3-7-2017

Distinct Kinase-Independent Role of RIPK3 in CD11c+ Mononuclear Phagocytes in Cytokine-Induced Tissue Repair

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Moriwaki, Kenta; Balaji, Sakthi; Bertin, John; Gough, Peter J.; and Chan, Francis Ka-Ming, "Distinct Kinase-Independent Role of RIPK3 in CD11c+ Mononuclear Phagocytes in Cytokine-Induced Tissue Repair" (2017). Open Access Articles. 3120.  
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Keywords
IL-1b, IL-23, RHIM, RIPK3, colitis, dextran sodium sulfate, inflammation, injury, necroptosis, tissue repair

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Distinct Kinase-Independent Role of RIPK3 in CD11c⁺ Mononuclear Phagocytes in Cytokine-Induced Tissue Repair

**Highlights**

- Generation of RIPK3-GFP reporter mice to determine the in vivo expression of RIPK3
- Generation of RIPK3-ΔRHIM mutant mice to determine functions of the RIPK3 RHIM
- The RHIM is crucial for RIPK3-dependent necroptosis and cytokine expression
- Kinase-independent function of RIPK3 drives intestinal CD11c⁺ MNPs cytokine production

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**In Brief**

Moriwaki et al. demonstrate that RIPK3 promotes cytokine production in CD11c⁺ mononuclear phagocytes in an RHIM-dependent, but kinase-independent manner. This necroptosis-independent function of RIPK3 is crucial for tissue repair in response to intestinal injury.
Distinct Kinase-Independent Role of RIPK3 in CD11c⁺ Mononuclear Phagocytes in Cytokine-Induced Tissue Repair

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http://dx.doi.org/10.1016/j.celrep.2017.02.015

SUMMARY

Receptor interacting protein kinase 3 (RIPK3) induces necroptosis, a type of regulated necrosis, through its kinase domain and receptor interacting protein (RIP) homotypic interaction motif (RHIM). In addition, RIPK3 has been shown to regulate NLRP3 inflammasome and nuclear factor κB (NF-κB) activation. However, the relative contribution of these signaling pathways to RIPK3-dependent inflammation in distinct immune effectors is unknown. To investigate these questions, we generated RIPK3-GFP reporter mice. We found that colonic CD11c⁺CD11b⁺CD14⁺ CD103⁻ mononuclear phagocytes (MNPs) expressed the highest level of RIPK3 in the lamina propria. Consequently, deletion of the RIPK3 RHIM in CD11c⁺ cells alone was sufficient to impair dextran sodium sulfate (DSS)-induced interleukin (IL)-23 and IL-1β expression, leading to severe intestinal inflammation. In contrast, mice expressing kinase inactive RIPK3 were not hypersensitive to DSS. Thus, a key physiological function of RIPK3 is to promote reparative cytokine expression through intestinal CD11c⁺ MNPs in a kinase- and necroptosis-independent manner.

INTRODUCTION

Necroptosis is a type of regulated necrosis induced by several cell surface immune receptors such as tumor necrosis factor (TNF) receptor (TNFR), Toll-like receptor (TLR) 3 (TLR3), TLR4, interferon receptor, and T cell receptor (Chan et al., 2015). Receptor interacting protein kinase 3 (RIPK3) is a cytosolic master regulator of necroptosis (Moriwaki and Chan, 2013). RIPK3 has an active serine-threonine kinase domain at the N terminus and a unique protein-protein interaction domain called the RIP homotypic interaction motif (RHIM) at the C terminus. Both kinase activity and RHIM are indispensable for necroptosis (Cho et al., 2009). RIPK3 interacts with other RHIM-containing proteins such as RIPK1 (Cho et al., 2009; He et al., 2009), Toll/interleukin-1 (IL-1) receptor domain-containing adaptor protein inducing interferon β (TRIF) (He et al., 2011), or DNA-dependent activator of interferon regulatory factor (DAI) (Upton et al., 2012). RHIM-RHIM interaction leads to amyloid-like conformational change and enhancement of RIPK3 kinase activity (Li et al., 2012). Activated RIPK3 phosphorylates the downstream adaptor MLKL (Sun et al., 2012), which triggers oligomerization and translocation of MLKL to the plasma membrane (Cai et al., 2014; Chen et al., 2014; Dondelinger et al., 2014; Wang et al., 2014).

