Cellular FLIP Inhibits Myeloid Cell Activation by Suppressing Selective Innate Signaling

Yu-Jung Wu, Yung-Hsuan Wu, Shu-Ting Mo, Huey-Wen Hsiao, You-Wen He and Ming-Zong Lai

*J Immunol* 2015; 195:2612-2623; Prepublished online 3 August 2015; doi: 10.4049/jimmunol.1402944
http://www.jimmunol.org/content/195/6/2612

---

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/08/01/jimmunol.1402944.DCSupplemental

**References**

This article cites 48 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/195/6/2612.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cellular FLIP Inhibits Myeloid Cell Activation by Suppressing Selective Innate Signaling

Yu-Jung Wu,*† Yung-Hsuan Wu, † Shu-Ting Mo, † Huey-Wen Hsiao, † You-Wen He, ‡ and Ming-Zong Lai*†

In this study, we generated mice with c-FLIP CKO in DCs and unexpectedly found an increased activation in DCs. Knockout of c-FLIP in DCs led to neutrophilia and splenomegaly, enhanced TLR2-, TLR4-, and dectin-1–induced TNF-α, IL-2, and G-CSF production, and increased p38 MAPK activation. Therefore, c-FLIP antagonizes selective activation signals transmitted by innate receptors. Our results demonstrate the suppressive nature of c-FLIP in the activation and signaling in myeloid cells. Moreover, the present study suggests that downregulation of c-FLIP during cancer therapy may enhance DC activation and T cell priming.
Materials and Methods

Reagents

LPS, Pam3CSK4, zymosan depleted, and curdlan were purchased from Invivogen (San Diego, CA). For ELISA assays, mouse IL-2 DuoSet, mouse IL-6 DuoSet, mouse TNF-α DuoSet, and mouse IL-1β DuoSet were obtained from R&D Systems (Minneapolis, MN). Anti-β2a, anti-phospho-β2a (Ser172/181), anti-β3a, anti-phospho-β3a (Ser172/181), anti-CD11c, anti-CD11b, anti-iCOS, anti-iCOS-ligand, anti-VEGFR2, anti-VEGFR3, anti-p38, anti-p38 MAPK, anti-phospho-p38 MAPK (T202/Y204), anti-mouse, anti-phospho-p53 (Ser15), anti-anti-CD86, anti-CD95, anti-CD11c, anti-iCOS, anti-p53, anti-CD40, anti-CD44, anti-CD62L, anti-CD25, anti-CD27, anti-CD8, anti-CD11c, anti-CXCR4, anti-CD45, anti-Gr1, anti-CD8, anti-CD4, anti-CD20, anti-CD19, anti-CD11b, anti-CD23, and anti-CD40L were purchased from BioLegend (San Diego, CA). Anti-fLAG was purchased from Covance (Princeton, NJ). Anti-CD11c-Pacific Blue (N418), anti–CD44-FITC (10G4), anti–CD4-v450 or -allophycocyanin (RM4-5), anti–CD25-FITC (7D4), and anti–CD62L-PE (MEL-14) were obtained from BioLegend (San Diego, CA). Anti–F4/80-allophycocyanin (C13/38) and anti–CD11c-Pacific Blue (N418) were obtained from BioLegend (San Diego, CA). Anti–F4/80-allophycocyanin (P3/93), anti–CD11b-PE-Cy7 (M1/70), anti–CD44-FITC (HTA1), anti–CD80-PE-Cy7 (3D32), anti–CD86-PE (GL3), anti–CD25-FITC (PC61), anti–CD127-FITC (BioLegend), anti–CD68-FITC (HCD120), anti–CD163-FITC (1B11/42), anti–CD11c-PE-Cy7 (M1/70), anti–CD68-FITC (20C1), anti–CD163-FITC (HCD120), anti–CD11c-FITC (HCD120), and anti–CD86-FITC (GL3) were purchased from eBioscence (San Diego, CA).

Preparation and purification of splenic DCs

Spleens were collected and digested for 30 min at 37°C with Liberase TL (1.67 U/ml; Roche) and DNase I (0.2 mg/ml; Roche) in RPMI 1640, followed by incubation with 0.5 mM EDTA for 5 min and then lysis of RBCs. Single-cell suspensions of splenocytes were enriched for CD11c cells by MACS CD11c microbeads (Miltenyi Biotec). Anti–CD11c treatment

c-FLIP KO was more extensive in DCs from c-FLIP f/f CD11c-Cre mice in breeding and survival but were smaller in size than control mice. We examined the genotype and c-FLIP expression in DCs from c-FLIP f/f CD11c-Cre mice. Genomic PCR indicated that the flox allele was exclusively removed in the splenic DC and bone marrow–derived DC (BMDC) populations from c-FLIP f/f CD11c-Cre mice (Fig. 1A). c-FLIP protein was profoundly reduced in c-FLIP f/f CD11c-Cre splenic DCs and c-FLIP f/f CD11c-Cre BMDCs, but a residual amount of c-FLIP remained detectable (Fig. 1B). The degree of reduction in c-FLIP flox allele and c-FLIP protein suggest that c-FLIP KO was more extensive in DCs from c-FLIP f/f CD11c-Cre mice than in macrophages in c-FLIP f/f Lyso-cre mice (28). The c-FLIP KO was DC specific, as the c-FLIP levels in the T cell population were not affected in c-FLIP f/f CD11c-Cre mice (Fig. 1A, 1B).

Knockdown of caspase-8 in DCs

Small interfering RNA (siRNA) duplexes were obtained from Dharmacon (Lafayette, CO). The siRNA sequence for caspase-8 is 5′-TGGCTACGTGTCATACGAT-3′. The nonspecific siRNA control was Silencer Cy3-labeled negative control no. 1 siRNA (Ambion, Austin, TX).

