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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

TNFSF15 Promotes Antimicrobial Pathways in Human Macrophages and These Are Modulated by *TNFSF15* Disease-Risk Variants

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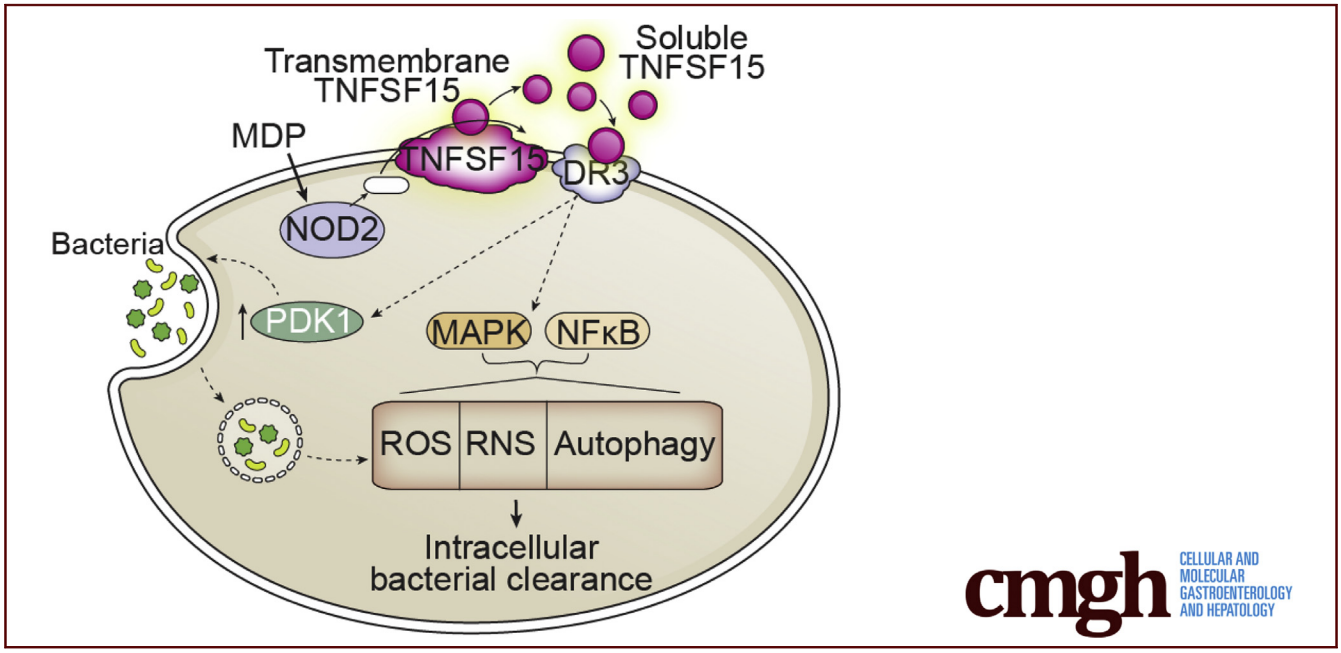

SUMMARY

TNF superfamily member 15 (TNFSF15) promotes bacterial uptake and intracellular bacterial clearance in human macrophages, and autocrine/paracrine TNFSF15 is required for optimal induction of antimicrobial pathways upon innate receptor stimulation. Macrophages from *TNFSF15* inflammatory bowel disease risk carriers show higher levels of these antimicrobial processes.

BACKGROUND & AIMS: *TNFSF15* genetic variants leading to increased TNF superfamily member 15 (TNFSF15) expression confer risk for inflammatory bowel disease (IBD), and TNFSF15 is being explored as a therapeutic target in IBD patients. Although the focus for TNFSF15-mediated inflammatory outcomes has been predominantly on its action on T cells, TNFSF15 also promotes inflammatory outcomes in human macrophages. Given the critical role for macrophages in bacterial clearance, we hypothesized that TNFSF15 promotes antimicrobial pathways in human macrophages and that macrophages from *TNFSF15* IBD risk carriers with higher TNFSF15 expression have an advantage in these antimicrobial outcomes.