Besides necroptosis, recent emerging evidence shows that RIPK3 also has necroptosis-independent functions (Moriwaki and Chan, 2014). For example, RIPK3 stimulates nuclear factor κB (NF-κB)-dependent Il23p19 (Il23a) expression and pro-IL-1β processing downstream of TLR4 in bone marrow (BM)-derived dendritic cells (DCs) (BMDCs) (Moriwaki et al., 2014). Similar RIPK3-dependent processing of pro-IL-1β has also been observed in BM-derived macrophages (BMDMs) treated with Smac mimetics and/or caspase inhibitors (Kang et al., 2013; Lawlor et al., 2015; Vince et al., 2012). However, the relative contribution of necroptosis-dependent and -independent effects of RIPK3 in physiological inflammation has not been clearly elucidated.

Germline Ripk3-deficient mice have been used extensively to investigate the pathophysiological roles of RIPK3 (Chan et al., 2015). The resolution of inflammation in Ripk3⁻/⁻ mice has often been attributed as a consequence of blocking necroptosis. Strikingly, RIPK3 has recently been shown to have paradoxical function in promoting injury-induced tissue repair (Godwin et al., 2015). In a mouse model of chemical-induced intestinal injury, RIPK3 facilitates expression of IL-23 and IL-1β, which in turn stimulates expression of the tissue repair cytokine IL-22. Thus, Ripk3⁻/⁻ mice developed sustained injury and inflammation (Moriwaki et al., 2014, 2016). Based on these results, we asked whether RIPK3 promotes cytokine-induced tissue repair mainly through DCs. To achieve our goal, we generated “knock-in” mice expressing an RIPK3-GFP fusion reporter, as well as mice with tissue-specific deletion of the essential RHIM (Ripk3ΔRHIM). Using the RIPK3-GFP reporter mice, we found that RIPK3 was highly expressed in intestinal CD11c⁺CD11b⁺CD14⁻CD103⁻.
mononuclear phagocytes (MNPs). Deletion of RIPK3 RHIM in CD11c+ cells fully recapitulated the severe intestinal inflammation observed in germline Ripk3−/−/− mice. In contrast, kinase inactive RIPK3 knock-in mice did not exhibit hypersensitivity in response to chemical-induced colitis. Collectively, these results reveal cell-type-specific functions of RIPK3 in inflammation as well as the importance of RHIM-RHIM-mediated interaction in biological responses beyond necroptosis.

RESULTS

Generation of RIPK3-GFP Reporter Mice

RIPK3 expression is often induced in tissues and cells during inflammation (Gautheron et al., 2014; Moriwaki et al., 2014; Vitner et al., 2014). To determine the physiological expression pattern of RIPK3 in quiescent and inflamed states, we generated RIPK3-GFP reporter mice in which the Ripk3 gene was fused in-frame with the eGfp sequence at the end of the RIPK3 translated sequence (Figure S1A). Proper targeting of the Ripk3 locus was confirmed by Southern blot analysis (Figures S1A–S1C). We confirmed that RIPK3-GFP and wild-type RIPK3 were expressed at similar levels in Ripk3-gfpfl/fl mouse embryonic fibroblasts (MEFs) (Figure 1A).

The percentage and number of T and B cells in the thymus and lymph node were normal in Ripk3+/+ and Ripk3-gfpfl/fl reporter mice (Figures S1D and S1E and data not shown). In addition, the composition of BM cells was unchanged in Ripk3-gfpfl/fl mice (Figure S1F).

Hence, the RIPK3-GFP fusion protein faithfully reports in vivo expression of RIPK3 without disrupting RIPK3 function.
Real-time PCR analysis using various mouse tissues showed the highest expression of the Ripk3 transcript in the spleen (Figure S2A), suggesting that RIPK3 is highly expressed in immune cells. Indeed, strong GFP fluorescence signal was detected in various immune cells in the spleen (Figure 1B; Figure S2B). In particular, splenic CD11c+CD11b+ GFP/F4/80low monocytes expressed RIPK3 at the highest level (Swirski et al., 2009) (Figure 1B). In contrast, CD11c+CD11b+CD103+ MNPbs, but not CD11c+CD11b+ macrophages, showed the highest RIPK3 expression in the colon (Figure 1C; Figure S2C). These results indicate that RIPK3 expression pattern in immune cells is highly variable in different cell types and tissues.