Ag presentation in DC–T cell cocultures

CD4+ T cells were isolated from the spleens of OT-II mice and were labeled with CellTracker (Invitrogen). Splenic CD11c+ DCs (8 × 104) were cultured with OT-II CD4+ T cells (5 × 105) in the presence of the OVA peptide 323–339 (0.1 μg/ml). Proliferation and IL-2 secretion were assessed on day 3 by CellTracker dilution and ELISA.

Quantitative PCR

Total RNA from control and c-FLIP CKO BMDCs was isolated using TRIzol (Invitrogen). cDNAs were prepared and analyzed for the expression of Cflar, Tofa, Csf3, and Il2 on a LightCycler 480 real-time PCR system (Roche). The PCR protocol is 95˚C for 10 min, followed by 45 cycles of 95˚C for 10 s, 60˚C annealing for 10 s, and 72˚C extension for 8 s. The PCR primers were as follows: Cflar forward, 5′-GGG AAG ATG GCC AAG GAC A-3′, reverse, 5′-GA CAC GTG CAG ATT CAT-3′; Tofa, forward, 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′, reverse, 5′-TTG TAG GAG TAG AGG TAG AAC C-3′; Il2, forward, 5′-ATC AGC AAC ATG AGA GTA ACT GGT-3′, reverse, 5′-CAT CTC CAG AGA TCC ACC-3′; Csf3, forward, 5′-GGA AGA CCG CCT GGG TTG-3′, reverse, 5′-GGA ATC AAT AGT TAT TTA CCC GTG CAC AAG-3′.

Cell viability analysis

Cell viability was assayed by determination of ATP levels. Wild-type (WT) and c-FLIP–deficient BMDCs were treated with LPS, TNF-α, or dectin-1 ligands for the indicated time periods. CellTiter-Glo reagent (Promega, Madison, WI) was then added in 1:1 volume and incubated for 30 min. Luminescent signal was determined using a Victor2 1420 multilabel counter (PerkinElmer, Shelton, CT).

Determination of caspase-8 activity

Caspase-8 activity was determined using luminogenic caspase-8 substrate containing the LETD sequence. BMDMs were treated with DMSO, IETD, or zVAD-fmk for 1 h. Equal volume of Caspase-Glo 8 reagent (Promega) was then added and incubated for an additional 1 h. Luminescent signal was determined using a luminescence reader Victor2 1420 multilabel counter (PerkinElmer, Shelton, CT).

Results

CKO of c-FLIP in DCs leads to an increase in neutrophils in bone marrow

We bred the c-FLIPf/f (Cflarf/f) mouse with the Cd11c-Cre mouse to generate mice with DC KO of c-FLIP (c-FLIPf/fCd11c-Cre). c-FLIPf/fCd11c-Cre mice were comparable to control (c-FLIP f/f) mice in breeding and survival but were smaller in size than control mice. We examined the phenotype and c-FLIP expression in DCs from c-FLIPf/fCd11c-Cre mice. Genomic PCR indicated that the flox allele was extensively removed in the splenic DC and bone marrow–derived DC (BMDC) populations from c-FLIPf/fCd11c-Cre mice (Fig. 1A). c-FLIP protein was profoundly reduced in c-FLIPf/fCd11c-Cre splenic DCs and c-FLIPf/fCd11c-Cre BMDCs, but a residual amount of c-FLIP remained detectable (Fig. 1B). The degrees of reduction in c-FLIP flox allele and c-FLIP protein suggest that c-FLIP KO was more extensive in DCs from c-FLIPf/fCd11c-Cre mice than in macrophages in c-FLIPf/fLyso-cre mice (28). The c-FLIP KO was DC specific, as the c-FLIP levels in the T cell population were not affected in c-FLIPf/fCd11c-Cre mice (Fig. 1A, 1B).

The c-FLIP KO in DCs did not affect total bone marrow cell numbers (Fig. 1F). The composition of the bone marrow macrophage (F4/80+ or F4/80+CD11b+pop) and cell number were comparable between control and c-FLIPf/fCd11c-Cre mice (Fig. 1C, 1G, 1H), with a slight reduction in the F4/80 intensity in c-FLIPf/fCd11c-Cre mice. The composition of the bone marrow microglia (F4/80+CD11b−) population and cell number were comparable between control and c-FLIPf/fCd11c-Cre mice (Fig. 1C, 1G, 1H), with a slight reduction in the F4/80 intensity in c-FLIPf/fCd11c-Cre mice.
There was a small increase in bone marrow stromal macrophages (F4/80+CD11bInt) (Fig. 1C, 1I). The most notable change was the prominent increase in both percentage and total numbers of neutrophil granulocytes (CD11b+Gr-1+) in bone marrows of c-FLIP f/fCD11c-Cre mice (Fig. 1D, 1J). Lineage (Lin²) cell number, but not percentage, was moderately decreased in c-FLIPf/fCD11c-Cre bone marrows (Fig. 1E, 1L). A decrease in the percentage and total number of bone marrow B220+ cells was detected in c-FLIP CKO mice (Fig. 1E, 1K). Therefore, deletion of c-FLIP in DCs led to elevation in polymorphonuclear neutrophils (PMNs) and reduction in B cells and Lin² cells in bone marrow, similar to mice with c-FLIP CKO in macrophages (28).
c-FLIP deficiency in DCs results in splenomegaly and peripheral neutrophilia