METHODS: We analyzed protein expression, signaling, bacterial uptake, and intracellular bacterial clearance in human monocyte-derived macrophages through flow cytometry, enzyme-linked immunosorbent assay, and gentamicin protection.

RESULTS: Autocrine/paracrine TNFSF15 interactions with death receptor 3 (DR3) were required for optimal levels of pattern-recognition-receptor (PRR)-induced bacterial clearance in human macrophages. TNFSF15 induced pyruvate dehydrogenase kinase 1-dependent bacterial uptake and promoted intracellular bacterial clearance through reactive oxygen species, nitric oxide synthase 2, and autophagy up-regulation. The TNFSF15-initiated TNF receptor-associated factor 2/receptor-interacting protein kinase 1/RIP3 pathway was required for mitogen-activated protein kinase and nuclear factor-κB activation, and, in turn, induction of each of the antimicrobial pathways; the TNFSF15-initiated Fas-associated protein with death domain/mucosa-associated lymphoid tissue lymphoma translocation protein 1/caspase-8 pathway played a less prominent role in antimicrobial functions, despite its key role in TNFSF15-induced cytokine secretion. Complementation of signaling pathways or antimicrobial pathways restored bacterial uptake and clearance in PRR-stimulated macrophages where TNFSF15:DR3 interactions were inhibited. Monocyte-derived

macrophages from high TNFSF15-expressing rs6478108 TT IBD risk carriers in the *TNFSF15* region showed increased levels of the identified antimicrobial pathways.

CONCLUSIONS: We identify that autocrine/paracrine TNFSF15 is required for optimal PRR-enhanced antimicrobial pathways in macrophages, define mechanisms regulating TNFSF15-dependent bacterial clearance, and determine how the *TNFSF15* IBD risk genotype modulates these outcomes. *(Cell Mol Gastroenterol Hepatol 2021;11:249–272; https://doi.org/10.1016/j.jcmgh.2020.08.003)*

Keywords: Pattern Recognition Receptors; Macrophages; Crohn's Disease; Genetics.

Immune-mediated diseases, such as inflammatory bowel disease (IBD),[1] show dysregulated cytokines. In the intestine, regulating pattern recognition receptor (PRR)-initiated signaling and cytokine secretion is crucial given ongoing host–microbial interactions.[2,3] One such cytokine pathway is TNF superfamily member 15 (TNFSF15). The TNFSF15 pathway promotes inflammation in a variety of diseases.[4–6] Moreover, genetic variants in the *TNFSF15* region are associated with multiple diseases,[7–9] including IBD[3,10–12] and leprosy.[13] Specifically, genetic variants in the *TNFSF15* region leading to an increase in TNFSF15 expression[14–17] are associated with increased risk for IBD.[3,10–12] *TNFSF15* risk variants in IBD are frequent (40%–50% in European ancestry individuals per Single Nucleotide Polymorphism Database). Moreover, IBD risk variants in *TNFSF15* are notable for leading to a particularly large effect size in Asian ancestry individuals (odds ratio, 1.75).[18] Consistent with the increased TNFSF15 expression with *TNFSF15* region genetic risk variants, TNFSF15 expression is upregulated in intestinal tissues of IBD patients.[19,20] Furthermore, blocking/reducing TNFSF15–death receptor 3 (DR3) interactions ameliorates inflammation and/or fibrosis in mouse models of experimental colitis.[21–24] As such, reducing TNFSF15 pathway responses currently is under active investigation as a therapeutic approach, including in IBD patients (ClinicalTrials.gov). Understanding the mechanisms and cell subsets through which TNFSF15 contributes to immune homeostasis and disease is important in the context of such therapeutic interventions, as well as in considering the manner in which the genetic variants in *TNFSF15* modulating TNFSF15 expression may regulate immunity.