We next examined how inflammation might affect RIPK3 expression. To this end, we first injected lipopolysacharride (LPS) intraperitoneally and analyzed splenocytes for RIPK3 expression. To this end, we first injected lipopolysacharride (LPS) intraperitoneally and analyzed splenocytes for RIPK3 expression. Whereas RIPK3 expression in CD11c+CD11b+CD103+ MNPbs, but not CD11c+CD11b+ macrophages, showed the highest RIPK3 expression in the colon (Figure 1C; Figure S2C). These results indicate that RIPK3 expression pattern in immune cells is highly variable in different cell types and tissues.

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The RIPK3 RHIM Is Critical for RIPK3-Mediated Cell Death

RIPK3 has two distinct functional domains: the kinase domain and the RHIM. Studies in knock-in mice expressing kinase inactive RIPK3 (Ripk351A/51A and Ripk351/51) showed that RIPK3 kinase activity is crucial for necroptosis (Mandal et al., 2014; Newton et al., 2014). In contrast, the physiological function of the RHIM has not been fully explored. To examine the physiological importance of the RHIM, we generated mice specifically lacking RIPK3 RHIM at the C terminus using Cre-loxP-mediated recombination (Figure S3A). We crossed the Ripk3-gfpCre reporter mice with Sox2-Cre deleter mice to generate mice with germline deletion of the RHIM (Ripk3ΔRHIM) (Figure S3B). Loss of GFP fluorescence signal from all immune cell subsets from Ripk3ΔRHIM mice was confirmed by flow cytometry (Figure 1A). Because the stop codon of the Ripk3 gene was removed in the Ripk3Δ allele, we performed 3′-rapid amplification of cDNA ends (RACE) experiment using total RNA from Ripk3ΔMEFs to determine the C-terminal amino acid sequence of the RIPK3-ΔRHIM protein. The result revealed two possible mRNA transcripts generated from the Ripk3Δ allele (Figure S3C). DNA sequencing confirmed that both transcripts are expected to encode truncated and shorter RIPK3 that lack the RHIM (Figure S3D). A widely used rabbit polyclonal antibody that recognizes the C-terminal end of mouse RIPK3 revealed loss of full-length RIPK3 expression from Ripk3Δ/Δ cells and tissues (Figures 2B and 2C; Figure S3E, middle, RIPK3-P). In contrast, the anti-RIPK3 antibody clone 1G6.1.4 (Ripk3-G) recognized both wild-type RIPK3 and the RIPK3-ΔRHIM proteins that were absent in Ripk3Δ/Δ cells (Figures 2B and 2C; Figure S3E, top, RIPK3-G) (Newton et al., 2014). It should be noted that the RIPK3-G antibody detected two bands, both of which were specific for RIPK3 (Newton et al., 2014), and that a single band of RIPK3-ΔRHIM protein was detected between these two wild-type (WT) bands (Figures 2B and 2C). This suggests that WT RIPK3 has two isoforms with different C-terminal sequences. Similar to mice expressing kinase inactive RIPK3 (Mandal et al., 2014), RIPK3-ΔRHIM was expressed at a lower level compared with wild-type RIPK3 (Figures 2B and 2C, top). This suggests that the RHIM and kinase activity are both required to stabilize RIPK3 protein expression.

To evaluate the impact of the RHIM on necroptosis, we treated Ripk3Δ/Δ MEFs with TNF, the pan-caspase inhibitor zVAD-fmk (zVAD), and cycloheximide (CHX). In contrast with wild-type MEFs, Ripk3Δ/Δ MEFs were resistant to TNF-induced necroptosis (Figure 2D). Ripk3Δ/Δ BMDCs were also resistant to necroptosis induced by LPS and zVAD-fmk (Figure 2E) and zVAD-fmk and the Smac mimetic BV6 (Figure 2F), which induces autocrine TNF production (McComb et al., 2012). In addition, Ripk3Δ/Δ BMDCs were resistant to necroptosis induced by TNF, LPS, or polyinosinic-polycytidylic acid (polyIC) in the presence of the pan-caspase inhibitor zVAD-fmk (Figure 2G). Similar resistance to necroptosis was observed in Ripk3Δ/Δ 3T3 cells reconstituted with HA-tagged RIPK3-ΔRHIM proteins (Figure S3F). By contrast, Ripk3Δ/Δ BMDCs were equally sensitive to apoptosis induced by TNF, LPS, or poly(I:C) (Figure S3G). Moreover, Ripk3Δ/Δ MEFs were resistant to apoptosis induced by the RIPK3 kinase inhibitor GSK’843 (Figure S3H), which causes caspase-8 activation through RHIM-mediated formation of the ripoptosome (Mandal et al., 2014; Moriwaki and Chan, 2016). Furthermore, Ripk3Δ/Δ BMDCs were resistant to LPS- and CHX-induced caspase-3/8 activation and apoptosis (Figures S3I and S3J) (Moriwaki et al., 2015). These results indicate that the RIPK3 RHIM is crucial for RIPK3-mediated necroptosis and apoptosis.