Splenomegaly and lymphadenopathy were found in all c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Fig. 2A). The splenic cellularity increased 2-fold in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Fig. 2D). A unique feature of c-FLIP<sup>fl/+</sup>CD11c-Cre mice was the abundant presence of splenic DC populations (Fig. 2B), in direct contrast to the depletion of macrophages in c-FLIP<sup>fl/+</sup>Lysm-Cre mice (28). The percentage of pDCs and CD11b<sup>+</sup>cDCs was in fact increased in spleens from mice with c-FLIP KO in DCs (Fig. 2B). An increase in total numbers of CD11b<sup>+</sup>cDCs, but less so with pDCs, was found in c-FLIP<sup>fl/+</sup>CD11c-Cre spleen (Fig. 2B, 2E, 2F). There was a small decrease in the percentage, but not the total cell numbers, of splenic CD8<sup>+</sup>cDC number in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Fig. 2B, 2G). Notably, the deletion of c-FLIP in the CD8<sup>+</sup>cDC population was not as extensive (Supplemental Fig. 1A) as those seen in total splenic CD11c<sup>+</sup>DC cells from c-FLIP KO mice (Fig. 1A). Whether the modest phenotype of CD8<sup>+</sup>cDCs is associated with the incomplete c-FLIP deletion in c-FLIP<sup>fl/+</sup>CD11c-Cre mice remains to be determined. Splenic cDC precursors (Lin<sup>−</sup>CD11c<sup>+</sup>F4/80<sup>+</sup>Flt3<sup>+</sup>SIRP<sup>a</sup>int) (34) in c-FLIP<sup>fl/+</sup>CD11c-Cre mice were not significantly affected (Supplemental Fig. 2A). A decrease in the floxed allele in c-FLIP KO DC precursors was clearly visible, yet the deleted allele was not detectable (Supplemental Fig. 1A), likely due to the low number of DC precursors. Because of such uncertainty, whether the c-FLIP<sup>fl/+</sup>CD11c-Cre cDC precursor population is affected by c-FLIP KO awaits further characterization.

Similar to bone marrow, neutrophils were profoundly elevated in c-FLIP<sup>fl/+</sup>CD11c-Cre spleen in both percentage and total numbers (Fig. 2C, 2H). A prominent increase in neutrophils was readily detectable in blood from c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 3A). Expansion of the peritoneal PMN population was also observed in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 3B). Therefore, similar to c-FLIP<sup>fl/+</sup>Lysm-Cre mice, neutrophilia was characteristic of c-FLIP<sup>fl/+</sup>CD11c-Cre mice. Additionally, the populations of inflammatory monocytes (SSC<sup>hi</sup>CD11b<sup>+</sup>Ly6C<sup>hi</sup>) and resident monocytes (SSC<sup>hi</sup>CD11b<sup>+</sup>Ly6C<sup>lo</sup>) were increased in c-FLIP<sup>fl/+</sup>CD11c-Cre spleen (Fig. 2C). A similar increase in blood inflammatory monocytes was found in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 3A). The splenic F4/80<sup>+</sup> population was not apparently disturbed (Supplemental Fig. 2B), but conventional macrophages (F4/80<sup>+</sup>CD11b<sup>+</sup>) were increased in both frequency and numbers in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 2C, 2G). No change in peritoneal macrophages was found in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 3B). Consistent with a cross-reactivity of Cd11c-Cre, a very weak deletion of c-FLIP<sup>fl</sup> allele in splenic macrophages (F4/80<sup>+</sup>CD11b<sup>+</sup>) was detected (Supplemental Fig. 1B).

For lymphocytes, the frequency, but not the cellularity, of splenic B cell, CD4<sup>+</sup>T cell, and CD8<sup>+</sup>T cell populations was modestly decreased in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 2B, 2D, 2F, 2H, 2J). A small decrease in CD4<sup>+</sup>CD62L<sup>+</sup> naive T cells and proportional increase in CD4<sup>+</sup>CD62L<sup>−</sup> effector T cells was observed in splenic CD4<sup>+</sup>T cells from c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 2E). The blood T cell population was not affected by c-FLIP deficiency in DCs (Supplemental Fig. 3C), consistent with the observations that thymic development was nearly normal in c-FLIP<sup>fl/+</sup>CD11c-Cre mice (Supplemental Fig. 3D).

We also examined bone marrow and the splenic population in c-FLIP hemizygotic (c-FLIP<sup>fl</sup>) mice. None of the aforementioned phenotypes observed in c-FLIP<sup>fl/+</sup>CD11c-Cre mice could be detected in c-FLIP<sup>fl</sup> mice (data not shown). These results suggest that hemizygous deficiency of c-FLIP in DCs was insufficient to generate inflammation. Therefore, depletion of c-FLIP in DCs led...
to PMN elevation in bone marrow and the periphery, similar to c-FLIP CKO in macrophages (28).

**Increased expression of MHC class II, CD80, and CD86 in c-FLIP–deficient DCs**

In addition to an increased splenic DC population in c-FLIP f/f CD11c-Cre mice, the expression of costimulatory molecules was enhanced in c-FLIP CKO DCs. Elevated cell surface CD80 and CD86 was observed in CD11b⁺ cDCs, CD8⁺ cDCs, and pDCs from c-FLIP f/fCD11c-Cre mice compared with those from control mice (Fig. 3A). Increased MHC class II levels were also found in c-FLIP f/fCD11c-Cre CD8⁺ cDCs and pDCs (Fig. 3A). c-FLIP-deficient DCs exhibited a better ability to prime T cells. Enhanced IL-2 production was found in OT-II T cells stimulated by OVA peptide and c-FLIP-deficient CD11c⁺ cDCs (Fig. 3B). A modest increase in OT-II proliferation was also observed when primed with c-FLIP f/fCD11c-Cre DCs, relative to WT DCs (Fig. 3B).

The survival of DCs through c-FLIP KO allowed us, to our knowledge for the first time, to examine the role of c-FLIP in myeloid cell activation. We found an increase in the expression of proinflammatory cytokine by c-FLIP f/fCD11c-Cre DCs. The generation of TNF-α and G-CSF was higher in dectin-1–stimulated c-FLIP f/fCD11c-Cre cDCs than in the control cDCs, correlating with an enhanced expression of Tnfa and Csf3 transcripts in c-FLIP CKO cDCs (Fig. 3C, 3D). Similarly, the expression of IFN-α was increased in c-FLIP f/fCD11c-Cre pDCs before and after CpG (for TLR9) stimulation (Fig. 3E).

