

# RIP2 promotes FcγR-mediated ROS production

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Running Title: *RIP2 promotes FcγR-mediated ROS production*

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## ABSTRACT

Receptor-interacting protein 2 (RIP2)<sup>2</sup> is a kinase which mediates signaling downstream of the bacterial peptidoglycan sensors NOD1 and NOD2. Genetic loss or pharmaceutical inhibition

of RIP2 has been shown to be beneficial in multiple inflammatory disease models with the effects largely attributed to reducing pro-inflammatory signaling downstream of peptidoglycan recognition. However, given the

<sup>2</sup> The abbreviations used are: ANOVA, analysis of variance; Bcl 10, B cell leukemia 10 protein; BMDMs, bone marrow derived macrophages; Btk, Bruton's tyrosine kinase; CCL2, C-C chemokine ligand 2; CCL3, C-C chemokine ligand 3; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; Erk, extracellular signal-regulated kinase; FcγR, Fc gamma receptor; FcRn, neonatal Fc receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S-transferase; GWAS, genome-wide association study; HEK-293, human embryonic kidney 293 cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBD, inflammatory bowel disease; iEDAP, γ-D-glutamyl-meso-diaminopimelic acid; IFN-γ, interferon gamma; IκBα, inhibitor of NF-κB, alpha; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; IP, immunoprecipitation; ITAM, immunoreceptor tyrosine-based activation motifs; ITIM, immunoreceptor tyrosine-based inhibitory motifs; IVK, in vitro kinase; JNK, Jun N-terminal kinase; LAT, linker for activation of T cells; Lpd, lammellipodin; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAPKs, mitogen-activated protein kinases; MΦ, macrophages; MDP, N-Acetylmuramyl-L-Ala-D-Isoglutamine; MFI, mean fluorescence intensity; mIgG, mouse immunoglobulin G; MS, multiple sclerosis;

NAC, N-acetyl-L-cysteine; NBD, nucleotide binding domain; NGF, nerve growth factor; NOD1 and 2, nuclear oligomerization domain proteins 1 and 2; NO, nitric oxide; p75<sup>NTR</sup>, p75 neurotrophin receptor; PI(3,4,5)P<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; PI(3,4)P<sub>2</sub>, phosphatidylinositol-3,4-bisphosphate; PLCγ, phospholipase C gamma; pTh17, pathogenic IL-17A-producing T cells; PMA, Phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PolyI:C, polyinosinic:polycytidylic acid; pY, phosphotyrosine; qRT-PCR, quantitative RT-PCR; RA, rheumatoid arthritis; RIP2, receptor-interacting protein 2; RIP2 KO, RIP2 knock out; ROS, reactive oxygen species; SEM, standard error of the mean; SH2, Src homology 2; SHIP1, SH2 domain-containing inositol 5'phosphatase 1; SFKs, Src family kinases; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; sRBCs, sheep red blood cells; TBS, Tris-buffered saline; TCR, T-cell receptor; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha; TRAF6, TNF receptor-associated factor 6; TRIM, tripartite motif; UTR, untranslated region; XIAP, X-linked inhibitor of apoptosis protein.

widespread expression of this kinase and its reported interactions with numerous other proteins, it is possible that RIP2 may also function in roles outside of peptidoglycan sensing. In this work, we show that RIP2 undergoes tyrosine phosphorylation and activation in response to engagement of the FcγR. Using bone marrow derived macrophages from WT and RIP2 KO mice, we show that loss of RIP2 leads to deficient FcγR signaling and ROS production upon FcγR cross linking without affecting cytokine secretion, phagocytosis, or nitrate/nitrite production. The FcγR-induced ROS response was still dependent on NOD2, as macrophages deficient in this receptor showed similar defects. Mechanistically, we find that different members of the Src family kinases (SFKs) can promote RIP2 tyrosine phosphorylation and activation. Altogether, our findings suggest that RIP2 is functionally important in pathways outside of bacterial peptidoglycan sensing and that involvement in such pathways may depend on the actions of SFKs. These findings will have important implications for future therapies designed to target this kinase.

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The RIP2 kinase is a dual-specificity kinase which functions downstream of the pattern recognition receptors Nuclear Oligomerization Domain 1 (NOD1) and 2 (1-7). NOD1 and NOD2 recognize the bacterial peptidoglycan breakdown products γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and N-Acetylmuramyl-L-Ala-D-Isoglutamine (MDP), respectively (8-10). The functional relevance of RIP2 within these peptidoglycan sensing pathways has been demonstrated extensively by studies showing a crucial role for NOD2 and RIP2 in host defense against *Mycobacterium tuberculosis* (11), *Legionella pneumophila* (12), *Listeria monocytogenes* (3), *Salmonella enterica* (13) and *Chlamydomphila pneumoniae* (14), to name a few.

RIP2 is not only more highly expressed in various tissues when compared to NOD1 or NOD2, but its expression is also less restricted (15). This, coupled with the fact that RIP2 also has numerous reported binding partners which

are associated with a variety of signal transduction pathways, leads to an appealing scenario for the involvement of RIP2 in pathways outside of, or in conjunction with, NOD signaling. Previous reports studying this kinase implicated RIP2 as acting downstream of Toll-like receptor (TLR) 2, 3, and 4, demonstrating defects in cytokine production as a result of lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan, and polyI:C stimulation (3). However, this was later disproven as the results were found to be a consequence of NOD agonists within the TLR agonist preparations (5). Recent studies suggest that nerve growth factor (NGF) acting through the p75 neurotrophin receptor (p75<sup>NTR</sup>) can utilize RIP2 to promote survival in cerebellar granule neurons (16). This was demonstrated to occur by RIP2-mediated displacement of TRAF6 from the p75<sup>NTR</sup> leading to NFκB activation and neuronal survival. Other studies have proposed a NOD1/2-independent, T cell intrinsic role for RIP2, with some groups suggesting that RIP2 prevents the generation of pathogenic IL-17A-producing T cells (pTh17) (17), and others that RIP2 can interact with Bcl10 and directly mediate its phosphorylation leading to activation of NFκB (18). However, multiple studies also exist demonstrating that RIP2 deficient T cells suffer no apparent defects in proliferation and cytokine secretion upon TCR ligation (19,20). Thus, the role of RIP2 in mediating TCR signaling is still somewhat controversial.

Recognizing a potential Src SH2 binding motif within RIP2 led us to speculate that RIP2 may be involved in Src-family kinase mediated signaling pathways. In this work, we describe a specific functional role for RIP2 downstream of FcγR engagement. We show that RIP2 specifically affects reactive oxygen species (ROS) generation but is dispensable for other functions such as cytokine secretion or phagocytosis. We additionally propose a potential mechanism by which this kinase is activated and discuss implications for IgG-mediated inflammatory diseases and RIP2-targeted therapies.

## Results

### ***RIP2 is tyrosine phosphorylated and activated upon FcγR cross-linking***

Identification of the tyrosine autophosphorylation site on RIP2 brought to our attention that, when phosphorylated, this site could serve as a Src-family kinase (SFK) Src homology 2 (SH2) binding motif [pY]-[E]-[X]-[I/L/P/V] (Fig 1A) (21). With this in mind, we sought to determine the involvement of RIP2 in SFK-mediated signal transduction pathways, such as downstream of the Fcγ receptor (FcγR). In order to determine whether RIP2 could be involved in transducing signals in response to FcγR engagement, we examined if RIP2 underwent posttranslational modification and/or activation upon FcγR stimulation. We used RAW 264.7 macrophages and wild-type bone marrow-derived macrophages (WT BMDMs) and induced FcγR cross-linking using IgG antibodies against BSA + BSA. We then immunoprecipitated RIP2 and assessed tyrosine phosphorylation, a surrogate for RIP2 activity, via Western Blot. Our results show that for both RAW 264.7 macrophages and WT BMDMs, RIP2 underwent tyrosine phosphorylation (Fig 1 B, and C), concurrent with activation of other downstream signaling cascades such as NFκB and MAPK pathways. To ensure that this tyrosine phosphorylation event indicated RIP2 enzymatic activity was increased, we performed a similar stimulation of the FcγRs, immunoprecipitated RIP2, and performed an in vitro kinase (IVK) assay using RIP2 tyrosine autophosphorylation as a readout for enzymatic activity. We find that, indeed, FcγR crosslinking increased RIP2 kinase (Fig 1 D and E). We confirmed this further using a second type of IVK assay which measures ADP generated during a kinase reaction. Using this assay, stimulation of RAW 264.7 macrophages and WT BMDMs through the FcγR resulted in a significant increase in enzymatic activity (Fig 1 F and G). We also made use of a previously defined panel of genetic RIP2 activation markers (22) and demonstrate that FcγR stimulation of WT BMDMs resulted in a significant upregulation of all 9 genes (Fig 1H). We observed a similar trend, although a reduced effect size, for THP-1 cells differentiated into macrophages (THP-1 MΦ) and stimulated with human IgG + anti-human IgG (S1). Altogether, using multiple readouts for

measuring RIP2 activity, the data indicate that RIP2 undergoes tyrosine phosphorylation and activation in response to engagement of FcγRs.

