we tested whether RORγt+ cells accumulated in the SI at E17.5. Although we hardly detected or only detected very few apoptotic cells in the SI of CFLARs Tg mice (Figures 2C and S3D), a number of RORγt+ cells were already recruited to the SI along with an increase in the expression of CCL20 (Figure S3D). As we hardly detected CD3+ T cells in the SI of CFLARs Tg mice at E17.5 and E18.5 by IHC (data not shown), accumulated RORγt+ cells were ILC3s, but not T H17 cells. Accumulation of neutrophils appeared to delay compared with that of ILC3s (Figures 5C and 5D), suggesting that neutrophils might not be primarily responsible for apoptosis induction, but accumulated in response to apoptotic cells. The expression of CCL20 was elevated in the SI of CFLARs Tg mice along with an appearance of necrotic cells, whereas its expression was abrogated in CFLARs Tg mice on a Ripk3−/− or Mkl1−/− background (Figure S5E). Notably, although only small numbers of IECs underwent necroptosis (Figure 1H), CCL20 was ubiquitously expressed in all IECs (Figure S5C). Inflammatory cytokines such as TNF and IL-1β have been shown to induce CCL20 production by IECs (Kwon et al., 2002), and macrophages and dendritic cells might produce these cytokines in response to danger-associated molecular pattern (DAMPs) released from necrotic IECs. Taken that the expression of Il1b was elevated in the SI of CFLARs Tg mice (Figure S6C), IL-1β might be responsible for the production of CCL20 by IECs. Consistently, the expression of both Il1b and Ccl20, but not Il22, was significantly elevated in the SI of CFLARs Tg mice at E17.5 (Figure S6D), suggesting an intimate cross talk between the expression of Il1b and Ccl20. Moreover, IL-1β and IL-23 have been shown to activate ILC3s (Manta et al., 2013; Mortha et al., 2014). Together, IL-1β might be responsible for CCL20 production by IECs and activation of ILC3s.
Figure 5. ILC3s Accumulate in the SI of CFLARs Tg Mice

(A) Percentages of neutrophils, but not T cells, B cells, or RORγt+ cells are increased in the SI of CFLARs Tg mice at E18.5 compared with WT mice. Cells were prepared from the SI of WT or CFLARs Tg mice at E18.5, and pooled samples (approximately 3–4 fetal SI per each genotype) were stained with the indicated antibodies and analyzed by flow cytometry. Results are representative of four independent experiments.

(B) Cells were prepared as in (A); absolute cell numbers of the indicated populations were calculated and are expressed as mean ± SEM of four independent experiments. Statistical significance was determined by the two-tailed unpaired Student’s t test. ***p < 0.001; ns, not significant.
Deletion of Rorc or Il22 Partially Rescues Embryonic Lethality of CFLARs Tg Mice

We next tested whether ILC3s might contribute to the development of ileitis in CFLARs Tg mice. Deletion of Rorc partially rescued embryonic lethality of X<sup>CFY</sup> mice and blocked the destruction of villi structure of the SI of CFLARs Tg mice (Figures 6A and 6B). A large numbers of neutrophils and CC3-positive IECs were detected in the SI of X<sup>CFY</sup>;Rorc-gfp/+ mice, whereas infiltration of these cells almost completely disappeared in the SI of X<sup>CFY</sup>;Rorc-gfp/gfp mice (Figures 6C and 6D). CXCL2 is a chemokine that recruits neutrophils (Kobayashi, 2008). Consistently, the expressions of Reg3b, Reg3g, Il22, Cxcl2, and Ccl20 were downregulated in the SI of CFLARs Tg mice on a Rorc-gfp/gfp background (Figure S7A). pRIPK3-positive IECs were still detected in the SI of CFLARs Tg mice on a Rorc-gfp/gfp background (Figure 6E), further substantiating that activation of RORγ<sup+t</sup> ILC3s is a downstream event of necroptosis of IECs of CFLARs Tg mice.

Previous studies have reported that IL-22 has anti-colitogenic or colitogenic functions in a context-dependent manner (Eken et al., 2014; Ouyang and Valdez, 2008). To determine the contribution of IL-22 to the development of ileitis in CFLARs Tg mice, we finally crossed CFLARs Tg mice with Il22<sup>−/−</sup> mice. Deletion of Il22 partially rescued embryonic lethality and prevented the development of ileitis in CFLARs Tg mice (Figures 6F–6H). Notably, CC3-positive cells completely disappeared in the SI of CFLARs Tg;Il22<sup>−/−</sup> mice (Figure 6I), but pRIPK3-positive cells were still detected (Figure 6J). Moreover, the expression of Reg3b, Reg3g, Cxcl2, and Ccl20 was downregulated in the SI of CFLARs Tg;Il22<sup>−/−</sup> mice (Figure S7A). Thus IL-22 contributes, at least in part, to intestinal injury at perinatal stages. Moreover, deletion of Ripk3, Mlkl, or kinase activity of RIPK1 downregulated the expression of these genes (Figure S7A), suggesting that the execution of necroptosis finally converged on recruitment and activation of ILC3s and IL-22 production.

We finally investigated the mechanism underlying IL-22-dependent tissue injury. We found that the expression of reactive oxygen species (ROS)-producing enzyme, Duox2 and its regulatory subunit, Duoxa2, were significantly elevated in the SI of CFLARs Tg mice (Figure S7B). DUOX2 is upregulated in patients with inflammatory bowel disease (IBD) before the onset of inflammation and is a marker of perturbed mucosal homeostasis in patients with early-stage IBD (Grasberger et al., 2015). Taken that IL-22 induces the expression of Duox2 of IECs (Grasberger et al., 2013), it is reasonable to speculate that ILC3s might induce ROS-dependent apoptosis of IECs through the IL-22-Duox2 pathway. Together, these results indicate that a positive feedforward loop between cFLIPs-dependent necroptosis (Figure 7A) and ILC3-dependent apoptosis might critically contribute to the development of lethal ileitis in neonatal mice (Figure 7B).