Caspase-8−/− and Fadd−/− mice suffer from embryonic lethality caused by excessive necroptosis. This defect was completely rescued by inactivation of Ripk3 (Kaiser et al., 2011; Oberst et al., 2011). We set up intercrosses of Fadd−/− Ripk3Δ/Δ mice and found that Fadd−/−Ripk3Δ/Δ mice were born at a Mendelian ratio (Figure 2H). Moreover, Fadd−/−Ripk3Δ/Δ mice developed splenomegaly and lymphadenopathy (Figure 2I), and exhibited an expansion of CD3+ CD20+ CD4+ CD8+ T cells in the spleen (Figure 2J). These phenotypes are characteristic of lpr and gld mice and the human autoimmune lymphoproliferative syndrome (ALPS) (Lenardo et al., 1999). Hence, the RIPK3 RHIM is essential for necroptosis induced by various physiological stimuli, and deletion of the RHIM is functionally equivalent to the Ripk3 null allele.

The RHIM Is Essential for RIPK3-Dependent Cytokine Production

We previously reported that RIPK3 promotes NF-κB and inflammation activation in BMDCs, and that these necroptosis-independent signaling functions are crucial for cytokine production.
downstream of TLR4 (Moriwaki et al., 2014). In contrast with BMDCs treated with an RIPK3 kinase inhibitor (Moriwaki et al., 2014), LPS-induced Il23p19 expression was reduced in Ripk3ΔR/ΔR BMDCs (Figure 3A). TNF expression was also significantly diminished in LPS-treated Ripk3ΔR/ΔR BMDCs (Figure 3B). The RelB-p50 heterodimer potently induces Il23p19 expression by BMDCs in response to TLR stimulation (Shih et al., 2012). This led us to test whether the RIPK3 RHIM is required for optimal RelB-p50 activation and cytokine expression (Moriwaki et al., 2014). Indeed, LPS-induced nuclear translocation of...
The RHIM of RIPK3 Mediates Protection against DSS-Induced Colitis

DSS induces intestinal epithelial cell injury, and the resultant inflammation exhibits certain features resembling acute colitis. We previously showed that RIPK3-mediated expression of the cytokines IL-23, IL-1β, and IL-22 has a crucial role in the resolution of intestinal injury (Moriwaki et al., 2014). To determine whether this tissue repair function of RIPK3 requires an intact RHIM, we treated Ripk3ΔRΔR mice with DSS for 7 days, followed by recovery in normal drinking water for another 7 days. When compared with Ripk3+/+ littermates, Ripk3ΔRΔR mice suffered...
from more severe body weight loss (Figure 4A). In addition, colon length after DSS treatment was significantly reduced in Ripk3<sup>3K1A</sup> mice (Figure 4B). In contrast, Ripk3<sup>kd/kd</sup> (K51A) kinase inactive mice developed similar intestinal inflammation compared with co-housed Ripk3<sup>−/−</sup> controls, in which RIPK3 protein is expressed at the level similar to Ripk3<sup>K51A</sup> protein, as determined by body weight loss and colon length on day 15 (Figures 4C and 4D). Because the kinase activity of RIPK3 is essential for necroptosis, these results indicate that RIPK3 mediates reparative inflammation in the intestine through RHIM-dependent, but necroptosis- and kinase-independent, mechanisms.