### ***RIP2 is involved in signaling downstream of FcγR engagement***

In order to determine the involvement of RIP2 in transducing signals emanating from FcγR engagement, we stimulated WT or RIP2 KO BMDMs with anti-BSA IgG<sub>1</sub> + BSA, anti-BSA IgG<sub>2a</sub> + BSA, or murine IgG + anti-mIgG and assessed activation of known downstream pathway intermediates via Western Blot. We find that anti-BSA IgG<sub>1</sub> + BSA stimulation (engagement of FcγRIII and FcγRIIB) induces a robust activation of the pathway in WT macrophages but a defect in activation of Syk, PLCγ, and p38 in macrophages lacking RIP2 (Fig 2A). Interestingly, if we stimulate FcγRs by using IgG<sub>2a</sub> (engagement of FcγRI and FcγRIV) or “bulk” mouse IgG (of all subclasses) these signaling defects are not apparent (Fig 2B and C). These data suggest that RIP2 is involved in transducing signals resulting from FcγR cross-linking specifically when complexes are comprised primarily of IgG<sub>1</sub> or when FcγRIII/FcγRIIB are preferentially engaged.

### ***RIP2 is not involved in FcγR-mediated cytokine production***

Cytokine production is an important downstream effect of FcγR stimulation. In order to assess if RIP2 is involved in the FcγR-mediated production of cytokines, we stimulated WT or RIP2 KO BMDMs through their FcγRs for 4hrs to isolate RNA for analysis of cytokine expression through qRT-PCR, or for 16hrs to collect supernatants for analysis of cytokine secretion through ELISA. Although FcγR stimulation induced a significant production of CCL2, CCL3, TNF-α, and IL-6 from both genotypes compared to their respective controls when assessed by ELISA (Fig 3A), and a significant increase in the expression of CCL2, CCL3, TNF-α, and IL-6 from both genotypes compared to their respective controls when assessed by qRT-PCR (Fig 3B), there was no difference in the responses between WT and RIP2 KO BMDMs upon FcγR stimulation. These

data indicate RIP2 is not involved in FcγR-mediated cytokine production.

### ***RIP2 is not involved in FcγR-mediated phagocytosis***

Given that induction of phagocytosis is an important consequence of FcγR engagement, we assessed if RIP2 instead contributed to the phagocytic capacity of macrophages in response to FcγR stimulation. We stimulated WT or RIP2 KO BMDMs through their FcγRs using PKH26-labelled and antibody-opsonized sheep red blood cells (sRBCs) and measured phagocytosis via flow cytometry and confocal microscopy. Exposure of both WT and RIP2 BMDMs to opsonized sRBCs resulted in a significant increase in the percent of cells which had phagocytosed opsonized sRBCs compared to unopsonized sRBCs when assessed by flow cytometry (Fig 4A). However, no difference was observed in the number of cells undergoing phagocytosis between WT and RIP2 KO BMDMs. When phagocytosis was assessed by confocal microscopy, a similar trend was observed. Exposure of both WT and RIP2 BMDMs to opsonized sRBCs resulted in a significant increase in the number of particles phagocytosed per macrophage compared to unopsonized controls (Fig 4B). However, no difference was observed in the phagocytic capacity of WT and RIP2 KO BMDMs. These data suggest that RIP2 is not involved in FcγR-mediated phagocytosis.

### ***RIP2 does not affect FcγR-mediated inducible nitric oxide synthase (iNOS) expression and nitrate/nitrite production***

FcγR engagement results not only in phagocytosis of microorganisms and the secretion of pro-inflammatory mediators but also leads to the production of nitric oxide (NO), a free radical with recognized cytotoxic effects on various microorganisms. To assess whether RIP2 was involved in the generation of NO, we generated BMDMs from WT or RIP2 KO deficient mice, primed these with IFN-γ, and tested the expression of inducible nitric oxide synthase (iNOS) upon FcγR cross-linking. We tested both protein expression of iNOS via Western Blot (Fig 5A) and gene expression via

qRT-PCR (Fig 5B). In both cases, FcγR cross-linking induced iNOS in both genotypes, although it did so to the same extent (Fig 5A and B). Given that NO is rapidly oxidized into nitrite and nitrate, we also quantified the levels of these molecules using a Griess assay (Fig 5C). Again, although FcγR cross-linking induced significant increases in both nitrite and nitrate in both genotypes compared to unstimulated controls, no differences were observed when comparing nitrite/nitrate levels between FcγR stimulated WT and RIP2 KO BMDMs. These data suggest that RIP2 is not involved in FcγR-mediated iNOS expression or nitrite/nitrate production.

### ***RIP2 is involved in ROS production downstream of FcγR engagement***

In phagocytic cells, various immune stimuli including FcγR engagement can result in the generation of reactive oxygen species (ROS). Some ROS species include superoxide anion, peroxide, hydrogen peroxide, hydroxyl radical, and hydroxyl ions which mediate antimicrobial activity during the so-called respiratory burst. To assess if RIP2 is involved in FcγR-mediated ROS production, we generated BMDMs from WT or RIP2 KO deficient mice, primed these with IFN-γ, induced FcγR cross-linking, and assessed ROS production using a ROS-reactive fluorescent probe. In both genotypes, a significant amount of ROS production was observed upon FcγR cross-linking compared to unstimulated cells (Fig 6 A and B, represented as % of cells stained positive for the probe and as MFI in the FL-1 channel). However, FcγR cross-linking of RIP2 KO BMDMs led to a significantly lower amount of ROS generated when compared to FcγR cross-linking of WT BMDMs (Fig 6A and B). Specificity of the probe for FcγR-induced ROS was verified by using the ROS inhibitor N-acetyl-L-cysteine (NAC) in the presence of the Fc cross-linking stimulus, and observation of the subsequent loss of fluorescence. These data indicate that RIP2 selectively influences ROS production in response to FcγR engagement.

In order to determine if such FcγR-induced ROS production is important in the host response, we also assessed ROS production in WT and RIP2 KO BMDMs in response to unopsonized or opsonized *Borrelia burgdorferi*



(Bb), the bacterial agent of Lyme disease. We did observe a similar trend of decreased ROS production in RIP2 KO BMDMs in response to opsonized, but not unopsonized, Bb when compared to the response of WT BMDMs. However, this difference was not statistically significant (S2 A). We also sought to determine if such differences may contribute to the intracellular killing of Bb. As depicted in S2, although we saw clear defects in the ability of RIP2 KO BMDMs to kill unopsonized Bb, opsonization enhanced the killing efficiency of both WT and RIP2 KO BMDMs, so much so that we could not confidently conclude an effect of RIP2 on FcγR-mediated intracellular killing. (S2 B).

### ***Fgr directly tyrosine phosphorylates RIP2***

One of the first events to occur upon FcγR cross-linking is the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by members of the Src family kinase (SFKs). Given the potential SFK SH2 interaction motif on RIP2 (Fig 1A), we wanted to determine if SFKs could mediate RIP2 activation. To assess whether RIP2 may be modified and/or activated by SFKs, we transiently transfected HEK 293T cells with kinase dead RIP2 in the absence or presence of kinase live or kinase dead Fgr or Hck. Upon immunoprecipitation of RIP2, we observed that tyrosine phosphorylation of RIP2 occurred in a manner dependent on the kinase activity of Fgr or Hck (Fig 7A and B). In order to assess whether these SFKs were directly phosphorylating RIP2, we performed an in vitro kinase (IVK) assay using purified RIP2 or SFKs. These IVK experiments indicated that Fgr, but not Hck, directly mediated tyrosine phosphorylation of RIP2 (Fig 7C and D). Lastly, to determine whether the SFK-mediated phosphorylation influenced the kinase activity of RIP2, we transiently transfected HEK 293T cells with RIP2 in the absence or presence of either Fgr or Hck, immunoprecipitated RIP2, and subjected this to an IVK assay (Fig 7E and F). Using tyrosine autophosphorylation of RIP2 as a readout for kinase activity, these experiments demonstrated that both Fgr and Hck increased the kinase activity of RIP2. Thus, both Fgr and Hck promoted RIP2 tyrosine autophosphorylation and

activation. However, only Fgr mediated direct phosphorylation of RIP2.

### ***NOD2 is required for the observed effects of RIP2 on FcγR-mediated ROS production.***

In order to determine whether the ability of RIP2 to promote FcγR-mediated ROS production also required the involvement of the upstream receptor NOD2, we also generated BMDMs from WT and NOD2 KO mice. Similar to RIP2 KO BMDMs, no defects were observed in cytokine production in response to FcγR cross-linking when comparing WT and NOD2 KO BMDMs (Fig 8A and B). As was done previously, we also primed these BMDMs with IFN-γ, induced FcγR cross-linking, and assessed ROS production using a ROS-reactive fluorescent probe. Again, similar to that observed when using RIP2 KO BMDMs, FcγR cross-linking of NOD2 KO BMDMs led to a significantly lower amount of ROS generated when compared to FcγR cross-linking of WT BMDMs (Fig 8C, expressed as either % positive cells or MFI of the ROS-reactive probe). These data suggest that the involvement of RIP2 in FcγR signaling and ROS production is dependent on NOD2.

### **Discussion**

In this study, we report the functional involvement of RIP2 in FcγR signaling, specifically, downstream of FcγRIII/FcγRIIB engagement. We propose that activation of RIP2 occurs very early in this response. In the canonical FcγR-mediated signaling cascade, crosslinking of FcγRs leads to the activation of SFKs such as Fgr and Hck to mediate the phosphorylation of ITAMs on the signal-transducing Fc γ chain to positively influence downstream signaling events. In an as yet undefined mechanism, crosslinking of FcγRs also leads to the activation of NOD2. Both NOD2 and SFKs promote tyrosine phosphorylation and activation of RIP2. This leads to RIP2 selectively influencing FcγRIII/FcγRIIB – induced ROS production but not cytokine secretion, phagocytosis, or nitrate/nitrite production. How it manages to accomplish this is still unclear but is a subject of current investigation.