**Increased production of TNF-α and IL-2 in c-FLIP–deficient BMDCs**

Because of the limited number of purified cDCs and pDCs, we further investigated activation in c-FLIP f/fCD11c-Cre BMDCs. c-FLIP–deficient BMDCs similarly displayed enhanced activation to stimulation through PRRs. LPS treatment led to increased secretion of TNF-α in c-FLIP f/fCD11c-Cre BMDCs 5 and 20 h after stimulation, accompanied with decreased production of IL-6 (Fig. 4A). Dectin-1 engagement, by curdlan or zymosan depleted for 5 and 20 h, also resulted in increased generation of

---

**FIGURE 3.** Increased expression of MHC class II, CD80, and CD86 in DCs isolated from c-FLIP–deficient mice. (A) Increased surface expression of CD80 and CD86 on c-FLIP–deficient CD8⁺ cDCs and pDCs. Flow cytometric analysis of the surface expression of CD86, CD80, and I-Aα on total CD11c⁺ DCs, CD11b⁺ cDCs, CD8⁺ cDCs, and pDCs from spleen of c-FLIP f/f and c-FLIP f/fCD11c-Cre mice. Data are representative of at least three independent experiments. (B) Increased T cell activation by c-FLIP–deficient CD11c⁺ DCs. IL-2 production and proliferation (measured by CellTracker dilution) of OT-II CD4⁺ T cells were determined 3 d after coculture with splenic c-FLIP f/f and c-FLIP f/fCD11c-Cre CD11c⁺ DCs in the absence or presence of OVA233–359 peptide. Open curve indicates stimulation with c-FLIP f/f DC cells; shadowed curve indicates stimulation with c-FLIP f/fCD11c-Cre DCs. (C–E) Enhanced expression of TNF-α, G-CSF, and IFN-α in c-FLIP f/fCD11c-Cre cDCs and pDCs. CD11c⁺ DCs and pDCs were isolated from c-FLIP f/f and c-FLIP f/fCD11c-Cre mice and were stimulated with curdlan (100 μg/ml) (C and D) or CpG (E). The expression of Tnfa (C), Csf3 (D), and Ifna4 (E) was determined by quantitative PCR, and the production of TNF-α (C) and G-CSF (D) was measured by ELISA. Values are the means ± SD of triplicate samples. Data are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 for paired t test.
TNF-α in c-FLIP-deficient BMDCs (Fig. 4B). A more prominent increase in dectin-1–induced IL-2 was observed in c-FLIP KO CD11c-Cre BMDCs and 20 h after activation, relative to control BMDCs (Fig. 4C). In contrast, c-FLIP deficiency did not affect IL-10 production (Fig. 4D). IL-6 production triggered by curdlan, similar to those induced by LPS, was reduced in c-FLIP-deficient BMDCs (Fig. 4B). Increased LPS- and dectin-1–induced production of TNF-α and IL-2 in c-FLIP KO CD11c-Cre BMDCs could be partly attributed to increased induction of *Tnfa* and *Il2* transcripts (Fig. 4E, 4G). Similarly, the decrease in IL-6 secretion in LPS- and curdlan-stimulated c-FLIP KO BMDCs correlated with a reduction in *Il6* mRNA (Fig. 4F). The enhanced production of TNF-α production in c-FLIP-deficient BMDCs was not restricted to TLR4 and dectin-1. Stimulation of TLR2 by Pam3CSK4 also led to enhanced secretion of TNF-α as well as elevated induction of *Tnfa* in c-FLIP KO CD11c-Cre BMDCs (Fig. 4H). Note that treatment with LPS induced extensive cell death in c-FLIP-deficient BMDCs, which could be largely prevented by necrostatin-1 (Nec-1; Supplemental Fig. 1C). In contrast, c-FLIP-deficient BMDCs were relatively resistant to cell death induced by TNF-α, curdlan, or zymosan depleted (Supplemental Fig. 1D, data not shown for zymosan depleted).

A recent study revealed that T cell proliferation defects in c-FLIP−/− T cells could be restored by inhibition of necrosis (10). The presence of Nec-1 did not affect the excess secretion of IL-2 by c-FLIP KO BMDCs stimulated by zymosan depleted (Fig. 4I), suggesting that the increased activation in c-FLIP–deficient BMDCs is dissociated from necrosis and activation of RIP-1. We also examined the activation of c-FLIP-deficient BMDCs, as we have previously found that the presence of hemizygous c-FLIP prevents cell death in myeloid cells (29). Increase in dectin-1–induced TNF-α production and IL-2 secretion was similarly observed in c-FLIP−/− BMDCs (Fig. 4J). Enhanced induction of *Tnfa* and *Il2* mRNA was also detected in c-FLIP−/− BMDCs stimulated with curdulan (Fig. 4K). Inhibition of TLR4 and dectin-1 activation by c-FLIP could be detected in other myeloid cells. Stimulation of c-FLIP−/− bone marrow–derived macrophages (BMMs) through dectin-1 also led to an increase in the generation of TNF-α (Fig. 4L). Taken together, these results suggest that c-FLIP suppresses TLR2-, TLR4-, and dectin-1–stimulated secretion of TNF-α and IL-2 in myeloid cells.