DISCUSSION

In the present study, we generated transgenic mice expressing human CFLARs on the X chromosome and investigated cellular responses triggered by necroptosis in vivo. As expected, all male CFLARs Tg mice died in utero, but female CFLARs Tg mice survived due to X chromosome inactivation. Thus the expression of cell-death-promoting gene on the X chromosome might be one of the strategies to evaluate cellular responses triggered by cell death in vivo. Although cFLIPs blocks caspase-dependent apoptosis but promotes necroptosis in vitro (Feoktistova et al., 2011; Oberst et al., 2011), IECs died by necroptosis and apoptosis in CFLARs Tg mice. Deletion of Ripk3 or Mlkl, or inhibition of kinase activity of RIPK1, partially prevented embryonic lethality of CFLARs Tg mice by suppressing both apoptosis and necroptosis of IECs. We finally showed that ILC3s induced apoptosis of IECs in an IL-22-dependent manner, culminating in the development of lethal ileitis in CFLARs Tg mice.

In sharp contrast to in vitro studies including ours (Feoktistova et al., 2011; Oberst et al., 2011; Shindo et al., 2013), overexpression of cFLIPs in vivo resulted in both apoptosis and necroptosis of IECs in mice. Taken together these findings, we conclude that necroptosis in vivo is not a distinct form of cell death that occurs independent of caspase-dependent apoptosis.
Figure 6. Deletion of Rorc or Il22 Partially Rescues Embryonic Lethality of CFLARs Tg Mice

(A and F) The progeny of crossing male Rorc-gfp/gfp (A) or Il22–/– (F) mice with XCFX mice. The genotypes of 3- to 4-week-old mice were determined by PCR.

(B–E and G–J) Intestinal sections of mice of the indicated genotypes at E18.5 were stained with H&E (B and G), anti-Gr-1 (C and H), anti-CC3 (D and I), or anti-pRIPK3 (E and J) antibodies (n = 3–4 mice per group).

Scale bars, 100 μm. See also Figure S7.
that cFLIP inhibits death receptor-induced apoptosis in vitro (Feoktistova et al., 2011; Oberst et al., 2011), and deletion of Tnfrsf1a did not attenuate embryonic lethality of CFLARs Tg mice, death receptor-induced caspase 8-dependent pathway is not primarily responsible for the execution of apoptosis of IECs of CFLARs Tg mice. Notably, the expression of Duox2 and its regulatory subunit, Duoxa2, were elevated in the SI of CFLARs Tg mice compared with control mice. Taken that oxidative stress has been shown to induce apoptosis under certain conditions (Circu and Aw, 2010), ROS-dependent activation of effector caspases might promote apoptosis of IECs in CFLARs Tg mice.

Figure 7. A Model for cFLIPs-Dependent Lethal Ileitis

(A) The complex IIb spontaneously forms in IECs overexpressing cFLIPs. As cFLIPs blocks activation of caspase 8, the complex IIb evolves into the necrosome, resulting in oligomerization of MLKL and subsequent membrane permeabilization.

(B) Necroptotic cells of IECs release DAMPs that subsequently activate nearby macrophages or dendritic cells, resulting in IL-1β production. In addition to the release of DAMPs, IECs in response to IL-1β produce CCL20 that recruits CCR6-positive ILC3s into IECs. Recruited ILC3s are activated by IL-1β and release IL-22 that acts on IECs, resulting in upregulation of Reg3b, Reg3g, Duox2, and Duoxa2. Importantly, aberrantly activated ILC3s induce apoptosis of IECs possibly through upregulation of several ROS-producing enzymes. Apoptotic IECs might enhance inflammation and further activate ILC3s. Thus blockade of the necroptotic pathway and depletion of ILC3s or IL22 suppress lethal ileitis.

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Histological analysis revealed that severe tissue injury was restricted to the SI of CFLARs Tg mice. We found that ILC3s were only detected in the SI and the liver, but not other tissues. Intriguingly, ILC3s were detected even in the liver of wild-type mice, suggesting that these cells were resident ILC3s, but not infiltrated ILC3s in response to certain stimuli observed in the SI of CFLARs Tg mice. These results suggest that cFLIPs-dependent tissue injury is correlated with infiltration of ILC3s in response to necroptotic cells. Although male CFLARs Tg;Ripk3−/− mice did not show any abnormality of the SI at E18.5, deletion of Ripk3 did not completely rescue embryonic lethality of CFLARs Tg mice. This suggests that the cause of lethality of CFLARs Tg;Ripk3−/− mice was not ileitis. A recent study showed that white blood cells are markedly increased in the peripheral blood of Ripk1−/− mice and Ripk1−/− hematopoietic cells fail to engraft efficiently in lethally irradiated wild-type (WT) mice (Peltzer et al., 2018; Rickard et al., 2014). Thus bone marrow (BM) failure might induce embryonic lethality of CFLARs Tg;Ripk3−/− mice when intestinal tissue injury was attenuated. Moreover, percentages of survived XcF y mice on an Mikl−/− background were lower than those on Ripk3−/− or Ripk1DN/DN background. Notably, RIPK1 kinase activity and RIPK3 are involved in inflammation and apoptosis under certain conditions (Pasparakis and Vandenabeele, 2015), suggesting that the MLKL-independent pathways also contribute to embryonic lethality of CFLARs Tg mice. Further study will be required to address these issues.