Although there have been some reports of peptidoglycan-independent mechanisms of activation of NOD1 and NOD2 such as ER stress inducers (23), pathogen effector proteins (24), and even viral triggers (25), to our knowledge, this is the first study suggesting that stimulation of FcγRs might somehow crosstalk with NOD2 signaling. So far, no data exists describing a role for NOD2 in binding to immune complexes, in mediating or synergizing with FcγR signaling, or in associating with the various members of the FcγR signaling pathway. IgG<sub>1</sub> complexes, although bound primarily by FcγRIII and FcγRIIB, can also theoretically be detected by FcRn and TRIM21 (26,27). However, antibody-bound particles do not generally cross plasma membranes unless infectious (i.e. antibody-bound viral particles) and can therefore access the cytosolic compartments where TRIM21 resides. Likewise, FcRn only binds IgG under acidic conditions (within endolysosomal compartments of cells or on the cell surface of enterocytes or other antigen presenting cells in the acidic gut lumen) leading us to conclude this is not a likely scenario in our system. Furthermore, both FcRn and TRIM21 lack association with an ITAM-bearing γ chain through which SFKs can be recruited and activated. NOD2 has been described to associate with another TRIM family member, TRIM27, which has been demonstrated to promote NOD2 ubiquitination and proteasomal degradation (28). Interaction between NOD2 and TRIM27 was mapped to occur between the TRIM27 PRY-SPRY domain and NOD2 NBD/NACHT domain. There is ~60% homology at the amino acid level between the PRY-SPRY domains of TRIM21 and TRIM27. Even if NOD2 and TRIM21 were somehow able to associate in the cytosol and these TRIM family members shared a similar function, then engagement would lead instead to downregulation of the response rather than activation due to the degradation of NOD2. Given this, the exact mechanisms underlying the crosstalk between NOD2 and FcγR signaling still remains to be determined. Furthermore, how RIP2 working downstream of FcγR and NOD2 might synergize or regulate the opposite pathway to influence the immune response would be a worthwhile topic for future study.

A number of outstanding questions still remain. For example, why is there selective participation of RIP2 downstream of some FcγRs, but not others? We observed a defect in FcγR signaling when we specifically used immune complexes generated using antibodies of the IgG<sub>1</sub> isotype but not when antibodies of the IgG<sub>2a</sub> isotype or mixed antibody isotypes were used. It is known that depending on the isotype, the primary activating Fc receptors can be differentially regulated by the inhibitory Fc receptor, FcγRIIB. Given the higher affinity for FcγRIIB over FcγRIII, in the case of IgG<sub>1</sub>, would mean that IgG<sub>1</sub>-mediated responses may be more strictly regulated compared to other isotypes (IgG<sub>2a</sub> or IgG<sub>2b</sub>) (29). In the classical paradigm of negative regulation mediated by FcγRIIB, receptor crosslinking results in the phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) by SFKs, leading to the recruitment of Src homology 2 (SH2) domain-containing inositol 5'phosphatase 1 (SHIP1) (reviewed in (30)). SHIP1, in turn, dephosphorylates phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P<sub>3</sub>] resulting in the generation of phosphatidylinositol-3,4-bisphosphate [PI(3,4)P<sub>2</sub>]. This dephosphorylation event not only prevents the recruitment of effector proteins such as Btk and Vav, but also promotes the specific recruitment of a different set of effector proteins such as TAPP1/2 and lamellipodin (Lpd). Whether these regulatory events are affected by the presence and activity of RIP2 is something which remains to be determined. Interestingly, although RIP2 and SHIP1 have not previously been shown to directly interact, SHIP1 has been shown to bind XIAP1, disrupt RIP2:XIAP1 interactions, and regulate NOD2 mediated signaling (31).

Lastly, demonstrating a functional role for RIP2 downstream of the FcγR suggests that manipulation of RIP2 activity (whether that be enzymatic or scaffolding activity) may have wider implications than previously appreciated. Given that dysfunctional FcγR responses have been linked to a number of diseases such as bacterial and viral infections (in concert with complement), vasculitides, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), inflammatory bowel

disease (IBD), allergic disease and others, the present work suggests that RIP2 can potentially influence the pathogenesis of these diseases as well. There has been one study which found a significant association between RIP2 SNPs and SLE in a Chinese population (32). One SNP resulted in a synonymous change within exon 6 of RIP2 while the other occurred within the 3'UTR of RIP2. Similarly, a RIP2 promoter polymorphism was found to associate with asthma severity in a Japanese population (33). The functional relevance of each of these polymorphisms has, so far, not been tested. Outside of linkage disequilibrium, case-control and GWAS studies, RIP2 has also been implicated in the pathogenesis of MS, IBD, RA and asthma using genetic knock-outs and pharmacological inhibition of RIP2 in animal models of these diseases (22,34-36). Combined with our findings, this suggests a potential additional mechanism through which loss or inhibition of RIP2 might be conferring therapeutic efficacy. As numerous RIP2-targeted therapies are continually being discovered and developed and one is currently being tested in clinical trials (37-40), this study broadens their potential use while also shedding light on the possible consequences of RIP2 inhibition in pathways outside of canonical NOD1/2 mediated responses to peptidoglycan.

## Experimental Procedures

### *Animals, Cells, and Antibodies*

C57BL/6J (000664), RIP2 KO (007017), and NOD2 KO (005763) mice were purchased from Jackson Laboratories. Male and female mice between 6-10 weeks of age were used for all experimentation. Animals were bred and housed in the Animal Facility at the UCF Health Science Campus at Lake Nona, an AALAC-accredited rodent barrier facility with a strong record of being a specific pathogen-free environment. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Central Florida and using a reviewed and approved animal protocol. The numbers of animals for each strain used per experiment are indicated within the figure legends.

Bone marrow-derived macrophages (BMDMs) were generated by culturing bone marrow for 7 days in DMEM containing 10% heat-inactivated FBS, 1% sodium pyruvate, 1% HEPES, 1% antibiotic-antimycotic, 0.05mM β-mercaptoethanol and 25% Ladmec conditioned media (Ladmec cells were a gift from Clifford Harding, CWRU). Cells were rested in DMEM containing 10% FBS and 1% antibiotic-antimycotic overnight before using in experiments. All media were purchased from Corning, all FBS from VWR, and supplements were from Invitrogen/ThermoFisher. Appropriate differentiation of BMDMs were verified by expression of F4/80 and CD11b by flow cytometry.

The RAW 264.7 (TIB-71), THP-1 (TIB-202), and 293T (CRL-3216) cell lines were purchased from ATCC and cultured using the recommended medium and culture conditions. For differentiation of THP-1 cells into macrophages, THPs were resuspended at a concentration of  $1 \times 10^6$  cells/mL and plated in 10 cm dishes with 100ng/ml PMA. Media was changed on days 2,4, and 6 and then used at day 8 for experiments.

Anti-phosphotyrosine (clone P-Tyr-100), anti-phospho Syk (Tyr352), anti-phospho LAT (Tyr191), anti-phospho-PLCγ1 (clone D6M9S), anti-phospho PLCγ2 (Tyr759), anti-phospho IκBα (clone 5A5), anti-phospho p44/42 MAPK (Erk1/2) (clone E10), anti-phospho p38 MAPK (clone 12F8), anti-phospho JNK (clone 81E11), anti-mouse iNOS, anti-GST tag (clone 91G1), and anti-Myc tag (clone 71D10) were obtained from Cell Signaling Technologies. Anti-RIP2 (clone H-300), and anti-tubulin (clone TU-02) were obtained from Santa Cruz Biotechnology. Protein G agarose was obtained from Invitrogen.

### *Human and murine FcγR cross-linking*

For murine FcγR cross-linking, cells were serum starved with or without 2.5μg/ml of anti-BSA antibody (IgG<sub>1</sub> clone 9E2C2, Innovative Bioresearch; IgG<sub>2a</sub> clone BSA-33, Sigma) for 4 hours. Afterwards, media was aspirated, cells were washed once with PBS and then stimulated in low serum DMEM containing 1μg/ml BSA for the specific time points indicated. In some experiments, cross-linking

was performed using 5μg/ml of murine IgG (Southern Biotechnologies) followed by 10μg/ml of goat anti-mouse IgG (Sigma).

For human FcγR cross-linking, THP-1 derived macrophages were serum starved for 4 hours then treated with 20μg/ml human IgG (Sigma) for 30 minutes on ice. Afterwards, cells were washed with PBS and fresh media containing 50μg/ml of cross linking anti-human IgG (Sigma) was added for the time points indicated.

#### *Transfection, immuno-precipitation and Western Blotting*

pEBG-RIP2, pEBG-RIP2 K47A, pcDNA3.1 myc-His Hck, pcDNA3.1 myc-His Hck K269R, pcDNA3.1 myc-His Hck Y501F, pcDNA3.1 myc-His Fgr, pcDNA3.1 myc-His Fgr K291M, and pcDNA3.1 myc-His Fgr Y523F were all kind gifts from D. Abbott (CWRU). All cDNA constructs used were human. Transient transfection was performed using calcium phosphate transfection of HEK 293T cells. Cells were lysed after 24 hours using cell lysis buffer (50 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1mM PMSF, 1 mM NaVO<sub>4</sub>, 10nM Calyculin A, protease inhibitor cocktail (Sigma). For analysis of RIP2 tyrosine phosphorylation, pervanadate treatment was performed 5 minutes prior to harvesting the cells. Immunoprecipitation (IP) was carried out by incubating the cleared cell lysates with the respective antibody for 1 hour, followed by addition of Protein G sepharose beads and rotation overnight at 4°C. IPs were washed at least 3 times prior to analysis via SDS-PAGE and Western Blotting. SDS-PAGE equipment, transfer systems, and nitrocellulose membranes were all from Bio-Rad.