**RIPK3 Promotes Injury-Induced Cytokine Expression by CD11c<sup>+</sup> Cells**

Radiation BM chimera experiments revealed that RIPK3 expression in hematopoietic cells was important for protection against DSS-induced colitis (Moriwaki et al., 2014). Adoptive transfer of LPS-treated Ripk3<sup>+/+</sup> BMDCs, but not Ripk3<sup>−/−</sup> BMDCs, rescued production of the tissue-repair-associated cytokines IL-22, IL-23, and IL-1β. Because RIPK3 promotes optimal cytokine expression in BMDCs, we reasoned that DC-like cells are responsible for the protective effect of RIPK3 in DSS-induced colitis and tissue repair. To test our hypothesis, we generated DC-specific Ripk3<sup>3K1AR</sup> (<sup>CD11c</sup>:Ripk3-gfp<sup>fl/fl</sup>) mice by crossing the Ripk3-gfp<sup>fl/fl</sup> reporter mice with CD11c<sup>(ltgax)-Cre</sup> transgenic mice. Flow cytometry confirmed that the GFP fluorescence signal was lost in CD11c<sup>+</sup> cells (Figure 5A). Consistent with the reported weak activity of the CD11c-Cre transgene in T and B cells (Catton et al., 2007), we also observed slight reduction in GFP signals in these cells. When the CD11c:Ripk3-gfp<sup>fl/fl</sup> mice were challenged with DSS, they exhibited much more severe body weight loss and shortening of the colon than Ripk3-gfp<sup>fl/fl</sup> littermates (Figures 5B and 5C). Similar to Ripk3<sup>−/−</sup> mice (Moriwaki et al., 2014), CD11c:Ripk3-gfp<sup>fl/fl</sup> mice produced significantly reduced IL-22, a critical promoter of tissue repair, in the colon after DSS treatment than littermate controls (Figure 5D). In addition, expression of IL-23 and IL-1β, which stimulate IL-22 production by type 3 innate lymphoid cells (Lee et al., 2013), was also significantly suppressed in the colon of DSS-treated CD11c:Ripk3-gfp<sup>fl/fl</sup> mice (Figure 5D). By contrast, TNF expression, which was normal in DSS-treated Ripk3<sup>−/−</sup> mice (Moriwaki et al., 2014), was also unaffected in CD11c:Ripk3-gfp<sup>fl/fl</sup> mice (Figure S4A). The number of T cells, B cells, and CD11c<sup>+</sup> and CD11b<sup>+</sup> MNPs in the intestinal lamina propria was similar in Ripk3-gfp<sup>fl/fl</sup> and CD11c:Ripk3-gfp<sup>fl/fl</sup> mice (Figures S4B and S4C). In addition, the percentage of type 3 innate lymphoid cells and RIPK3 in these cells was also unchanged in CD11c:Ripk3-gfp<sup>fl/fl</sup> mice (Figures S4D and S4E). These results are consistent with those observed in germline Ripk3<sup>−/−</sup> mice and indicate that RIPK3 promotes optimal expression of repair-associated cytokines by CD11c<sup>+</sup> cells in a cell-intrinsic manner.

**The Role of Colonic CX3CR1<sup>+</sup> MNPs in RIPK3-Dependent Cytokine Expression**

Colonic CD11c<sup>+</sup> cells are subdivided into two main populations: CD11c<sup>+</sup>:CD11b<sup>-/−</sup>:CD103<sup>+</sup> and CD11c<sup>+</sup>:CD11b<sup>-/−</sup>:CD103<sup>+</sup> cells (Figure S2C) (Denning et al., 2011). Whereas CD11c<sup>+</sup>:CD11b<sup>-/−</sup>:CD103<sup>+</sup> cells are considered to be conventional DCs developed from common DC precursors, CD11c<sup>+</sup>:CD11b<sup>-/−</sup>:CD103<sup>−</sup> cells are developed from monocytes and express both DC and macrophage markers (Cerovic et al., 2014). Within the CD11c<sup>+</sup>:CD11b<sup>-/−</sup>:CD103<sup>−</sup> MNPs, the chemokine receptor CX3CR1 was reported to mark a population of cells that are the main source of IL-23 during intestinal inflammation (Longman et al., 2014). We therefore tested the role of RIPK3 in cytokine production by the CX3CR1<sup>+</sup> MNPs. Because no reliable CX3CR1 antibody is available for flow cytometry, we used CD14<sup>+</sup> as a marker to test RIPK3 expression in this population. Consistent with a previous report (Diehl et al., 2013), we confirmed by using CX3CR1<sup>gfp<sup>+</sup></sup> reporter mice that the CD11c<sup>+</sup>:MHC-II<sup>−/−</sup>:CD14<sup>+</sup> MNPs were indeed exclusively positive for CX3CR1 expression (Figure S5A). Interestingly, these CD11c<sup>+</sup>:CD11b<sup>-/−</sup>:CD103<sup>+</sup> CD14<sup>+</sup> MNPs also expressed high levels of RIPK3 compared with CD11c<sup>+</sup>:CD11b<sup>-/−</sup>:CD103<sup>−</sup> DCs (Figure 6A). We isolated CX3CR1<sup>+</sup> MNPs and CD103<sup>+</sup> DCs from colonic lamina propria of untreated Ripk3<sup>+/+</sup>:Cx3cr1<sup>gfp<sup>+</sup></sup> and Ripk3<sup>−/−</sup>:Cx3cr1<sup>gfp<sup>+</sup></sup> mice or the mice challenged with DSS for 7 days and compared their cytokine expression profile (Figure S5B). Consistent with a published report (Longman et al., 2014), Il23p19 and Il1b expression were induced in CX3CR1<sup>+</sup> MNPs in response to DSS (Figure 6B). In contrast, the cytokine expression was undetectable in CD103<sup>−</sup>
RIPK3 is a key signal adaptor for necroptosis. Recently, RIPK3 has also been shown to promote activation of the NLRP3 inflammasome, NF-κB, and apoptosis. The role of RIPK3 in necroptosis-independent signaling is especially prominent in innate immune sentinels such as macrophages and DCs. However, tools that allow examination of RIPK3 functions in distinct cell populations have been lacking. In this study, we report two mouse models that address this deficiency. Using the RIPK3-GFP reporter mice, we found that RIPK3 expression is dynamically regulated in different immune subsets. For example, RIPK3 expression was increased in macrophages in response to LPS stimulation. In contrast, RIPK3 expression was strongly induced in T cells during intestinal inflammation. T-cell-receptor-dependent induction of RIPK3 expression suggests that RIPK3 may regulate T cell responses and functions. Indeed, previous studies showed that Fadd<sup>-/-</sup> Ripk3<sup>-/-</sup> and Casp8<sup>-/-</sup> Ripk3<sup>-/-</sup> mice developed an lpr/gld-like autoimmune disease (Dillon et al., 2012; Kaiser et al., 2011; Oberst et al., 2011), indicating that RIPK3 cooperates with FADD/caspase-8 to enforce peripheral T cell tolerance. Whether RIPK3 regulates other functions in T cells is unknown at present.