Although approximately 10% of XcF y mice died perinatally, other XcF y mice survived until adulthood. One might surmise that IECs susceptible to cFLIPs-induced cell death have been largely eliminated during the development in utero or soon after birth. Therefore few IECs might be positive for CC3 staining. On the other hand, Treg cells are very few in the SI during embryonic stages and then gradually expand along with colonization of commensal bacteria (Honda and Littman, 2016). We found that the expression of Foxp3 in the SI of either wild-type or CFLARs Tg mice at E18.5 was very low compared with that of adult mice. Intriguingly, LAG3+ regulatory T cells restrain IL-23- and IL-1β-producing macrophages, thereby suppressing activation of ILC3s and the development of intestinal injury (Bauche et al., 2018). Aberrant expression of ILC3 by IL-23 has been shown to drive IL-22-dependent intestinal inflammation (Buonocore et al., 2010). Moreover, Il23 transgenic mice spontaneously develop severe intestinal inflammation along with accumulation of ILC3s and neutrophils in the SI at neonatal stages (Chen et al., 2015). The development of intestinal inflammation of Il23 Tg mice is blocked when ILC3s are depleted, or mice are treated with antibiotics, suggesting that commensal bacteria-dependent production of IL-23 might contribute to the development of severe intestinal inflammation at the neonatal stage. Given that numbers of matured Treg cells were very few in CFLARs Tg at E18.5, Treg cells did not attenuate ILC3s-dependent ileitis in neonatal CFLARs Tg mice. In contrast, ileitis might be attenuated by Treg cells in adult XcF y mice. Together, these studies have revealed the critical contribution of ILC3s to the development of severe ileitis in mice at prenatal stages.

Previous studies have shown that germline deletion of Caspase 8 or Fadd in mice promotes necroptosis in utero, resulting in embryonic lethality due to a defect in formation of yolk sac vasculature at E10.5 (Kaiser et al., 2011; Oberst et al., 2011; Varfolomeev et al., 1998; Zhang et al., 2011). As CFLARs Tg mice did survive at least until E16.5, the phenotype of CFLARs Tg mice is completely different from that of Caspase 8−/− or Fadd−/− mice. This might come from the timing of the expression of cFLIPs driven by the CAG promoter during development. In contrast, IEC-specific deletion of Caspase 8 or Fadd (Caspase 8EC-KO or FaddEC-KO) results in severe intestinal inflammation in mice at after birth, suggesting that the phenotype of CFLARs Tg mice might be more severe than that of these murine models (Gunther et al., 2011; Welz et al., 2011). There are several differences among CFLARs Tg mice and these murine models. First, the development of ileitis in CFLARs Tg mice occurs earlier than other murine models, where the colitis usually starts after birth. Second, CFLARs Tg mice only developed ileitis, but Caspase 8EC-KO or FaddEC-KO mice develop both ileitis and colitis. Third, deletion of Tnfsh1a did not rescue embryonic lethality of CFLARs Tg mice, whereas deletion of Tnf or Tnfsh1a attenuates intestinal tissue injury in Caspase 8EC-KO or FaddEC-KO mice. Finally, large numbers of apoptotic cells were detected in the intestinal lumen, but very few apoptotic cells were detected in villi of CFLARs Tg mice. Although necroptosis was detected in various tissues, necroptotic cells per se were not sufficient to induce severe tissue damage in CFLARs Tg mice. Severe tissue damage of the SI of CFLARs Tg mice is tightly correlated with infiltration of ILC3s. Intriguingly, a recent study has reported that IL-22 enhances proliferation of IECs, but inhibits the expansion of intestinal stem cell (ISCs), resulting in a decrease in organoid survival (Zwarycz et al., 2019). Thus it might be plausible that aberrantly produced IL-22 increases the turnover of IECs and suppresses the
expansion of ISCs, resulting in anoikis-dependent apoptosis of IECs in the SI of CFLARs Tg mice. Further study will be required to address this issue.

Approximately 10% population of extremely preterm infants spontaneously develops severe necrotizing enterocolitis (Lim et al., 2015; Tanner et al., 2015). The histology of necrotizing enterocolitis in human is characterized by the dilatation of the intestine, destruction of the villi structures, and intestinal bleeding. Intriguingly, these futures are reminiscent of the histological features of the SI of CFLARs Tg mice. Immaturity of host defense, type of infant feeding, ischemia, anatomical anomaly, and bacterial infection are considered to be responsible for the development of necrotizing enterocolitis. However, the detailed mechanisms remain unclear. As overexpression of cFLIPs induced ILC3s-dependent lethal ileitis, it would be interesting to test whether the expression of cFLIPs was increased in such patients. Given that IECs produce CCL20 in response to inflammatory cytokines such as TNF or IL-1β (Kwon et al., 2002), IECs might further recruit ILC3s, culminating in the development of lethal ileitis. Therefore it would be also intriguing to test whether ILC3s accumulate in the lesions of necrotizing enterocolitis in preterm infants.

Limitations of the Study
Our results demonstrate that blockade of necroptosis completely suppresses apoptosis of IECs, but only partially rescues embryonic lethality of CFLARs Tg mice. We do not currently know which pathway(s) other than necroptosis induces embryonic lethality of CFLARs Tg mice. Moreover, we cannot show genetic evidence that IL-22-dependent ROS production induces apoptosis of IECs of CFLARs Tg mice. Another limitation of our current study is the inability to answer whether the RORγt+ cells/IL-22 axis operates and contributes to the exacerbation of intestinal diseases in human patients. Further investigation will be required to address these issues.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.05.011.

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AUTHOR CONTRIBUTIONS
Conceptualization, R.S., M.O., and H.N.; Investigation, R.S., S.K-S., S.M., Y.D., S.Y., T.N., S.K., and K.M.; Resources, M.O., T.Y., H. Konishi, H. Kiyma, T.M., and K.A.; Writing-Original Draft, R.S. and H.N.; Writing-Review & Editing, R.S., S.Y., K.M., and H.N.; Supervision, Y.U.