#### *ELISA*

Supernatants from stimulated BMDMs were harvested 16h after FcγR cross linking for assessing the levels of different cytokines by ELISA. Mouse CCL2, CCL3, IL-6 and TNF-α ELISA kits were purchased from eBioscience/ThermoFisher and used according to the manufacturer's instructions. A Cytation5 Plate Reader (BioTek) was used for reading

absorbance values at 450nm (with 570nm background reading subtracted). Cytokine concentrations were calculated by analyzing standard and sample values using a sigmoidal dose-response curve in Prism (Graphpad).

#### *Phagocytosis assay*

RBCs (MP Biomedical) were washed twice with PBS and then stained with PKH26 Red Fluorescent Cell Linker Kit (Sigma) using a final dye concentration of  $3 \times 10^{-6}$  M to stain  $1 \times 10^8$  RBCs per 1 ml volume. Cells were stained for 3 minutes, followed by quenching with an equal volume of FBS. The stained cells were washed twice with PBS and subsequently left unopsonized or were opsonized with 1:1600 dilution of rabbit IgG fraction to sheep RBCs (MP Biomedical) for 1 hour at 37°C. Excess antibody was removed by washing the cell suspension twice with PBS. Erythrocytes were added to macrophages in the 12-well plates in which they were cultured at a ratio of 1:1. Macrophages were incubated on ice prior to addition of the opsonized sRBC, after which, the plates containing macrophages and sRBCs were spun down for 1 minute (1000 rpm). The plates were incubated on ice for 10 minutes. Afterward, PBS was aspirated, warm media was added, and phagocytosis was allowed to occur at 37°C for 30 minutes. Plates were then washed with ice-cold PBS twice, followed by hypotonic lysis of any remaining uningested sRBCs. Cells were washed an additional three times with PBS, trypsinized, and then analyzed for phagocytosis by flow cytometry. For flow cytometric analysis of phagocytosis, % phagocytosis represents the percentage of PKH26<sup>+</sup> macrophages with the gating determined by signal obtained using macrophages alone.

For analysis of phagocytosis via confocal microscopy, BMDMs were cultured in slide chambers overnight (LabTek). The same procedure using sRBCs was performed as above. Cells were then fixed with 4% formaldehyde, blocked with blocking buffer (1X PBS/5% normal serum/0.3% Triton X-100) for 1 hour. Thereafter, cells were incubated overnight with anti-tubulin antibody (Santa Cruz) diluted in antibody dilution buffer 1:100 (1X PBS/1% BSA/0.3% TritonX-100). The next day, the cells were washed with 1xTBS-Tween and stained



with 1:1000 of DAPI (Sigma) and 1:1000 of Alexa 488 anti-mouse antibody (Molecular Probes) in antibody dilution buffer. The slides were analyzed using a Zeiss 710 confocal microscope and associated Zen software. Quantitation for confocal analysis of phagocytosis is presented as particles per 100 macrophages (a total of (3) 40x fields counted per condition).

#### *Nitrite and total nitrate assay*

To measure nitrite and total nitrate production from macrophages, a Greiss reagent kit (ThermoFisher) was used. BMDMs from WT and RIP2 KO mice were generated and primed with 100ng/ml of IFN-γ overnight. Cells were stimulated via Fc cross-linking as indicated above. After 24hrs, cell culture supernatants were collected and assayed using the Greiss reagent kit according to the manufacturer's instructions. Absorbance was measured at 548nm using a Cytation5 plate reader (BioTek). The nitrite concentration was calculated based on a nitrite standard curve made in the same culture media. For total nitrate/nitrite determination, nitrate reductase as well as enzyme cofactor mixture (obtained from a Nitrate/ Nitrite detection kit from Cayman) were added to the collected supernatant to convert nitrate to nitrite. The samples were incubated for 1 hour at room temperature to complete the reaction. Total nitrate was then quantified using Greiss reagent kit with reference to the nitrite standard curve (which was also converted to nitrate). In addition to collecting the supernatant for nitrite/nitrate analysis, cell lysates were also collected for analysis of iNOS expression using Western Blot.

#### *Reactive oxygen species (ROS) assay*

To measure real time production of ROS from living macrophages, a ROS-ID Total ROS detection kit (Enzo Life Sciences) was used. BMDMs derived from WT and RIP2 KO mice were plated in 6cm dishes at a concentration of  $1 \times 10^6$  cells/ml and then primed overnight with 100ng/ml of IFN-γ. The following day, cells were serum starved in the absence or presence of 2.5 μg/ml IgG<sub>1</sub> anti-BSA. After washing cells with PBS, Fc cross-linking was induced by addition of 1μg/ml BSA in low serum, phenol red-free

DMEM containing 2μM oxidative stress detection green dye. Cells were incubated for 30 mins at 37°C and immediately analyzed by flow cytometry. The percentage of FL-1<sup>+</sup> cells was determined with gates set based on unstained cells as a negative control.

Assessment of ROS production in response to opsonized Bb was performed similarly as above with the following modifications. Macrophages were serum-starved for 4 hours, scraped off the plates, washed once with PBS, then left unstimulated or incubated with mock-opsonized or opsonized Bb at a 0.5:1 MOI. Incubation of macrophages with Bb was done in low serum, phenol red-free DMEM containing 2μM of an oxidative stress detection green dye. Cells were incubated for 20 mins at 37°C and immediately analyzed by flow cytometry.

#### *RNA extraction and real time PCR*

Macrophages were stimulated for 4 hours prior to harvesting the cells for isolation of RNA using an RNeasy kit (Qiagen). RNA extraction was performed according the manufacturer's instructions. cDNA was synthesized using a Quantitect reverse transcription kit (Qiagen). Real-time PCR reactions were carried out using iQ SYBR Green Supermix (Bio-Rad) and run on a CFX96 C1000 Real-Time Thermal Cycler (Bio-Rad). The data shown are fold changes compared to control cells (unstimulated of each genotype) using delta delta Ct analysis. The following primer pairs were used for amplification:

Mouse	CCL2	(forward,	5'-
		CTGCTGTTACAGTTGCCG-3';	reverse, 5'-
		GCACAGACCTCTCTCTTGAGC-3');	mouse
	CCL3	(forward,	5'-
		GAAGGATACAAGCAGCAGCGA-3';	reverse, 5'-
		GTCTCTTTGGAGTCAGCGCA-3');	mouse
	TNF-α	(forward,	5'-
		GGTGCCTATGTCTCAGCCTC-3';	reverse, 5'-
		GCTCCTCCACTTGGTGGTTT-3');	mouse
	IL-6	(forward,	5'-
		CTCTGGGAAATCGTGGAAAT-3';	reverse, 5'-
		CCAGTTTGGTAGCATCCATC-3');	mouse
	iNOS	(forward,	5'-
		TTGGTGAAGGGACTGAGCTG-3';	reverse, 5'-
		TCCAAATCCAACGTTCTCCGT-3'); and	
mouse	GAPDH	(forward,	5'-

TGCCCCCATGTTTGTGATG-3'; reverse, 5'-TGTGGTCATGAGCCC TTCC-3').

Primers for mouse RIP2 genetic activation markers were used as described previously (22). All primers were synthesized by Integrated DNA technologies (IDT).

#### *In vitro kinase (IVK) assay*

To assess enhancement of RIP2's enzymatic activity upon FcγR cross-linking, RAW macrophages, WT BMDMs, or THP-1 macrophages were stimulated through the FcγR for 5 minutes. RIP2 was immunoprecipitated and subjected to an IVK assay. To assess enhancement of RIP2's enzymatic activity in the presence of Fgr or Hck, RIP2 was transiently transfected into HEK 293T cells with or without Fgr or Hck. RIP2 was immunoprecipitated and subjected to an IVK assay. To assess direct phosphorylation of RIP2 by SFKs, wild-type or kinase dead Fgr or Hck and kinase-dead RIP2 were individually transiently transfected into HEK 293T cells. SFKs or RIP2 were immunoprecipitated by their respective tags and combined together in an IVK as indicated. A kinase buffer containing 25 mM Tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 10 mM MgCl<sub>2</sub> and 0.1 mM Na<sub>3</sub>VO<sub>4</sub> was used. IPs were washed thrice in lysis buffer and then thrice in kinase buffer. The reaction was performed in a 40 μL volume using 10 mM of ATP (ThermoFisher). IVK assays were performed for 30 min at 30°C, after which an equal amount of sample buffer was added and the entire mixture boiled for 5 minutes before analysis of tyrosine phosphorylation by Western Blotting.

To assess kinase activity using ADP-glo (Promega), the IVK reaction was performed in a 25 μL volume using 1 mM of Promega Ultrapure ATP. After the assay, ADP Glo reagent and Kinase Glo reagent were added and incubated as suggested by the manufacturer. The resulting reactions were then read on a Cytation 5 Multimode Plate Reader (Biotek).