In contrast with T cells, RIPK3 has a key role in cytokine expression by DCs and macrophages. RIPK3 expression in CD11c<sup>+</sup> DCs and CD11b<sup>+</sup> macrophages was not dramatically altered during LPS- or DSS-induced inflammation, although its expression in these innate immune effectors in different anatomical locations is highly variable. For instance, CD11c<sup>+</sup>MHC-II<sup>+</sup> CD11b<sup>+</sup> mononuclear cells from mice of the indicated genotypes were isolated from Ripk3<sup>-/-</sup>Cx3cr1<sup>fl/fl</sup> and CD11c<sup>+</sup>Cx3cr1<sup>fl/fl</sup> mice. Indeed, the cells from CD11c<sup>+</sup>Cx3cr1<sup>fl/fl</sup> mice also exhibited reduction in Il1b and Il23p19 expression (Figure S4), although the difference was not statistically significant. Collectively, these results suggest that CX3CR1<sup>+</sup> MNP in the lamina propria contribute to injury-induced cytokine expression.

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RIPK3 is a key signal adaptor for necroptosis. Recently, RIPK3 has also been shown to promote activation of the NLRP3 inflammasome, NF-κB, and apoptosis. The role of RIPK3 in necroptosis-independent signaling is especially prominent in innate immune sentinels such as macrophages and DCs. However, tools that allow examination of RIPK3 functions in distinct cell populations have been lacking. In this study, we report two mouse models that address this deficiency. Using the RIPK3-GFP reporter mice, we found that RIPK3 expression is dynamically regulated in different immune subsets. For example, RIPK3 expression was increased in macrophages in response to LPS stimulation. In contrast, RIPK3 expression was strongly induced in T cells during intestinal inflammation. T-cell-receptor-dependent induction of RIPK3 expression suggests that RIPK3 may regulate T cell responses and functions. Indeed, previous studies showed that Fadd<sup>-/-</sup> Ripk3<sup>-/-</sup> and Casp8<sup>-/-</sup> Ripk3<sup>-/-</sup> mice developed an lpr/gld-like autoimmune disease (Dillon et al., 2012; Kaiser et al., 2011; Oberst et al., 2011), indicating that RIPK3 cooperates with FADD/caspase-8 to enforce peripheral T cell tolerance. Whether RIPK3 regulates other functions in T cells is unknown at present.