DECLARATION OF INTERESTS
The authors declare that they do not have competing financial interests. Data and Software Availability The accession number for the microarray data reported in this paper is NCBI GEO: GSE120982.

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Necroptosis of Intestinal Epithelial Cells Induces Type 3 Innate Lymphoid Cell-Dependent Lethal Ileitis

Ryodai Shindo, Masaki Ohmuraya, Sachiko Komazawa-Sakon, Sanae Miyake, Yutaka Deguchi, Soh Yamazaki, Takashi Nishina, Takayuki Yoshimoto, Soichiro Kakuta, Masato Koike, Yasuo Uchiyama, Hiroyuki Konishi, Hiroshi Kiyama, Tetuo Mikami, Kenta Moriwaki, Kimi Araki, and Hiroyasu Nakano
Figure S1. pRIPK3-, but not CC3-positive cells are detected in various tissues of CFLARs Tg mice, Related to Figure 1.

(A-C) Tissue sections from mice of the indicated genotypes at E18.5 were stained with H&E (A), anti-pRIPK3 (B), or anti-CC3 (C) antibodies (n=3 to 5 mice per each genotype). The tyramide signal amplification (TSA) method was used to enhance pRIPK3-positive signals. Scale bars, 50 µm.

(D) The expression of endogenous cFLIP_L and exogenously introduced human cFLIPs in various tissues of 8-week-old X^CF^X mice. Extracts were prepared from various tissues and analyzed by immunoblotting with anti-cFLIP and anti-tubulin antibodies. C9 and C28 indicate two independently generated Tg lines. Results are representative of three independent experiments. W and Tg indicate WT and CFLARs Tg mice, respectively.

(E-G) Small numbers of IECs still undergo apoptosis in the SI of adult X^CF^X mice. Tissue sections of 6- to 8-week-old XX and X^CF^X mice were stained with H&E (E) or anti-CC3 antibody (F) (n= 3 to 5 mice per each genotype). The black box is an enlarged image of CC3^+^ cells. CC3^+^ cells are indicated by red arrows. Numbers of CC3^+^ cells in the small intestine or colon were calculated in randomly selected fields (G). Results are expressed as numbers of CC3^+^ cells per villi. Results are mean ± SEM (n=3 mice per each genotype). Statistical significance was determined by the two-tailed unpaired Student’s t test. **P<0.01; ns, not significant.
Figure S2. Inactivation of one allele of Diap2 loci does not induce perinatal lethality, Related to Figure 1.

(A) Diagram of a vector for Cre-ERT2 Tg mice.

(B) $X^{cr}Y$ mice are born at the expected Mendelian ratio and develop normally. The progeny of crossing male wild-type mice with $X^{cr}X$ mice. The genotypes of 2- to 4-week-old mice were determined by PCR.

(C-E) Small intestinal or colon sections of mice of the indicated genotypes at E18.5 were stained with H&E (C) (n=10 mice per each genotype) or anti-CC3 antibody (D) (n=3 to 4 mice per each genotype). Scale bars, 100 µm. Numbers of CC3$^+$ cells were counted and are expressed as in Figure S1G (E). Results are mean ± SEM (n=3 mice per group). Statistical significance was determined by the two-tailed unpaired Student’s $t$ test. ns, not significant.
Figure S3. Male CFLARs Tg;Ripk3−/− mice grow without apparent abnormality, Related to Figure 3.

(A) Transmission electron microscopic analysis of the SI of mice of the indicated genotypes at E18.5. Results are representative of two independent experiments. Scale bars, 5 µm. Red arrowheads and arrows indicate apoptotic and necroptotic cells, respectively.

(B-D) Small intestinal sections from 6- to 8-week-old mice of the indicated genotypes were stained with H&E (B) or anti-CC3 antibody (C) (n= 3 mice per each genotype). Scale bars, 100 µm. Numbers of CC3+ cells were counted and are expressed as in Figure S1G (D). Results are mean ± SEM (n=3 mice).
per each genotype). Statistical analysis was determined by the one-way ANOVA test. ns, not significant.

(E) Expression of cFLIPs in various tissues. Tissue extracts from mice of the indicated genotype were analyzed by immunoblotting with anti-cFLIP and anti-tubulin antibodies. Results are representative of two independent experiments.
Figure S4. Transcriptome analysis of the SI of CFLARs Tg mice, Related to Figure 5.

(A) RNAs were prepared from the SI of mice of the indicated genotype at E18.5, and the expression of genes was determined by microarray analysis. Heat map of microarray gene expression of the SI of the indicated mice and their clustering are shown. Gene-expression color normalized by Z score transformation is shown at the right.

(B) Heat map of 30 genes upregulated more than two-fold in CFLARs Tg mice compared to WT mice. Red and blue indicate higher and lower relative expression, respectively.

(C) Signaling pathways significantly enriched in the SI of CFLARs Tg mice compared to wild-type mice.
Figure S5. Expression of Reg3b and Reg3g is elevated in the SI of CFLARs Tg mice, Related to Figure 5.
(A) mRNAs were extracted from the SI of mice of the indicated genotypes at E18.5, and the expression of Reg3b and Reg3g were determined by qPCR. Results are mean ± SEM (n=8 mice per each genotype). Statistical significance was determined by the two-tailed unpaired Student’s t test. *P<0.05; ***P<0.001.
(B) Small intestinal sections of mice of the indicated genotypes at E18.5 were stained with anti-Reg3β or anti-Reg3γ antibodies (n=3 mice per each genotype). Scale bars, 100 µm. Results are representative of three independent experiments.