**Enhanced p38 MAPK activation in c-FLIP–deficient BMDCs and BMMs**

The increased TLR- and dectin-1–mediated induction of *Tnfa* and *Il2* transcripts in c-FLIP-deficient BMDCs suggests that c-FLIP deficiency enhances expression of these cytokines by selective TLR and dectin-1 activation signals. We next delineated the stage of the signal cascade downstream of PRRs targeted by c-FLIP in c-FLIP−/−CD11c-Cre BMDCs. Dectin-1–triggered SYK phosphorylation was not altered in c-FLIP-deficient BMDCs (Fig. 5A). The activation of JNK, ERK, and AKT by dectin-1 was similar between control and c-FLIP KO BMDCs (Fig. 5B). A comparable NF-κB activation, measured by nuclear p65 (RelA) translocation and total IκB phosphorylation, was also detected in control and c-FLIP−/− CD11c-Cre BMDCs (Fig. 5C). Among the activation signals examined, dectin-1–mediated p38 MAPK activation was clearly increased in c-FLIP−/−CD11c-Cre BMDCs, relative to control BMDCs (Fig. 5D). Similarly, LPS-induced p38 MAPK phosphorylation was higher in c-FLIP KO BMDCs than in WT BMDCs (Fig. 5D). The specific elevated p38 MAPK activation was also observed in c-FLIP−/− BMDCs and c-FLIP−/− BMMs stimulated by curdlan and LPS (Supplemental Fig. 4). In contrast, the phosphorylation of AKT, IKK, IκBα, JNK, and ERK were comparable between c-FLIP−/− and c-FLIP−/− BMDCs stimulated by curdlan and LPS (Supplemental Fig. 4A, 4B). Consistent with previous reports that p38 MAPK is involved in the expression of TNF-α and IL-2 (32, 35–37), LPS- and dectin-1–induced TNF-α and IL-2 expression in WT DCs was attenuated by p38 inhibitor SB203580 (Fig. 5E). c-FLIP–deficient BMDCs were more sensitive to SB203580 inhibition in IL-2 production than were WT BMDCs (Fig. 5F). These results suggest that c-FLIP–inhibited PRR-mediated TNF-α and IL-2 production may be p38 MAPK–dependent.

As previously reported (20), we found that c-FLIP was associated with p38 MAPK (Fig. 6A). Active MKK3 (MKK3e–)–induced p38 activation was attenuated by c-FLIP (Fig. 6B). Additionally, ASK1-induced p38 MAPK activation was inhibited by c-FLIP overexpression (Fig. 6C). Notably, an association between ASK1 and c-FLIP was also observed (Fig. 6D). These results suggest that c-FLIP directly antagonizes p38 MAPK activation by interaction with p38 MAPK or MAP3K.

**Differential production of G-CSF may account for neutrophilia in c-FLIP KO mice**

Increased p38 MAPK activation and TNF-α/IL-2 production were found in both c-FLIP−/− and c-FLIP−/−CD11c-Cre BMDCs, but neutrophilia was detected in only c-FLIP−/−CD11c-Cre mice. We looked into the difference between these two strains of mice that led to the inflammatory phenotype in c-FLIP−/−CD11c-Cre mice. G-CSF plays a pivotal role in the excess granulopoiesis in c-FLIP−/−Lysm-Cre mice (28). We found a profound increase in serum G-CSF levels in c-FLIP−/−CD11c-Cre mice (Fig. 7A). Anti–G-CSF resolved the inflammation in c-FLIP−/−CD11c-Cre mice (Fig. 7B), confirming a similar critical role of G-CSF in the spontaneous neutrophilia in c-FLIP−/−CD11c-Cre mice. Dectin-1–induced G-CSF production was increased in c-FLIP−/−CD11c-Cre BMDCs, which was correlated with a similar extent of *Csf3* transcript induction (Fig. 7C). We then examined whether there was a difference in the production of G-CSF between c-FLIP−/− and c-FLIP−/−CD11c-Cre BMDCs. LPS and dectin-1 induced robust G-CSF generation in c-FLIP−/−CD11c-Cre BMDCs relative to c-FLIP−/− BMDCs and c-FLIP−/− BMDCs (Fig. 7D). Consistent with the involvement of p38 MAPK in LPS-induced G-CSF production (38), the dectin-1–triggered secretion of G-CSF was clearly p38 MAPK–dependent in DCs (Fig. 7E). These results suggest the excess G-CSF expression in c-FLIP KO DCs may partly account for neutrophilia and inflammatory phenotypes observed in c-FLIP−/−CD11c-Cre mice.