In contrast with T cells, RIPK3 has a key role in cytokine expression by DCs and macrophages. RIPK3 expression in CD11c<sup>+</sup> DCs and CD11b<sup>+</sup> macrophages was not dramatically altered during LPS- or DSS-induced inflammation, although its expression in these innate immune effectors in different anatomical locations is highly variable. For instance, CD11c<sup>+</sup>MHC-II<sup>+</sup> CD11b<sup>+</sup> mononuclear cells from mice of the indicated genotypes were isolated from Ripk3<sup>-/-</sup>Cx3cr1<sup>fl/fl</sup> and CD11c<sup>+</sup>Cx3cr1<sup>fl/fl</sup> mice. Indeed, the cells from CD11c<sup>+</sup>Cx3cr1<sup>fl/fl</sup> mice also exhibited reduction in Il1b and Il23p19 expression (Figure S4), although the difference was not statistically significant. Collectively, these results suggest that CX3CR1<sup>+</sup> MNP in the lamina propria contribute to injury-induced cytokine expression.
et al., 2015). It is therefore striking that unlike caspase-8 and FADD, or multiple cellular inhibitors of apoptosis proteins (cIAPs), all of which are strong inhibitors of RIPK3 pro-necroptotic function (Chan et al., 2015). It is therefore striking that unlike 

RIPK3 is widely believed to induce inflammation through necroptosis-associated release of damage-associated molecular patterns (Kaczmarek et al., 2013). This current dogma is mostly based on studies from mice lacking caspase-8 or FADD, or multiple cellular inhibitor of apoptosis proteins (cIAPs), all of which are strong inhibitors of RIPK3 pro-necroptotic function (Chan et al., 2015). It is therefore striking that unlike Ripk3−/− or Cd11c-Cre:Ripk3−/− mice, Ripk3+/- mice did not develop more severe colitis. Thus, in injury-induced cytokine expression and tissue repair, RIPK3 functions exclusively as an inducer of cytokine expression rather than as a cell death adaptor. A necroptosis-independent role for RIPK3 in NLRP3 inflammasome activation has been observed in in vitro studies with BMDMs and BMDCs (Kang et al., 2015; Lawlor et al., 2015; Moriwaki et al., 2015; Vine et al., 2012). However, as in the case of necroptosis, maximal RIPK3-mediated inflammasome activation also requires FADD, caspase-8, or cIAPs inhibition. Thus, our result demonstrates a necroptosis-independent role for RIPK3 in physiological inflammation without pharmacological or genetic manipulation of FADD, caspase-8, or cIAPs. Our results also reveal that the non-necroptotic functions of RIPK3 may be confined to specific cell compartments. In this scenario, its function can only be revealed using tissue-specific inactivation approaches.

We previously reported that the RIPK3-RIPK1-FADD-caspase-8 complex was formed upon LPS stimulation in BMDCs. Assembly of this ripoptosome-like complex also requires another RHIM-containing adaptor TRIF (Moriwaki et al., 2015). Results from this present study indicate that an intact RHIM is not only crucial for necroptosis, but also for RIPK3-dependent ripoptosome assembly and pro-IL-1β processing. However, this function of RIPK3 does not require its pro-necroptotic kinase activity. In contrast, the mechanism by which RIPK3 promotes RelB-p50 activation is less understood. RIPK3 has been implicated to shutle between the cytoplasm and nucleus (Yang et al., 2004; Yoon et al., 2016), suggesting that RIPK3 may directly engage the transcriptional machinery under certain conditions.

**EXPERIMENTAL PROCEDURES**

**Mice**

To generate Ripk3-gfplox/lox reporter mice, we created a targeting construct in which an egfp sequence was inserted at the end of the coding region of the Ripk3 gene. The neomycin resistance gene flankned by flipase recognition target (FRT) sites was inserted in intron 9 for positive selection. For negative selection, thymidine kinase was added outside the 5’ homology arm. LoxP sites were also inserted before the neomycin cassette and after the egfp sequence. Embryonic stem (ES) cells were transfected with the targeting construct and subsequently selected by neomycin in the transgenic animal core at the University of Massachusetts Medical School (UMMS). ES clones in which proper recombination occurs were selected by Southern blot analysis, with probe 1 located in the 5’ homology arm and probe 2 located outside the 3' homology arm and subsequently used for injection into albino C57BL/6 blastocysts to generate chimeric mice. Germline transmission of the transgene was confirmed by Southern blot analysis. The neomycin cassette was removed by crossing the Ripk3-gfplox/lox mice with the flippase transgenic mice [Gt(ROSA)26SCreERT2(T2a); kindly provided from S. Jones in UMMS]. To generate mice constitutively deficient for the Ripk3 RHIM, we crossed Ripk3-gfplox/lox mice with Sox2-Cre transgenic mice (kindly provided from S. Jones in UMMS). The resultant Ripk3−/− and Ripk3+/- mice were backcrossed with C57BL/6 mice for 8-10 generations. To delete the RHIM specifically in CD11c+ cells, we crossed Ripk3-gfplox/lox mice with CD11c-Cre transgenic mice obtained from The Jackson Laboratory. Ripk3+/- mice were obtained from Genentech. Fadd+/- mice were described before (Zhang et al., 2011). Ripk3 K51A kinase dead knock-in (Ripk3K51A) mice were generated in GlaxoSmithKline (Mandal et al., 2014). Sox2-Cre;Ripk3floxed reporter mice were obtained from The Jackson Laboratory. All animal experiments were approved by the institutional animal care and use committee. The Ripk3-GFP floxed mice are available at The Jackson Laboratory as Stock No. 030284.