(C) Tissue extracts of the SI of mice of the indicated genotypes at E18.5 were analyzed by immunoblotting with the indicated antibodies (n=2 mice per each genotype). Each number indicates an individual mouse. Results are representative of two independent experiments.

(D) The expression of Reg3b and Reg3g mRNAs is abolished in the SI of mice on an Il22−/−, or Rorc−gfp/gfp, but not Rag2−/− background. mRNAs were prepared from the SI of 8- to 12-week-old mice of the indicated genotypes, and the expressions of Reg3b and Reg3g was determined by qPCR. Results are mean ± SEM (n= 4 to 12 per group). Statistical significance was determined by the two-tailed unpaired Student’s t test. ***P<0.001; ns, not significant. (E) The expression of Il22 mRNAs is abolished in the SI of Rorc−gfp/gfp, but not Rag2−/− mice. mRNAs were prepared as in (D), the expressions of Il22 was determined by qPCR. Results are mean ± SEM (n= 4 to 12 per group). Statistical significance was determined by the two-tailed unpaired Student’s t test. **P<0.01; ns, not significant.
Figure S6. Gating strategy to analyze RORγt+ ILCs and expression of ILC3 signature genes in the SI of CFLARs Tg mice, Related to Figure 5.

(A, C, D) RNAs were prepared from the SI of mice of the indicated genotypes at E18.5 (A, C) or E17.5 (D) or 8- to 12-week-old wild-type adult mice (A), and the expression of the indicated genes was determined by qPCR. Results are mean ± SEM (n=6 to 8 mice per each genotype). Statistical significance was determined by the two-tailed unpaired Student's t test. *P<0.05; **P<0.01; ***P<0.001; ns, not significant.

(B) Gating strategy of analysis of RORγt+ ILC3s. Intestinal lamina propria cells were isolated as described in Transparent Methods. Live and CD45.2+ cells were gated, and then lineage-negative cells were gated. CD127+ cells were analyzed by the expression of GFP and CCR6. Representative results of three independent pooled experiments.
Figure S7. Deletion of genes associated with necroptosis, *Il22*, or *Rorc* downregulates the expression of various genes in the SI of CFLARs Tg mice, Related to Figure 6.

(A, B) mRNAs were prepared from the SI of mice of the indicated genotypes at E18.5, and the expression of the indicated genes was determined by qPCR. Results are mean ± SEM (n=5 to 15 mice per group). Statistical analysis was determined by the one-way ANOVA test (A) or the two-tailed unpaired Student’s t test (B). *P<0.05; **P<0.001; ***P<0.001; ns, not significant.
Table S1. Upregulated genes in the SI of CFLARs Tg mice compared to wild-type mice, Related to Figure 5.

| Accession No. | Symbol   | Gene                                      | Fold change |
|---------------|----------|-------------------------------------------|-------------|
| NM_011260     | Reg3g    | regenerating islet-derived 3 gamma        | 48.5        |
| NM_011036     | Reg3b    | regenerating islet-derived 3 beta         | 35.1        |
| NM_008204     | H2-M2    | histocompatibility 2, M region locus 2    | 21.4        |
| NM_009140     | Cxcl2    | chemokine (C-X-C motif) ligand 2          | 21.1        |
| NM_011315     | Saa3     | serum amyloid A 3                         | 19.6        |
| NM_0011685    | Pla2g4c  | phospholipase A2, group IVC (cytosolic,   | 18.7        |
|               |          | calcium-independent)                      |             |
| NM_008599     | Cxcl9    | chemokine (C-X-C motif) ligand 9          | 12.8        |
| NM_010927     | Nos2     | nitric oxide synthase 2, inducible        | 11.6        |
| NM_0011682    | Gsdmc2   | gasdermin C2                              | 11.3        |
| NM_177610     | Duox2    | dual oxidase 2                            | 11.0        |
| NM_028992     | Gsdmc4   | gasdermin C4                              | 10.4        |
| NM_173869|     Stfa2l1 | stefin A2 like 1                         | 10.3        |
| NM_0011682    | Gsdmc2   | gasdermin C2                              | 9.3         |
| NM_016960|     Ccl20 | chemokine (C-C motif) ligand 20           | 8.5         |
| NM_008324     | Ido1     | indoleamine 2,3-dioxygenase 1             | 8.4         |
| NM_0010825    | Stfa2    | stefin A2                                 | 8.3         |
| NM_025777     | Duoxa2   | dual oxidase maturation factor 2          | 7.8         |
| NM_010260     | Gbp2     | guanylate binding protein 2               | 7.6         |
| NM_021274     | Cxcl10   | chemokine (C-X-C motif) ligand 10         | 7.3         |
| NM_008147     | Gp49a    | glycoprotein 49 A                         | 7.1         |
| NM_011410     | Slfn4    | schlafen 4                               | 6.9         |
| NM_011337     | Ccl3     | chemokine (C-C motif) ligand 3            | 6.9         |
| NM_011579|     Tgtp1 | T-cell specific GTPase 1                 | 6.7         |
| NM_009114     | S100a9   | S100 calcium binding protein A9 (calgranulin B) | 6.6         |
| NM_0010825    | BC100530 | cDNA sequence BC100530                   | 6.5         |
| NM_009264     | Sprr1a   | small proline-rich protein 1A             | 6.5         |
| NM_011579     | Tgtp1    | T-cell specific GTPase 1                 | 6.4         |
| Accession | Symbol  | Description                                                      | Score |
|-----------|---------|-----------------------------------------------------------------|-------|
| NM_013650 | S100a8  | S100 calcium binding protein A8 (calgranulin A)                 | 6.4   |
| NM_144544 | 2210407C| RIKEN cDNA 2210407C18 gene                                      | 6.3   |
| NM_0010014 | Tnip3  | TNFAIP3 interacting protein 3                                    | 5.8   |
| NM_008361 | Il1b    | interleukin 1 beta                                              | 5.7   |
| NM_013532 | Lirb4   | leukocyte immunoglobulin-like receptor, subfamily B, member 4   | 5.6   |
| NM_008392 | Irg1    | immunoresponsive gene 1                                          | 5.4   |
| NM_009909 | Cxcr2   | chemokine (C-X-C motif) receptor 2                               | 5.4   |
| NM_010742 | Ly6d    | lymphocyte antigen 6 complex, locus D                            | 5.3   |
| NM_008530 | Ly6f    | lymphocyte antigen 6 complex, locus D                            | 5.1   |
| NM_011113 | Plaur   | plasminogen activator, urokinase receptor                        | 5.0   |
| NM_153564 | Gbp5    | guanylate binding protein 5                                      | 4.9   |
| NM_0011462 | ligg1  | interferon inducible GTPase 1                                    | 4.9   |
| NM_010416 | Hemt1   | hematopoietic cell transcript 1                                  | 4.8   |
| NM_0011360 | Anxa10  | annexin A10                                                     | 4.7   |
| NM_0011682 | Serpina3f| serine (or cysteine) peptidase inhibitor, clade A, member 3F    | 4.7   |
| NM_0010819 | Gm11428| predicted gene 11428                                            | 4.7   |
| NM_009903 | Cldn4   | claudin 4                                                       | 4.7   |
| NM_0010256 | Ii1r1  | interleukin 1 receptor-like 1                                    | 4.6   |
| NM_009704 | Areg    | amphiregulin                                                    | 4.6   |
| NM_007607 | Car4    | carbonic anhydrase 4                                             | 4.6   |
| NM_008620 | Gbp4    | guanylate binding protein 4                                      | 4.5   |
| NM_010555 | Ii1r2   | interleukin 1 receptor, type II                                  | 4.4   |
| NM_008611 | Mmp8    | matrix metalloproteinase 8                                       | 4.4   |
| NM_008491 | Lcn2    | lipocalin 2                                                     | 4.3   |
| NM_009252 | Serpina3n| serine (or cysteine) peptidase inhibitor, clade A, member 3N    | 4.3   |
| NM_0010332 | Nlrc5   | NLR family, CARD domain containing 5                            | 4.1   |
| NM_011452 | Serpinb9b| serine (or cysteine) peptidase inhibitor, clade B, member 9b     | 4.1   |
| NM_181596 | Retnlg  | resistin like gamma                                             | 4.1   |
RNAs were extracted from the SI of the indicated mice at E18.5 and analyzed using oligonucleotide arrays. Fold changed were calculated using the expression levels of each gene in the SI of CFLARs Tg mice compared to those of wild-type mice and top 55 genes are shown. Experiments were performed using two mice per each genotype and the average values of fold change are shown.
### Table S2. Primers used for qPCR in the study, Related to Figure 5.