#### *Borrelia burgdorferi (Bb) growth conditions and opsonization*

The Bb clone used in this study was the low passage, infectious clone B31 A3-68 Δ*bbe02* (41). Bb cultures were grown at 35°C in liquid Barbour-Stoenner-Kelly II (BSKII) medium containing gelatin and 6% rabbit serum. For measuring viability, Bb was plated in solid BSK-agarose medium incubated at 35°C and 2.5% CO<sub>2</sub>.

Fifty milliliter cultures of Bb in BSK-II were grown to mid-log phase, pelleted, and washed twice with HN buffer (50 mM Hepes, 50 mM NaCl, pH 7.5). The final Bb density was determined using a Petroff-Hauser chamber under darkfield microscopy and adjusted to 10<sup>8</sup>/mL using HN buffer. For opsonization, 63 μg of rabbit polyclonal anti-Bb antibody (Abcam) was added to 0.5 mL of the 10<sup>8</sup>/mL Bb and incubated at 37°C for 30 mins with end-over-end mixing. Opsonized Bb was collected by centrifugation and was washed twice with HN buffer. The opsonized Bb was resuspended in 0.5 mL HN buffer and the density determined using a Petroff-Hauser chamber under darkfield microscopy. Mock opsonization of Bb was performed as above but without the addition of the opsonizing antibody.

#### *Measurement of intracellular killing of Bb by macrophages*

BMDMs were serum starved for 4 hours. Prior to the start of the assay, BMDMs were washed once with ice-cold PBS. Ice-cold opsonized or mock-opsonized Bb were added to each well at an MOI of 1:1 followed by a brief centrifugation at 4°C. The supernatant was removed and the macrophages were washed with ice cold PBS to remove any unbound Bb. Pre-warmed low serum DMEM was added to the macrophages and the cells incubated at 37°C with 5% CO<sub>2</sub> for the indicated times to allow for intracellular killing. Macrophages were then washed three times with ice-cold PBS and lysed by addition of 1 mL dH<sub>2</sub>O for 4 mins. The total lysate was then immediately added to 2 mL of BSKII to halt the lytic effect of dH<sub>2</sub>O on the released Bb. The number of viable Bb was determined by quantification of the number of colony-forming units in solid BSK medium.

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**Author Contributions:** MS, OC, GA, MJ and JTA were responsible for designing and performing the experiments, analyzing the resulting data, and for writing and editing of the manuscript.

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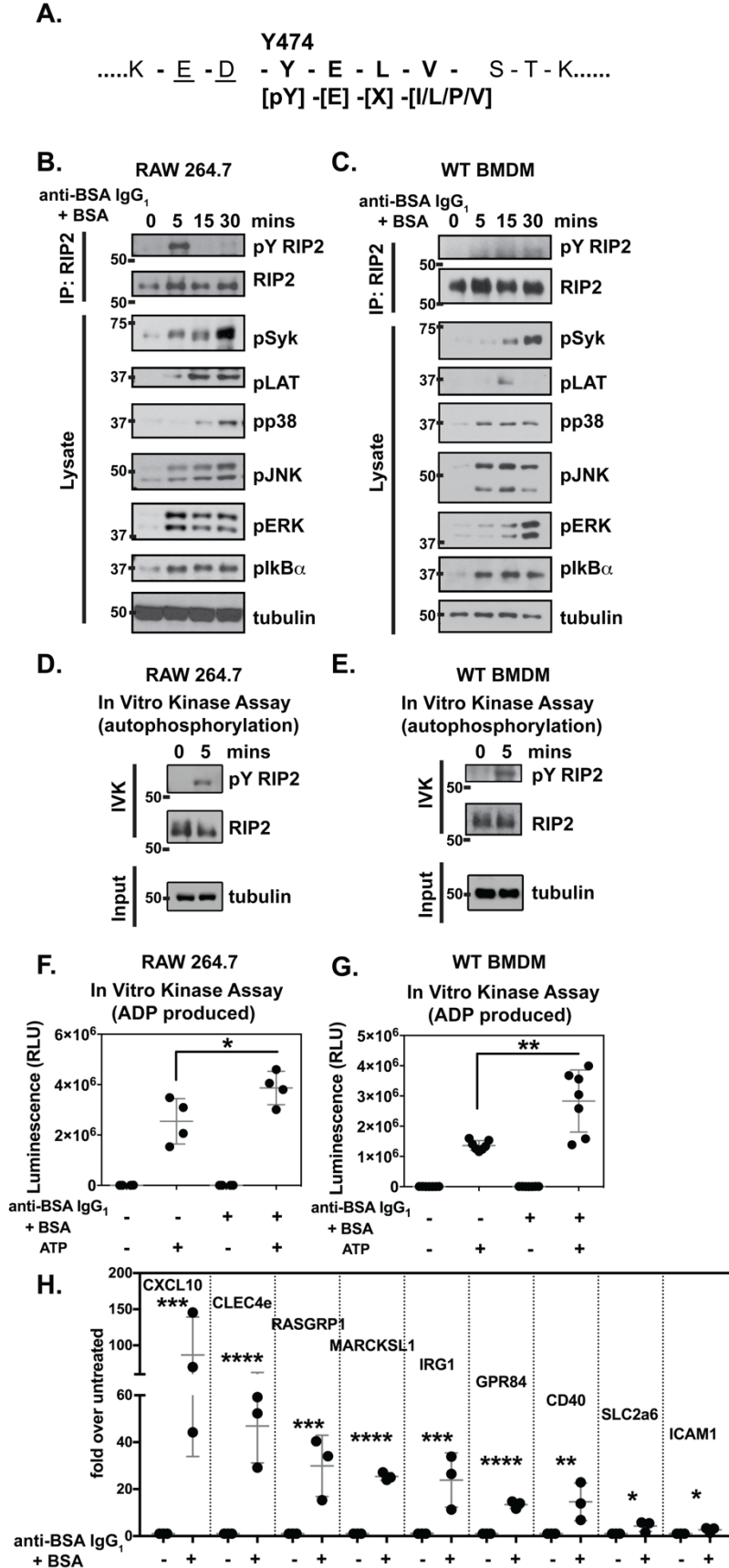


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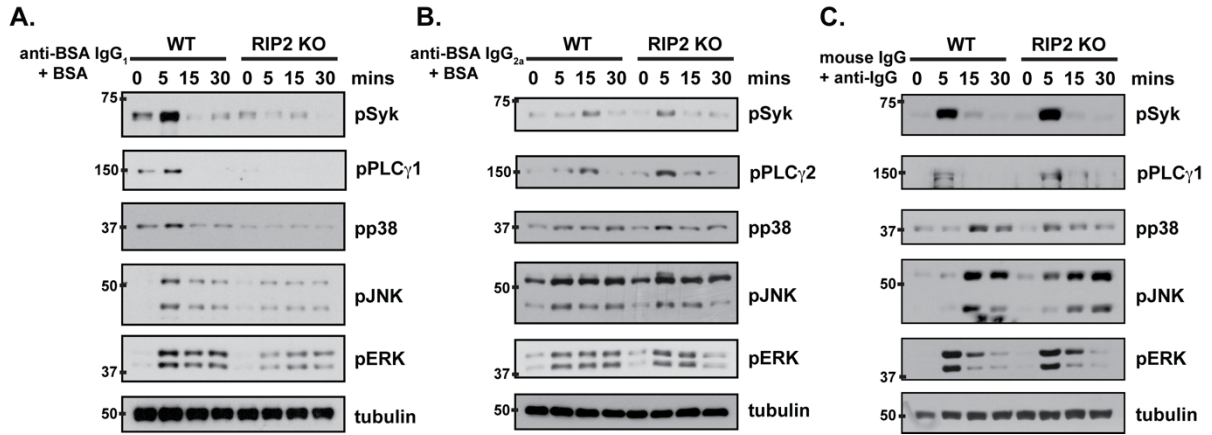
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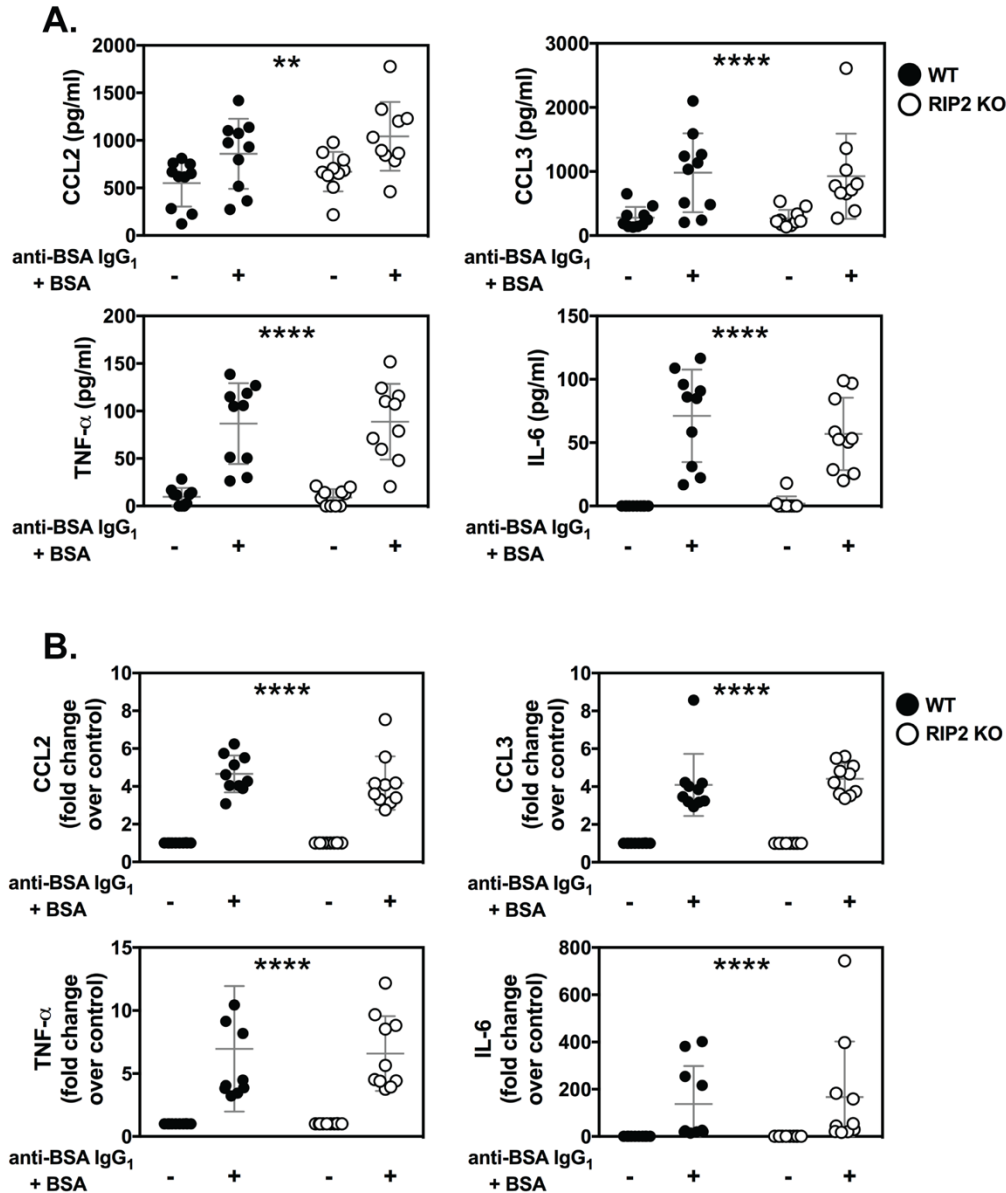