**DSS Treatment**

Female mice (9-12 weeks old) were treated with 3% DSS (molecular mass 36,000-50,000 Da; MP Biomedicals) for 7 days. The DSS water was replaced with fresh DSS water on days 3 and 5 and with regular water on day 8. Body weight was monitored for 15 days, and the weight at the beginning of the experiments was normalized as 100%. To obtain Ripk3+/− control mice with a minimum difference of genetic background, we crossed Ripk3+/- mice with Ripk3−/− mice. The resultant Ripk3+/− or Ripk3−/− mice were intercrossed to obtain Ripk3+/lox or Ripk3−/− mice. After weaning, these mice were co-housed in the same environment. To analyze neutrophil migration, mice were treated with DSS for 7 days and subjected to RNA extraction and qPCR analysis (n = 2-6). Results shown are mean ± SEM. *p < 0.05. See also Figure S5.
to minimize the influence of intestinal microflora. All experiments, except for the ones using Ripk3ΔFL mice, were performed using littermates. To minimize the differences in intestinal microbial environment among cages, we mixed bedding materials from different cages once a week after weaning.

Flow Cytometry
Colon was harvested from untreated or DSS-treated mice. After removing feces with PBS, the colon was longitudinally opened and cut into three pieces. The tissues were washed with PBS and subsequently incubated in 1 mL DTT for 10 min at room temperature (RT). Then, the tissues were shaken in 10 mL HEPES buffer containing 30 mM EDTA for 10 min at 37°C (225 rpm). After washing, the tissues were digested with 0.5 mg/mL collagenase IV (Sigma) and 150 µg/mL DNase I (Sigma) for 90 min at 37°C. Tissues were broken by vigorously shaking, filtered, and subjected to Percoll density gradient separation. LPMCs at the interphase of the two Percoll solutions were collected and subjected to flow cytometric analysis. To isolate splenocytes, we incubated spleen with 2 mg/mL collagenase D solution (10 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM KC1, 1 mM MgSO4, 1.8 mM CaCl2) for 30 min at 37°C. After gliding the spleen, red blood cells were lysed with ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA). Splenocytes were filtered and subjected to flow cytometric analysis. Prior to incubation with primary antibodies, cells were incubated with anti-Fc receptor 2.4G2 antibody for 10 min. PE-labeled anti-CD3 (145-2C11), PerCP-Cy5.5-labeled anti-CD11b (M1/70), Pacific blue-labeled anti-F4/80 (BM8), and PE-labeled anti-CD124 (YTS/56.7.7) were used. PE-labeled anti-MHCII I-Ab (AF6-120.1) antibodies were obtained from BioLegend. PE-Cy7-labeled CD19 (eBio10-3), PE-Cy7-labeled CD44 (IM7), PE-Cy7-labeled anti-CD14 (Sal2-6), and allophycocyanin (APC)-fluor780-labeled streptavidin were obtained from Biolegend. APC-Cy7-labeled anti-CD8a (53-6.7), APC-Cy7-labeled anti-CD3 (145-2C11), APC-Cy7-labeled anti-CD4 (GK1.5), FITC-labeled anti-CD19 (1D3), PE-labeled anti-B220 (RA3-6B2), Alexa Fluor 647-labeled CCR6 (14076), PE-labeled Ter119 (TER-119), and Pacific blue-labeled anti-CD4 (R45-5) antibodies were obtained from BD Biosciences. Cells were analyzed by LSRII (BD Biosciences). For cell sorting, FACSAria II (BD Biosciences) was used.
REFERENCES