| Gene   | Forward Primer                               | Reverse Primer                             |
|--------|----------------------------------------------|--------------------------------------------|
| Ccl20  | 5'- GCCTCTCTGATACAGACGC-3'                   | 5'- CCAGTTCTGCTTTGGATCAGC-3'               |
| Csf2   | 5'- CTTTGAATGCAAAAACCAGTCC-3'                | 5'- TCCTGGCTCATTACGCAAGGC-3'               |
| Cxcl2  | 5'- CCAACCACCAGCTACAGG-3'                    | 5'- GCGTCACACTCAAGCTCTG-3'                 |
| Duox2  | 5'- ACRCAGCTCTGTGTCAAAGGT-3'                 | 5'- TGATGAACGAGACTCGACAGC-3'               |
| Duoxa2 | 5'- GACGGGGGTGCTACCTTTAC-3'                  | 5'- GCTAAGAAGGACTTCAACAA-3'                |
| Foxp3  | 5'- GCGAAAAAGTGGCAGAGG-3'                    | 5'- AAGGCAGAG TCAGGAG-3'                   |
| Hprt   | 5'- AACAAAGTCTGGCTATCAGA -3'                 | 5'- GCAGTACAGCCCCAAATGG-3'                 |
| Il1b   | 5'- GCAACTGTTCTCAGACTCAACT-3'                | 5'- ATCTTTTTGGGTCGGTTCAACT-3'              |
| Il6    | 5'- GTATGAACAGATGATGACTG-3'                  | 5'- ATGGTACTCCAGAAAGACCAGAGA-3'           |
| Il11   | 5'- CTGCACAGATGAGAGACAATTCG-3'               | 5'- GAAGCTGCAAGATCCCGAAT-3'                |
| Il17a  | 5'- CTGGAGGATAAACACTGTGGAGAGT-3'             | 5'- TGCTGAATGGCGACGATTC-3'                 |
| Il17f  | 5'- CAAAACCCAGGGCATTCTGT-3'                  | 5'- ATGGTGCTGTCTCTGACC-3'                 |
| Il21   | 5'- AGCCCCCAAGGGCCAGATCG-3'                  | 5'- AGCTGCAATGCTGACAGTCCCTTTT-3'          |
| Il22   | 5'- TCCGAGGAGTGCTGCTAA-3'                    | 5'- AGAAGCGCTTCCAGGATGAA-3'               |
| Il22ra2| 5'- TCAGCAGCAAAGAGAAGAAAC-3'                 | 5'- GTGTCTCCAGCCAACTCTCA-3'               |
Il23: 5'-GGGGAACATTACCTTCCTGG-3'
       5'-CTAGATTCTGTAGAACTGAGG-3'
Il23r: 5'-CCCAGACAGTTCCAGGTTACAGC-3'
       5'-TGGCCAGAACATTTCCCGACA-3'
Nos2: 5'-GTTCGTCGACACAAATACAAGA-3'
       5'-GTGGACGTTTTCGATGTCAC-3'
Reg3b: 5'-CTCCTGCGATGCCCTTAT-3'
       5'-TGGTTACTCATTCCCATCC-3'
Reg3g: 5'-ACGAATCCTTCCTCTCAG-3'
       5'-GTCTTCACATTGGATTTG-3'
Transparent Methods