**Figure 1.** RIP2 is tyrosine phosphorylated and activated upon FcγR cross-linking. (A) Sequence surrounding the Y474 autophosphorylation site on RIP2, showing that it fulfils the criteria for a Src family kinase SH2 binding motif. (B) RAW 264.7 macrophages and (C) wild-type bone marrow-derived macrophages (WT BMDMs) were stimulated with murine anti-BSA IgG<sub>1</sub> + BSA for the indicated times. RIP2 was immunoprecipitated (IP), Western Blotting was performed and IPs were immunoblotted using an anti-phosphotyrosine antibody. Total cell lysates were immunoblotted with the indicated antibodies to assess activation of downstream signaling cascades. In vitro kinase (IVK) assays were performed using RIP2 immunoprecipitated from FcγR-stimulated (D) RAW 264.7 cells and (E) WT BMDMs using tyrosine autophosphorylation of RIP2 as a readout of kinase activity. In vitro kinase (IVK) assays were performed using RIP2 immunoprecipitated from FcγR -stimulated (F) RAW 264.7 cells and (G) WT BMDMs using an ADP Glo assay. (H) WT BMDMs were unstimulated or stimulated with murine anti-BSA IgG<sub>1</sub> + BSA for 4hrs. RNA was extracted and qRT-PCR was performed for previously defined genetic RIP2 activation markers. Data in graphs represent means ± SD. Data are aggregated from at least 3 independent experiments using n=3-7 mice for the unstimulated condition and n=3-7 mice for the FcγR stimulated condition. One-way ANOVA with Sidak's multiple comparisons test was used for statistical analysis of IVK assays and a Student t-test was used to analyze (log) fold changes in gene expression. (\* = p <0.05, \*\* = p <0.01, \*\*\* = p <0.001, \*\*\*\* = p <0.0001)

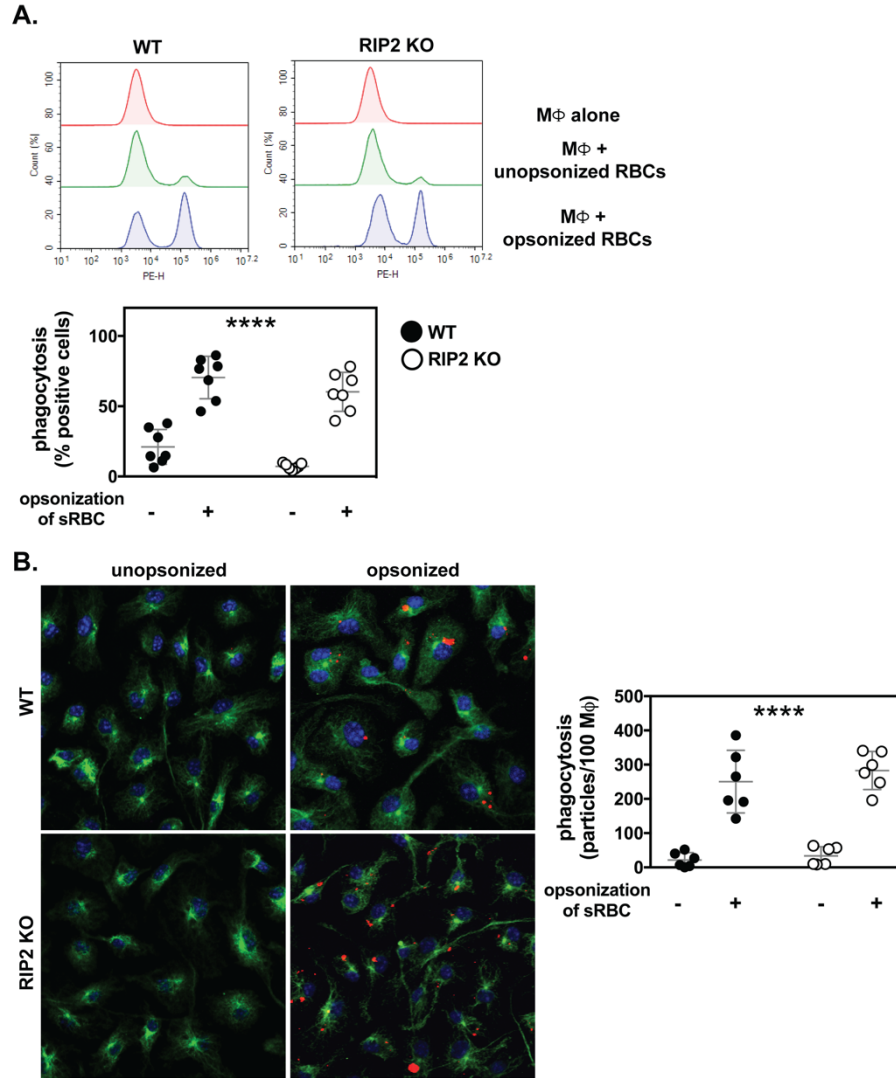




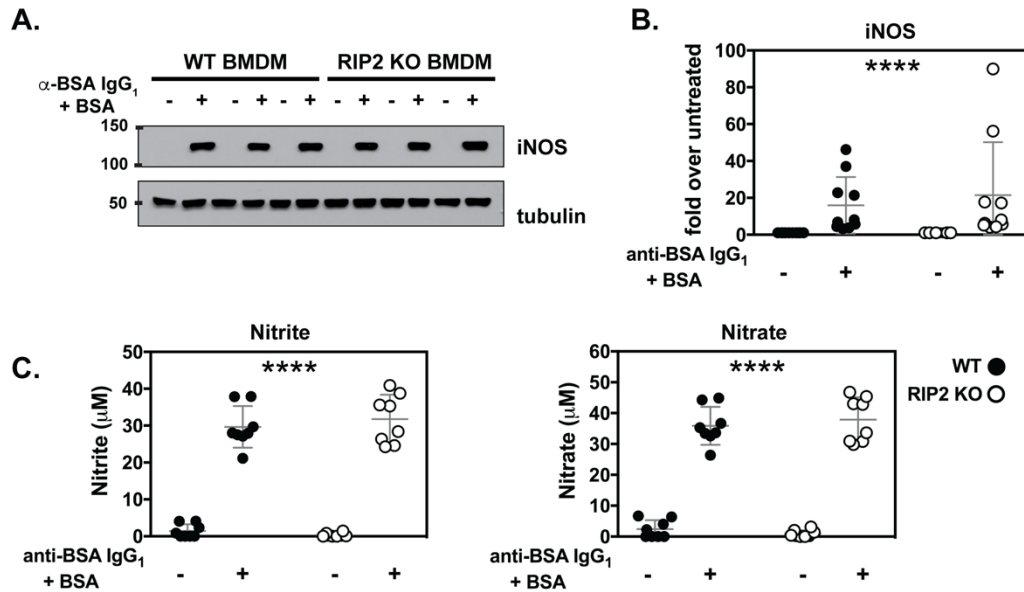
**Figure 2.** RIP2 is involved in signaling downstream of FcγR engagement. Bone marrow-derived macrophages (BMDMs) from WT and RIP2 KO mice were stimulated with (A) murine anti-BSA IgG<sub>1</sub> + BSA, (B) murine anti-BSA IgG<sub>2a</sub> + BSA or (C) murine IgG + anti-mIgG for the indicated times. Western Blotting was performed and lysates were immunoblotted with the indicated antibodies. Data shown are representative of at least 3 independent experiments performed.