**The suppressive activity of c-FLIP is caspase-8–independent**

c-FLIP is a direct inhibitor of caspase-8, whereas caspase-8 is known to transmit activation signals (39, 40). Additionally, TLR3, TLR4, and dectin-1 have been shown to trigger IL-1β production in the caspase-8–dependent pathway (41, 42). We recently found that c-FLIP suppresses dectin-1–induced IL-1β production by direct inhibition of caspase-8 (29). We thus examined whether the increased expression of TNF-α, IL-2, and G-CSF in c-FLIP–deficient DCs was due to enhanced activation of caspase-8. Caspase-8 inhibitor IETD was included in the culture of BMDCs stimulated with zymosan depleted. Fig. 8A illustrates that the inhibition of caspase-8 did not interfere with the enhanced production of IL-2 in c-FLIP–deficient BMDCs stimulated through dectin-1. The efficacy of IETD (10 μM) to inhibit caspase-8 was confirmed by the suppression of caspase-8 activity (Fig. 8B) and by the inhibition of LPS- and dectin-1–induced IL-1β production (Supplemental Fig. 1F). IETD did not affect LPS- and dectin-1–triggered expression of G-CSF in c-FLIP−/−CD11c-Cre BMDCs either (Fig. 7F). We also used caspase-8–specific siRNA to knock down caspase-8 in c-FLIP−/− BMDCs (Fig. 8C). Downregulation of caspase-8 in c-FLIP−/− BMDCs did not affect curdlan-induced expression of TNF-α and IL-2 (Fig. 8D, 8E). Additionally, the...
FIGURE 4. c-FLIP deficiency increases TLR2-, TLR4-, and dectin-1–induced production of TNF-α and IL-2 in BMDCs. (A) Increased LPS-induced TNF-α production and decreased IL-6 secretion in c-FLIP–deficient BMDCs. c-FLIPΔf and c-FLIPΔfCD11c-Cre BMDCs were stimulated with LPS (1 μg/ml) for 5 and 20 h, and TNF-α and IL-6 in supernatants were determined by ELISA. Nec-1 (30 μM) was included in the 20-h experiment. (B) Increased dectin-1–induced TNF-α and reduced IL-6 secretion in c-FLIP–deficient BMDCs. c-FLIPΔf and c-FLIPΔfCD11c-Cre BMDCs were stimulated with curdlan (100 μg/ml) for 5 and 20 h, and TNF-α and IL-6 in supernatants were determined by ELISA. (C and D) IL-2 secretion, but not IL-10 production, is increased in dectin-1–treated c-FLIP KO BMDCs. Control and c-FLIP–deficient BMDCs were stimulated with curdlan for 4 h or zymosan depleted (200 μg/ml) for 24 h, and the secretion of IL-2 (C) and IL-10 (D) was determined. (E–G) Increased Tfαf and Il2f transcript in LPS- and curdlan-stimulated c-FLIP–deficient BMDCs. c-FLIPΔf and c-FLIPΔfCD11c-Cre BMDCs were stimulated with LPS (1 μg/ml) or curdlan (100 μg/ml) for 3 h, and total RNA was isolated. The amounts of Tfα (E), Il6 (F), and Il2 (G) transcripts were determined by quantitative PCR. Values were normalized against Gapdh transcripts. (H) Increased Pam3CSK4-induced TNF-α protein and transcripts in c-FLIP–deficient BMDCs. c-FLIPΔf and c-FLIPΔfCD11c-Cre BMDCs were stimulated with Pam3CSK4 (1 μg/ml) for 3 h and RNA was harvested and for 5 h and cell supernatants were collected. The TNF-α secreted and the Tfαf mRNA induced were quantitated. (I) Nec-1 did not affect dectin-1–induced IL-2 production in c-FLIP–deficient BMDCs. c-FLIPΔf and c-FLIPΔfCD11c-Cre BMDCs were pretreated with or without Nec-1 (30 μM) for 1 h and stimulated with zymosan depleted (200 μg/ml) for 24 h with the continued presence of Nec-1, as indicated. The IL-2 produced was quantitated. (J and K) Increased dectin-1–induced TNF-α and IL-2 expression in c-FLIP hemizygotic BMDCs. c-FLIPΔf and c-FLIPΔfCD11c-Cre BMDCs were stimulated with curdlan for 3 h. The TNF-α and IL-2 secreted (J) and the Tfαf and Il2f mRNA induced (K) were quantitated. (L) Increased LPS-induced TNF-α expression in c-FLIP hemizygotic macrophages. c-FLIPΔf and c-FLIPΔfCD11c-Cre BMDCs were pretreated with curdlan for 3 h, and the TNF-α secreted and the Tfαf mRNA induced were quantitated. Values are means ± SD. Data in (A)–(F) are results of three to six pairs of mice. Data in (G)–(L) are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 for paired t test.
enhanced p38 MAPK activation in curdlan-stimulated c-FLIP-deficient BMDCs was not affected by caspase-8 knockdown (Fig. 8F). These results suggest that the suppression of PRR-induced TNF-α, IL-2, and G-CSF production and p38 MAPK activation by c-FLIP are not through caspase-8 inhibition.

Discussion
In the present study, we examined the effect of c-FLIP deficiency in the development and function of DCs. We found that even though the deletion of c-FLIP in DCs was incomplete in c-FLIPf/fCD11c-Cre mice, the ratio of deleted allele to floxed allele in DCs suggests that the degree of deletion of c-FLIP was extensive (Fig. 1A, 1B). The effects of c-FLIP KO in DCs were not as profound as in c-FLIP KO in macrophages (27, 28). The populations of cDCs and pDCs were not affected in c-FLIPf/fCD11c-Cre mice (Fig. 2), in contrast to loss of macrophages in c-FLIPf/fLysm-Cre mice (27, 28). Additionally, the extent of neutrophilia was much more profound in c-FLIPf/fLysm-Cre mice (27, 28) than in c-FLIPf/fCD11c-Cre mice (Figs. 1, 2). The differential effect of c-FLIP deficiency in macrophages and DCs is analogous to the effect of caspase-8 deficiency: whereas the differentiation of caspase-8−/− macrophages is impaired, caspase-8−/− DCs are less affected (30). Note that there
Spontaneous neutrophilia and inflammation in c-FLIP<sup>−/−</sup>Lysm-Cre mice are caused by deficiency of macrophages for clearance of apoptotic neutrophils in a G-CSF-dependent manner (28). In c-FLIP<sup>−/−</sup>CD11c-Cre mice, the macrophage population was not significantly affected (Fig. 2), suggesting that the uptake of apoptotic neutrophils by macrophages proceeded mostly normally. We found an excess G-CSF production in c-FLIP<sup>−/−</sup>CD11c-Cre DCs, but not in c-FLIP<sup>+/+</sup> BMDCs (Fig. 7C, 7D), correlating with the development of neutrophilia in c-FLIP<sup>−/−</sup>CD11c-Cre mice but not in c-FLIP<sup>+/+</sup> mice. These results suggest that aberrant G-CSF secretion in c-FLIP<sup>−/−</sup>CD11c-Cre mice likely accounts for the extra presence of neutrophils and the inflammatory phenotype.

TNF-α and dendin-1 ligands induced a weak cell death in c-FLIP-deficient BMDCs (Fig. 1D). In contrast, c-FLIP-CKO DCs were sensitive to LPS-induced cell death, which could be mostly prevented by Nec-1 (Supplemental Fig. 1C). We conducted analyses in the presence of Nec-1 but observed that dendin-1–induced IL-2 generation was still enhanced in c-FLIP<sup>−/−</sup>CD11c-Cre BMDCs (Fig. 4I). Additionally, Tafs, Il2, and Csf3 transcripts were increased whereas Il6 mRNA was reduced in the same experiments. This suggests that the stages targeted by c-FLIP are not at the TLR4/dectin-1 receptor levels or the signaling immediately downstream of TLR4/dectin-1.