Reagents
Murine TNF (34-8321, eBioscience), zVAD-fmk (3188-v, Peptide Institute), and Hoechst 33258 (Molecular Probes) were purchased from the indicated sources. The following antibodies were used in this study and were obtained from the indicated sources: anti-Reg3β (AF5110, R&D Systems, 1: 3000 for WB, 1: 200 for IHC), anti-CLIP (Dave-2, Adipogen, 1: 500 for WB, 1: 200 for IF), anti-caspase-3 (9662, Cell Signaling, 1: 1000), anti-cleaved caspase-3 (9661, Cell Signaling, 1: 1000 for WB, 1: 200 for IHC or IF), anti-caspase-8 (1G12, Alexis, 1: 1000), anti-CCL20 (MAB7601, R&D SYSTEMS, 1: 200), anti-F4/80 (BM8, Caltag, 1: 100), anti-RIPK3 (IMG-5523-2, IMGENEX, 1: 3000), anti-phospho-RIPK3 (57220, Cell Signaling, 1: 1000 for WB, 1: 200 for IF), anti-tubulin (T5168, Sigma-Aldrich, 1: 40000), anti-STAT3 (sc-482, Santa-Cruz, 1: 1000), anti-phospho-STAT3 (9131, Cell Signaling, 1: 1000), anti-CD3 (Ab5690, Abcam, 1: 200) were purchased from the indicated sources. Anti-CD3 (145-2C11, 1: 200), anti-CD4 (RM4-5, 1: 200), anti-CD8 (53-6.7, 1: 200), anti-CD11b (M1/70, 1: 200), anti-CD11c (N418, 1: 200), anti-CD19 (1D3, 1: 200), anti-B220 (RA3-6B2, 1: 200), anti-Gr-1 (RB6-8C5, 1: 200), anti-Ly-6G (1A8, 1: 200), anti-CD45.2 (104, 1: 100), anti-TER-119 (TER-119, 1: 200), anti-CD127 (A7R34, 1: 200) antibodies were purchased from TONBO Biosciences. Anti-CCR6 antibody (140706, 1: 200) and Fixable Viability Dye eFluor 506 (65-0816-14) were purchased from BD and eBioscience. Anti-Reg3β (1: 1000) and anti-Reg3γ (1: 1000) antibodies were described previously (Matsumoto et al., 2012). HRP-conjugated donkey anti-rabbit IgG (NA934, 1:5000), HRP-conjugated sheep anti-mouse IgG (NA931, 1: 5000), HRP-conjugated goat anti-rat IgG (NA935, 1: 5000) antibodies were purchased from GE Healthcare Life Science. Alexa Fluor 594–conjugated donkey anti-rabbit IgG (A21207, 1: 500) and Alexa Fluor 488-conjugated donkey anti-goat IgG (A11055, 1: 500) antibodies were from Invitrogen. Biotin-conjugated goat anti-rabbit IgG (E0432, Dako, 1: 200), Biotin-conjugated rabbit anti-rat IgG (BA-4001, VECTOR, 1: 200) and biotin-conjugated rabbit anti-sheep IgG (BA-6000, VECTOR, 1: 200) antibodies, and HRP-conjugated streptavidin (P0397, Dako, 1: 300) were purchased from the indicated sources.

Cells
Primary MEFs were prepared from mice of the indicated genotypes at E14.5 after coitus using a standard method. MEFs below ten passages were used as primary MEFs for experiments. MEFs were maintained with DMEM containing 10% fetal calf serum.

Cell viability assay
MEFs were plated onto 96-well plates and cultured for 12 hours in DMEM containing 10% FCS. Then, cells were stimulated with the indicated concentrations of TNF in the absence or presence of zVAD-fmk.
(20 μM) for 7 hours. Cell viability was determined by WST-1 (water soluble 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)[2H] tetrazolium monosodium salt-1) assay using a Cell Counting kit (343-07623, Dojindo).

**Mice**

*Ripk3−/−* (Newton et al., 2004) (provided by V. Dixit), *Milkl−/−* (Dannappel et al., 2014) and *Ripk1<sup>DN/DN</sup>* (Polykratis et al., 2014) (provided by M. Pasparakis), *Il22−/−* (Zheng et al., 2008) (provided by Genentech, Inc.), and *Tnfrsf1a−/−* (Pfeffer et al., 1993) (provided by T.W. Mak) mice were described previously. *Rorc-gfp/gfp* mice (Eberl et al., 2004) were provided by K. Honda under the third party transfer agreement of the Jackson Lab. *Rag2−/−* mice (Hao and Rajewsky, 2001) were purchased from the Jackson Lab. *C57/BL6* mice were purchased from Sankyo Lab. All experiments were performed according to the guidelines approved by the Institutional Animal experiments Committee of Kumamoto University, Juntendo University Graduate School of Medicine, Toho University School of Medicine, and Tokyo Medical University.