**Figure 3.** RIP2 is not involved in FcγR-mediated cytokine production. Bone marrow-derived macrophages (BMDMs) from WT and RIP2 KO mice were left unstimulated or were stimulated with murine anti-BSA IgG<sub>1</sub> + BSA for 4hrs or for 16hrs in low-serum media. (A) Supernatants were collected after 16hrs of stimulation for analysis of cytokine secretion by ELISA. (B) In a separate set of experiments, RNA was harvested from cells after 4hrs to perform qRT-PCR for determining expression of the indicated genes. Bars within graphs indicate means ± SD. Data are aggregated from at least 3 independent experiments using n=10 mice per group. Two-way ANOVA was used for statistical analysis. For (A) and (B), no interaction was observed (cytokine secretion and gene upregulation was similar for WT and RIP2 KO mice upon treatment). Therefore, no further testing was performed. The p value for the overall effect of FcγR stimulation is indicated within the graph. (\*\* = p < 0.01, \*\*\*\* = p < 0.0001)

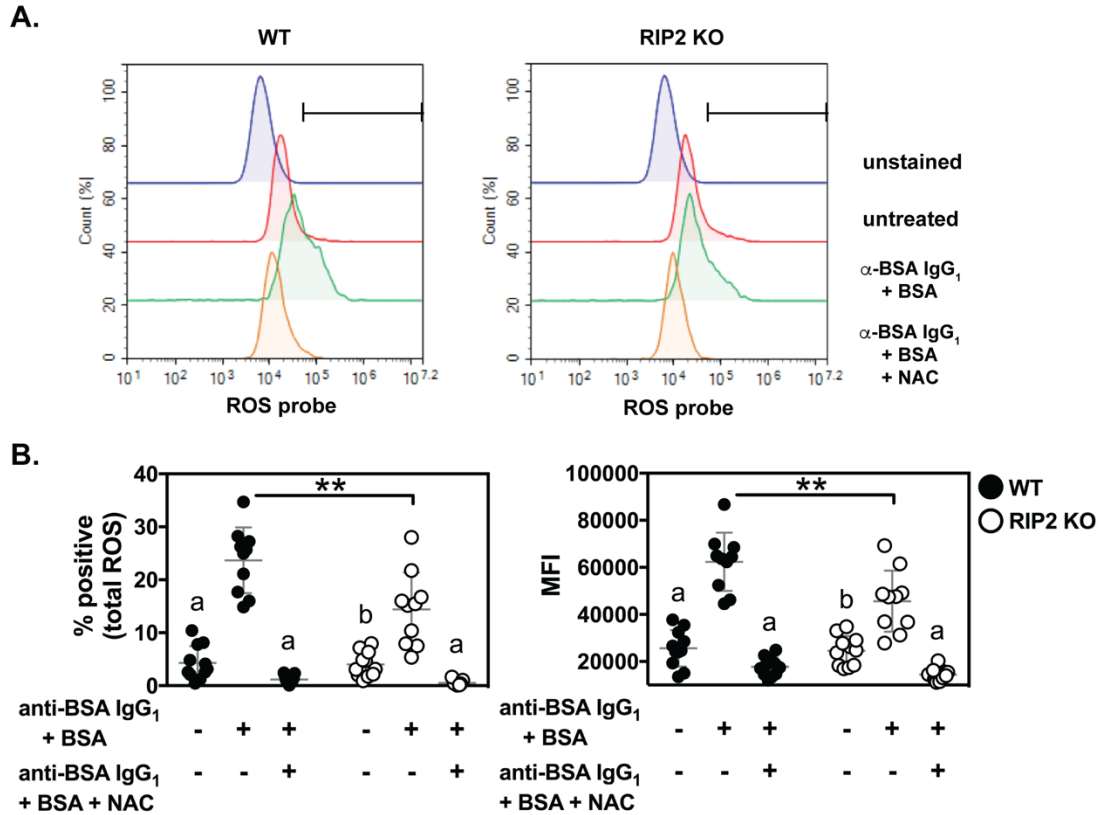


**Figure 4.** RIP2 is not involved in FcγR-mediated phagocytosis. Bone marrow-derived macrophages (BMDMs) from WT and RIP2 KO mice were incubated with an equal number of PKH26 red-labelled sheep red blood cells (sRBCs) which were previously unopsonized or opsonized with rabbit IgG against sRBC (1:1600). BMDMs were allowed to phagocytose sRBCs for 30 mins at 37°C. Unphagocytosed sRBCs were osmotically lysed and BMDMs were washed and analyzed by flow cytometry. (A) Red fluorescence from phagocytosed sRBCs appear as a second peak to the right in flow cytometry histograms. Quantitation for flow cytometric analysis of phagocytosis is presented as % of cells positive for red fluorescence and is shown below the histograms. (B) Phagocytosis of PKH26-labelled opsonized sRBCs by WT and RIP2 KO BMDMs was also analyzed by confocal microscopy. Images of BMDMs incubated with unopsonized or opsonized PKH26-labelled sRBCs were taken after allowing phagocytosis to occur for 30mins. Quantitation for confocal analysis of phagocytosis is presented as particles per 100 macrophages (total of (3) 40x fields counted) shown beside the images. Bars within graphs represent means ± SD. Data are aggregated from 2 independent experiments using n=7 mice per group for flow cytometric analysis of phagocytosis. Data are aggregated from 2 independent experiments using n=6 mice per group for confocal analysis of phagocytosis. Statistical analysis was performed using a two-way ANOVA. For (A) and (B), no interaction was observed (phagocytosis of the PKH26-labelled opsonized sRBCs was found to be similar for WT and RIP2 KO mice). Therefore, no further testing was performed. The p value for the overall effect of FcγR stimulation is indicated within the graph. (\*\*\*\* = p < 0.0001)