We found that the activation of several signal pathways, including Akt, JNK, and NF-κB, were not apparently affected in c-FLIP<sup>−/−</sup> CD11c-Cre BMDCs (Fig. 5, Supplemental Fig. 4). A signal in our analysis that was consistently increased in both c-FLIP<sup>−/−</sup>CD11c-Cre BMDC and c-FLIP<sup>+/+</sup> BMDCs was p38 MAPK (Fig. 5D, Supplemental Fig. 4). We and others have previously shown the requirement of p38 MAPK in T cell activation and IL-2 production (32, 35). p38 MAPK also participates in the generation of TNF-α in T cells and macrophages (36, 37). We also demonstrated that dendin-1–triggered G-CSF production was p38-dependent (Fig. 7D). SB203580 inhibited IL-2 production more effectively in c-FLIP–deficient BMDCs than in WT BMDCs (Fig. 5F). The increased production of TNF-α/IL-2/G-CSF in c-FLIP-deficient myeloid cells could be partly due to enhanced activation of p38 MAPK. However, we cannot exclude the involvement of additional signaling in promoting TNF-α/IL-2/G-CSF expression in c-FLIP-deficient DCs.

Previous studies have shown that overexpressed c-FLIP inhibits the TNF-α–activated JNK and ERK in cancer cells (19) and suppresses bile acid–triggered p38 activation in hepatoma cells (20). We failed to detect an increase in ERK or JNK signaling in c-FLIP-deficient BMDCs when stimulated through TLR4 or dendin-1 (Fig. 5). We did observe the binding of c-FLIP to p38 and ASK1 and the inhibition of ASK1-induced p38 activation by c-FLIP (Fig. 6), in agreement with a moderate suppression of ASK1-mediated p38 phosphorylation by c-FLIP previously observed (19). c-FLIP less effectively inhibited the MKK3-triggered p38 activation (Fig. 6B). These results suggest that the binding of c-FLIP to p38 MAPK/ASK1 may inhibit the signaling complex containing ASK1/MKK3/p38 but less so in suppressing the direct activation of p38 by active MKK3. Our studies therefore not only confirm the inhibitory activity of c-FLIP in p38 activation in an overexpression system, but also demonstrate, to our knowledge for the first time, the inhibition of p38 activation by c-FLIP in c-FLIP–deficient myeloid cells. Notably, earlier studies have also found that TCR-induced p38 activation is not affected by c-FLIP deficiency (16). These discrepancies suggest a possibility that the involvement of c-FLIP in p38 MAPK activation is receptor- and cell type–dependent.

The most prominent effect in c-FLIP–deficient BMDCs was the increase in dendin-1–induced IL-2 and G-CSF expression (Figs. 4, 7). c-FLIP deficiency moderately increased LPS- and dendin-1–triggered TNF-α production (Figs. 3, 4). Additionally, LPS-induced IL-6 generation was mildly decreased in c-FLIP<sup>−/−</sup>CD11c-Cre BMDCs (Fig. 4). The modest effect of c-FLIP deficiency in LPS-induced
inflammatory cytokine production, together with the stringent requirement of c-FLIP for macrophage survival, may explain why the inhibitory effect of c-FLIP on TLR-mediated inflammatory cytokine production in myeloid cells was previously overlooked. An increase in LPS-induced p38 activation in c-FLIP<sup>−/−</sup>/CD11c-Cre mice has been previously reported, even when LPS-induced B cell proliferation was suppressed in c-FLIP<sup>−/−</sup> B cells (43). Our demonstration that c-FLIP inhibited TLR- and dectin-1–induced production of IL-2, G-CSF, and TNF-α partly by inhibition of p38 MAPK and independent of caspase-8 therefore represents a novel apoptosis-independent biological activity of c-FLIP through the suppression of activation signals.