**Generation of CFLARs Tg mice at the Diap2 locus on the X-chromosome**

We previously generated one ES cell line designated B210, in which a promoter trap vector was integrated into the *Diaphanous homolog 2* (*Diap2*) gene on the X-chromosome (Taniwaki et al., 2005). We transfected a replacement vector for CFLARs where the expression of CFLARs was under the control of the *cytomegalovirus early enhancer/chicken b-actin* (CAG) promoter and flanked by two mutant lox sites into B210 cells (Figure 1A). The replacement vector was electroporated into B210 cells along with an expression vector for Cre recombinase to induce recombination between two mutant lox sites. Characterization of B210 ES cells harboring a gene trap vector at the *Diap2* locus on the X-chromosome will be published elsewhere. Selection was maintained for 5 days, and then colonies were picked into 48-well plates and expanded for freezing. The puromycin-resistant colonies were analyzed by Southern blotting and PCR to select ES cell lines showing successful integration of pCAGGS-CFLARs-pA. Positive clones were aggregated with ICR morula according to the protocol previously described. Germline transmission was obtained in three mouse lines, and two different lines were backcrossed onto *C57BL/6N* at least 5 generations. CFLARs Tg mouse lines, designated as C9 and C28, were used in this study.

*Cre-ERT2* Tg mice were generated by an essentially similar to the protocol of generation of CFLARs Tg mice as described above. Detailed information of a vector of pCAGGS-Cre-ERT2-pA and characterization of *Cre-ERT2* Tg mice will be published elsewhere.

**Western blotting**
Murine tissues were homogenized with a Polytron (KINEMATICA) or cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethanesulfonfonyl fluoride (PMSF), 1 µg/ml aprotinin, and 1 µg/ml leupeptin]. After centrifugation, tissue extracts or cell lysates were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were analyzed with the indicated antibodies. The membranes were developed with Super Signal West Dura Extended Duration Substrate (Thermo Scientific) and analyzed with a LAS4000 or Amersham Imager 600 (GE Healthcare Life Sciences).

Histological, immunohistochemical, and immunofluorescence analyses
Murine tissues were fixed in 10% formalin and embedded in paraffin blocks. Paraffin-embedded tissue sections were used for hematoxylin and eosin (H&E) staining. TUNEL staining was performed with ApopTag Fluorescein In Situ Apoptosis Detection kit (S7110, Millipore) (for Figure 1G) or the In Situ Detection kit (Roche Diagnostics) (for Figure 2E). Briefly, paraffin-embedded sections were incubated with a TdT reaction mixture containing digoxigenin nucleotide and biotin-16-dUTP followed by incubation with fluorescein-conjugated anti-digoxigenin antibody and HRP-conjugated streptavidin, respectively. For immunostaining, paraffin-embedded sections were incubated with the indicated primary antibodies and then visualized with respective secondary antibodies. To detect cFLIPs, pRIPK3, Gr-1, and CCL20, the tyramide signal amplification (TSA) method was applied according to the manufacturer’s instructions (NEL741001KT, PerkinElmer). Images were obtained by All In One analyzer (KEYENCE) or confocal microscopy (Nikon). Images were analyzed with KEYENCE software (KEYENCE) or NIS-Elements AR Analysis software (Nikon).

TEM analysis
Small intestines from mice of the indicated genotype at E17.5 or E18.5 were removed and fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Slices of these fixed tissues were postfixed with 2% OsO4, dehydrated in ethanol, and embedded in Epok 812 (Okenshoji Co.). Ultrathin sections were cut with an ultramicrotome (ultracut N or UC6: Leica), stained with uranyl acetate and lead citrate, and examined with a Hitachi HT7700 or JEOL JEM-1400 electron microscope.

Microarray analysis
We compared gene expression profiles of RNAs from the intestines of wild-type and CFLARs Tg mice at E18.5. Total RNAs were extracted from the small intestines of mice of the indicated genotype at E18.5 (n=2 mice per each genotype) using Sepasol-RNA I Super G according to the manufacturer’s
instructions (09379-55, Nacalai Tesque), then labeled with Cy3. Samples were hybridized to a Mouse Oligonucleotide Microarray (G4121B, Agilent) according to the manufacturer’s protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent). Data were analyzed using GeneSpring GX software (Agilent). The accession number for the microarray data reported in this paper is NCBI GEO: GSE120982.

Heat maps and principal component analysis plots were generated in the R-method. Gene Ontology (GO) enrichment analysis was performed using the ToppGene Suite (http://toppgene.cchmc.org/)(Chen et al., 2009).

**Quantitative polymerase chain reaction assays**

Total RNAs were extracted with the small intestines of mice of the indicated genotype at E18.5 and cDNAs were synthesized with the Reverta Ace qPCR RT Kit (Toyobo). Quantitative polymerase chain reaction (qPCR) analysis was performed with the 7500 Real-Time PCR detection system with CYBR green method of the target genes together with an endogenous control, murine Hprt with 7500 SDS software (Applied Biosystems). The primers used for qPCR were listed in Table S2.

**Flow cytometry**

After genotyping, three to four small intestines of the fetus of the same genotype at E18.5 were pooled and cut into small fragments and then digested with 1 mg/ml of collagenase (032-22364, Wako) in 10% FCS RPMI at RT for 30 min. Cells were passed through a 70 µm cell strainer, and single cell suspension was prepared. Cells were incubated with the indicated antibodies for 30 min. Fixable Viability Dye eFluor 506 (65-0816-14, eBioscience) was used to distinguish live cells from dead cells, and live cells were analyzed with the indicated antibodies.

Gating strategy for RORγt+ ILC3s analysis was shown in Figure S6B. Briefly, CD45.2-positive but all lineage markers-negative cells were gated, and GFP-positive cells were analyzed by the expression of CCR6. The lineage markers used here were CD3, CD19, CD11b, CD11c, Gr-1, and TER-119. Data were obtained on a Fortessa (GE Healthcare) and analyzed by a Flow-Jo software (GE Healthcare).

**Statistical analysis**

Statistical analysis was performed by the two-tailed unpaired Student’s t test or the one-way ANOVA test. P < 0.05 was considered to be statistically significant.
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