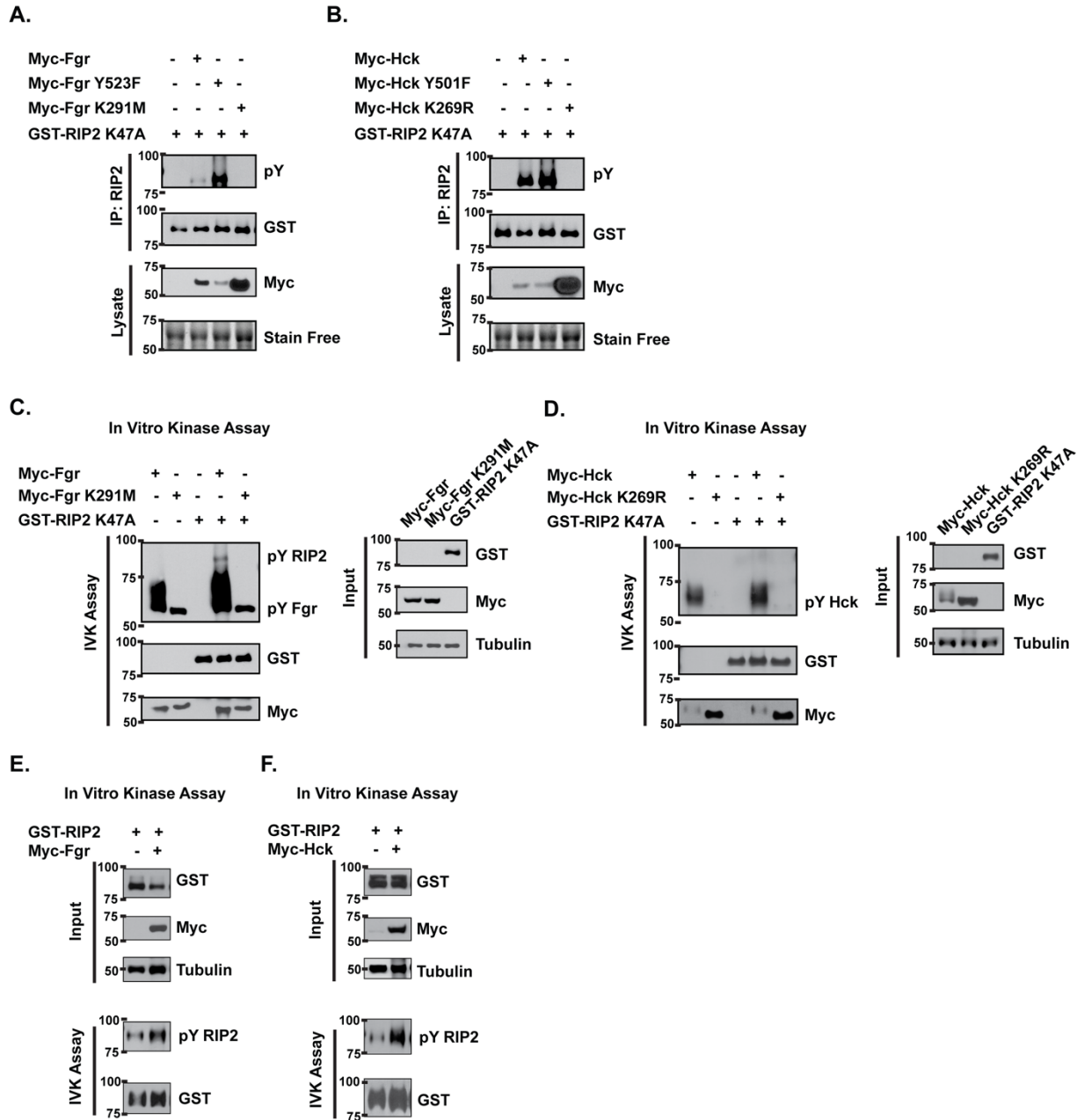


**Figure 5.** RIP2 does not affect FcγR-mediated iNOS expression and nitrate/nitrite production. (A) Bone marrow-derived macrophages (BMDMs) from WT and RIP2 KO mice were primed with 100ng/ml IFN-γ overnight prior to FcγR stimulation for 24hrs. Cell lysates were then harvested to perform a Western Blot. FcγR-mediated production of inducible nitric oxide synthase (iNOS) by WT and RIP2 KO BMDMs was assessed by immunoblotting using an anti-iNOS antibody and anti-tubulin antibody as a loading control. Three sets of BMDMs are shown for each genotype. (B) BMDMs from WT and RIP2 KO mice were left unstimulated or were stimulated with murine anti-BSA IgG<sub>1</sub> + BSA. RNA was harvested from cells after 4hrs to perform qRT-PCR to determine expression of inducible nitric oxide synthase (iNOS). (C) IFN-γ primed WT and RIP2 KO BMDMs were left unstimulated or were stimulated with murine anti-BSA IgG<sub>1</sub> + BSA for 24hrs. Cell supernatants were collected for assessment of nitrate/nitrite using a Griess assay. Bars within graphs represent means ± SD. For qRT-PCR analysis of iNOS expression, data are aggregated from 2 independent experiments using n=10 mice per group. For nitrate/nitrite quantification, data are aggregated from 2 independent experiments using n=8 mice per group. Statistical analysis was performed using a two-way ANOVA. For (B) and (C), no interaction was observed (iNOS expression and nitrate/nitrite production as a result of FcγR stimulation was found to be similar for WT and RIP2 KO mice). Therefore, no further testing was performed. The p value for the overall effect of FcγR stimulation is indicated within the graph. (\*\*\*\* = p < 0.0001)

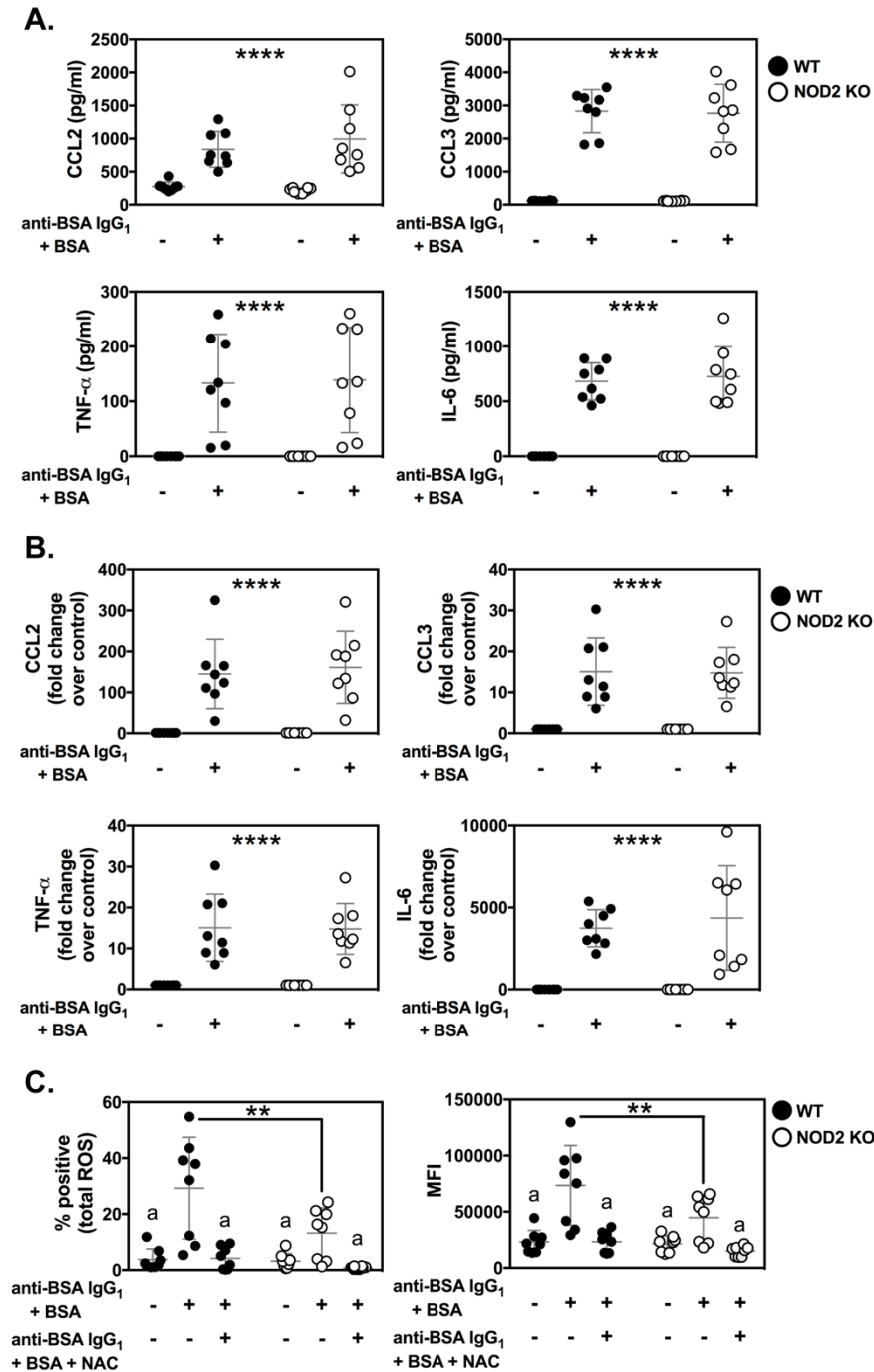




**Figure 6.** RIP2 is involved in ROS production downstream of FcγR engagement. (A) Bone marrow-derived macrophages (BMDMs) from WT and RIP2 KO mice were primed with 100ng/ml IFN-γ overnight prior to FcγR stimulation for 30mins in the presence of 2μM of an oxidative stress detection probe. The probe reacts with various ROS species to produce a green fluorescent product indicated by increased fluorescence in the FL-1 channel. Treatment of FcγR-stimulated cells with an ROS inhibitor, N-acetyl L-cysteine (NAC), results in a leftward shift and a decrease in the FcγR-induced fluorescence. (B) Quantitation for flow cytometric analysis of ROS production is presented as percent of cells with green fluorescence (compared to unstained control cells) or mean fluorescence intensity (MFI) in the FL-1 channel. Bars within graphs represent means ± SD. Data are aggregated from 3 experiments using n=11 mice/group. Statistical analysis was performed using a two-way ANOVA with Tukey's multiple comparisons test. (\*\*, b = p < 0.01; a = p < 0.0001 when compared to the FcγR-stimulated group of the same genotype)



**Figure 7.** Fgr directly tyrosine phosphorylates RIP2. HEK293 cells were transiently co-transfected with kinase-dead RIP2 and either wild-type, constitutively active or kinase-dead (A) Fgr or (B) Hck. RIP2 was immunoprecipitated and tyrosine phosphorylation was assessed. HEK293 cells were singly transfected with either kinase dead RIP2 or with either wild-type or kinase dead (C) Fgr or (D) Hck. RIP2, Fgr, and Hck were immunoprecipitated individually and combined together in an in vitro kinase (IVK) assay as indicated. HEK293 cells were transiently transfected with wild-type RIP2 with or without (E) Fgr or (F) Hck. RIP2 was immunoprecipitated and an in vitro kinase (IVK) assay (for tyrosine autophosphorylation) was performed. Data presented are representative of at least 3 independent experiments performed.



**Figure 8.** NOD2 is required for the observed effects of RIP2 in *FcγR*-mediated ROS production. Bone marrow-derived macrophages (BMDMs) from WT and NOD2 KO mice were left unstimulated or were stimulated with murine anti-BSA IgG<sub>1</sub> + BSA for 4hrs or for 16hrs in low-serum media. (A) Supernatants were collected after 16hrs of stimulation for analysis of cytokine secretion by ELISA. (B) In a separate set of experiments, RNA was harvested from cells after 4hrs to perform qRT-PCR for determining expression of the indicated genes. (C) BMDMs from WT and NOD2 KO mice were primed with 100ng/ml IFN-γ

overnight prior to FcγR stimulation for 30mins in the presence of 2μM of an oxidative stress detection probe with or without addition of a ROS inhibitor, N-acetyl L-cysteine (NAC). Quantitation for flow cytometric analysis of ROS production is presented as percent of cells with green fluorescence (compared to unstained control cells) or mean fluorescence intensity (MFI) in the FL-1 channel. Bars within graphs represent means ± SD. Data are aggregated from 3 experiments using n=8 mice/group. Statistical analysis was performed using a two-way ANOVA with Tukey's multiple comparisons test. For (A) and (B), no interaction was observed (cytokine secretion and gene upregulation was similar for WT and NOD2 KO mice upon treatment). Therefore, no further testing was performed. The p value for the overall effect of FcγR stimulation is indicated within the graph. (\*\*\*\* = p <0.0001, a = p <0.0001 when compared to the FcγR-stimulated group of the same genotype)

## **RIP2 promotes FcγR-mediated ROS production**

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