Notably, the present study has illustrated, to our knowledge for the first time, the anti-inflammatory function of c-FLIP in myeloid cells. c-FLIP is upregulated in many types of cancer cells. Once a tumor is formed, the presence of anti-inflammatory setting signals in the tumor microenvironment prevents lymphocyte activa-
DCs play a critical role in the generation of antitumor T cell response and in tumor immune tolerance (44). c-FLIP is a target in cancer therapy. Small-molecule drugs and c-FLIP–specific siRNA that downregulates c-FLIP have been developed (45, 46). Additionally, many chemotherapeutics exhibit the ability to inhibit c-FLIP expression. Our results show that downregulation of c-FLIP led to elevated activation of TLR and dectin-1. Importantly, the increased production of inflammatory cytokines and recruitment of neutrophils resulted in enhanced expression of CD80 and CD86 in DCs in vivo (Fig. 3A), leading to increased T cell activation (Fig. 3B). Recent studies have illustrated the potent efficacy of enhanced costimulation in the generation of tumor-specific T cell immunity (47). Even though downregulation of c-FLIP in cancer therapy is aimed primarily at increasing the sensitivity of cancer cells to apoptosis induction by TNF-α and TRAIL, our results suggest that c-FLIP degradation further increases the activation of DCs, accompanied by enhanced activation of T cells and the potential generation of tumor-specific T cell immunity. c-FLIP–targeting anticancer compounds therefore represent drug candidates that target both cancer cells for cell death and DCs for activating tumor-specific T cell responses (48). Further characterization will help assess the precise contribution of c-FLIP–downregulated DCs to the production of immune responses toward a tumor for an optimized regimen in cancer therapy.
Supplemental Figure 1. Differential knockout of c-FLIP in selective myeloid populations and sensitivity to cell death. (A) Partial deletion of c-FLIP in bone marrow pre-DCs and splenic CD8+ cDC. Genomic DNA from bone marrow pre-DCs and CD8+ cDCs were isolated, and c-FLIP floxed allele and deleted allele were detected by PCR. c-
FLIP\textsuperscript{fr}CD11c-Cre BMDCs was served as a control. A long exposure of PCR product from floxed allele in pre-DCs is shown, due to inability to detect the deleted allele. (B) A small deletion of c-FLIP in splenic macrophages from c-FLIP\textsuperscript{fr}CD11c-Cre mice. Genomic DNA from splenic F4/80\textsuperscript{+}CD11b\textsuperscript{+} cells was isolated, and c-FLIP floxed allele and deleted allele were detected by PCR. DNA from c-FLIP\textsuperscript{fr}CD11c-Cre BMDCs was used as a control. (C) Increased sensitivity to LPS-induced cell death in c-FLIP-deficient dendritic cells. c-FLIP\textsuperscript{fr} and c-FLIP\textsuperscript{fr}CD11c-Cre BMDCs were treated with LPS for 6 h in the absence or presence of Nec-1 (30 µM) and cell viability was determined by ATP contents. (D) Resistance to TNF-α- and curdlan-triggered cell death in c-FLIP-knockout dendritic cells. c-FLIP\textsuperscript{fr} and c-FLIP\textsuperscript{fr}CD11c-Cre BMDCs were treated with TNF-α or for curdlan for 6 h and cell viability was determined by ATP contents. **p < 0.01, ***p < 0.001 for paired t-test. (E) Increased Il2 expression in c-FLIP-deficient BMDCs. Results from two additional independent studies are shown for Fig. 4G. (F) IETD inhibits dectin-1-induced IL-1β production. WT bone marrow-derived macrophages were stimulated with LPS and zymosan-depleted in the absence or presence of IETD (10 µM) as indicated, and the production of IL-1β determined 6 h after stimulation. Data (A, B, C, D, F) are representative of three independent experiments.
Supplemental Figure 2. Conditional knockout of c-FLIP in dendritic cells does not affect cDC precursors and lymphocytes in spleen. (A) Flow cytometric analysis of spleen pre-DCs (Lin^L^-I^A^b^-SIRPa^int^Flt3^-^CD11c^high^) from c-FLIP^ff^ and c-FLIP^ff^CD11c-Cre mice. Data are representative of two independent experiments. (B) Normal F4/80^+^ fraction and reduced B220^+^ B cell percentage in c-FLIP-CKO spleen. Splenic cells from c-FLIP^ff^
and c-FLIP<sup>fl</sup>CD11c-Cre mice were analyzed for the expression of F4/80 and B220. (C) Splenic macrophage population is increased in c-FLIP<sup>fl</sup>CD11c-Cre mice. F4/80<sup>+</sup>CD11b<sup>+</sup> population was analyzed in spleens from c-FLIP<sup>fl</sup> and c-FLIP<sup>fl</sup>CD11c-Cre mice. (D, E) Moderate decrease in splenic T cell frequency in c-FLIP-CKO mice. Splenic CD4<sup>+</sup> and CD8<sup>+</sup> (D) or CD4<sup>+</sup> naïve (CD44<sup>−</sup>CD62L<sup>+</sup>) and CD4<sup>+</sup> effector (CD44<sup>+</sup>CD62L<sup>−</sup>) (E) T cells in c-FLIP<sup>fl</sup> and c-FLIP<sup>fl</sup>CD11c-Cre mice were analyzed by flow cytometry. (F, G, H, I) c-FLIP-deficiency increases the cellularity of macrophages but not lymphocytes. The absolute numbers of B cells (F), macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>) (G), CD4<sup>+</sup> T (H), and CD8<sup>+</sup> T cells (I) in spleens of c-FLIP<sup>fl</sup> and c-FLIP<sup>fl</sup> CD11c-Cre mice were calculated. Values are mean ± SD. n = 5. * p < 0.05 for paired t-test.
Supplemental Figure 3. Conditional knockout of c-FLIP in dendritic cells leads to increased PMNs in peripheral blood and peritoneal cavity. (A) Increased PMN frequency in peripheral blood in c-FLIP<sup>ff</sup> CD11c-Cre mice. The populations of neutrophils (CD11b<sup>+</sup>Ly6C<sup>int</sup>), inflammatory monocytes (SSC<sup>lo</sup>CD11b<sup>+</sup>Ly6C<sup>hi</sup>), or resident monocytes (SSC<sup>lo</sup>CD11b<sup>+</sup>Ly6C<sup>lo</sup>) in peripheral blood from c-FLIP<sup>ff</sup> and c-FLIP<sup>ff</sup> CD11c-Cre mice were determined. (B) Increased PMN in peritoneal cavity in c-FLIP<sup>ff</sup> CD11c-Cre mice. Peritoneal exudate cells (PECs) were analyzed for the populations of CD11b<sup>+</sup>Gr-1<sup>-</sup>
neutrophils and CD11b^F4/80^ macrophages. (C) Normal blood CD4^+ and CD8^+ T cell population in c-FLIP^{f/f} CD11c-Cre mice. Flow cytometric analysis of T cell populations in peripheral blood from c-FLIP^{f/f} and c-FLIP^{f/f} CD11c-Cre mice. (D) Normal thymus population in c-FLIP^{f/f} CD11c-Cre mice. Thymocytes from c-FLIP^{f/f} and c-FLIP^{f/f} CD11c-Cre mice were subjected to analysis by staining with CD4/CD8 and TCR\(\beta/C\)D69. CD4^- CD8^- double negative (DN) thymocytes were gated and analyzed for the populations of DN1 to DN4 by the expression of CD44 and CD25. Data (A-D) shown are representative of three mice.
Supplemental Figure 4. Enhanced p38 MAPK activation in dectin-1- and LPS-stimulated c-FLIP-hemizygotic BMDCs and BMMs. (A) Increased p38 MAPK activation and normal JNK, AKT, and IKK activation in c-FLIP+/− BMDCs stimulated by curdlan. c-FLIP+/− and c-FLIP−/− BMDCs were stimulated with curdlan, and the phosphorylation of p38 MAPK, IκBα, AKT, JNK, and IKK determined at the indicated time points. (b) Increased LPS-induced p38 MAPK activation in c-FLIP+/− BMDCs. c-
FLIP^{f/f} and c-FLIP^{f-} BMDCs were stimulated with LPS, and the phosphorylation of p38 MAPK, AKT, IKK, IκBα, ERK, and JNK were determined at the indicated time points. (c) Enhanced p38 MAPK activation in c-FLIP^{+/} BMM stimulated by LPS and curdlan. c-FLIP^{f/f} and c-FLIP^{f-} BMMs were stimulated with LPS or curdlan, and the phosphorylation of p38 MAPK were determined at the indicated time points. Data shown is representative of three independent experiments.