The beneficial effects of blocking TNFSF15 in inflammatory models has been attributed primarily to myeloid cell–derived TNFSF15 interactions with DR3-expressing T cells[22,25–27]; signaling from DR3 then regulates T-cell activation/differentiation.[21,28] Additional studies have shown that TNFSF15 can contribute to the production of interleukin (IL)22 by group 3 innate lymphoid cells, and, in turn, mucosal healing, thereby providing protective roles.[29–31] We previously found that TNFSF15 is not only produced by myeloid cells such as dendritic cells and macrophages, but also directly acts on these cells through DR3 to then amplify signaling and cytokines in the context of microbial stimulation of PRRs.[14] Because macrophages are essential for mediating microbial clearance, this raises the possibility that TNFSF15 also may promote various antimicrobial pathways in macrophages. As such, therapeutic blockade of TNFSF15 may increase the risk for infection. Furthermore, although individuals with genetic variants leading to low TNFSF15 expression may be relatively protected from IBD and other immune-mediated diseases, they may be at increased risk for select infections. To our knowledge, only a couple of reports to date have examined the role of TNFSF15–DR3 interactions on the efficacy of bacterial clearance.[32,33] DR3 deficiency led to less-effective clearance of *Salmonella enterica serova* Typhimurium in mice.[32,33] Consistent with the focus to date on TNFSF15 interactions with DR3 on T cells, DR3 on T cells was required for reducing the burden of *S* Typhimurium in mice in vivo.[32] How TNFSF15 regulates antimicrobial pathways in macrophages has not been defined to date. We hypothesized that TNFSF15–DR3 would regulate PRR-initiated antimicrobial pathways in myeloid cells and that disease-associated *TNFSF15* polymorphisms would modulate TNFSF15-dependent antimicrobial outcomes.

In this study, we found that TNFSF15–DR3 interactions on human macrophages promote bacterial clearance and, moreover, amplify PRR-initiated bacterial clearance. We define TNFSF15–DR3–initiated signaling pathways and mechanisms regulating this enhanced bacterial clearance and determine that the *TNFSF15* IBD risk genotype modulates these outcomes. Thus, TNFSF15–DR3 interactions amplify PRR-initiated antimicrobial pathways, thereby highlighting the potential for adverse infectious complications when therapeutically targeting the TNFSF15–DR3 pathway.

Results

TNFSF15 Promotes Intracellular Bacterial Clearance in Human Monocyte-Derived Macrophages

To assess if TNFSF15 contributes to intracellular bacterial clearance in human macrophages, we treated human monocyte-derived macrophages (MDMs) with TNFSF15 for a prolonged period of time to simulate conditions in the intestinal environment of ongoing cytokine exposure. We

Abbreviations used in this paper: **AIEC, adherent invasive *Escherichia coli*; BMDM, bone marrow–derived macrophage; DR3, death receptor 3; ERK, extracellular signal-regulated kinase; FADD, Fas-associated protein with death domain; IBD, inflammatory bowel disease; IL, interleukin; JNK, Janus kinase; LC3II, light chain 3-II; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MAPK, mitogen-activated protein kinase; MDM, monocyte-derived macrophages; MDP, muramyl dipeptide; NADPH, nicotinamide adenine dinucleotide phosphate; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NOD, nucleotide-binding oligomerization domain; NOS2, nitric oxide synthase 2; PDK1, pyruvate dehydrogenase kinase 1; PI3K, phosphoinositide 3-kinase; PRR, pattern-recognition receptor; RIP, receptor-interacting protein kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; siRNA, small interfering RNA; TACE, tumor necrosis factor converting enzyme; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFSF15, TNF superfamily member 15; TRADD, tumor necrosis factor receptor 1-associated death domain; TRAF2, TNF receptor associated factor 2.**

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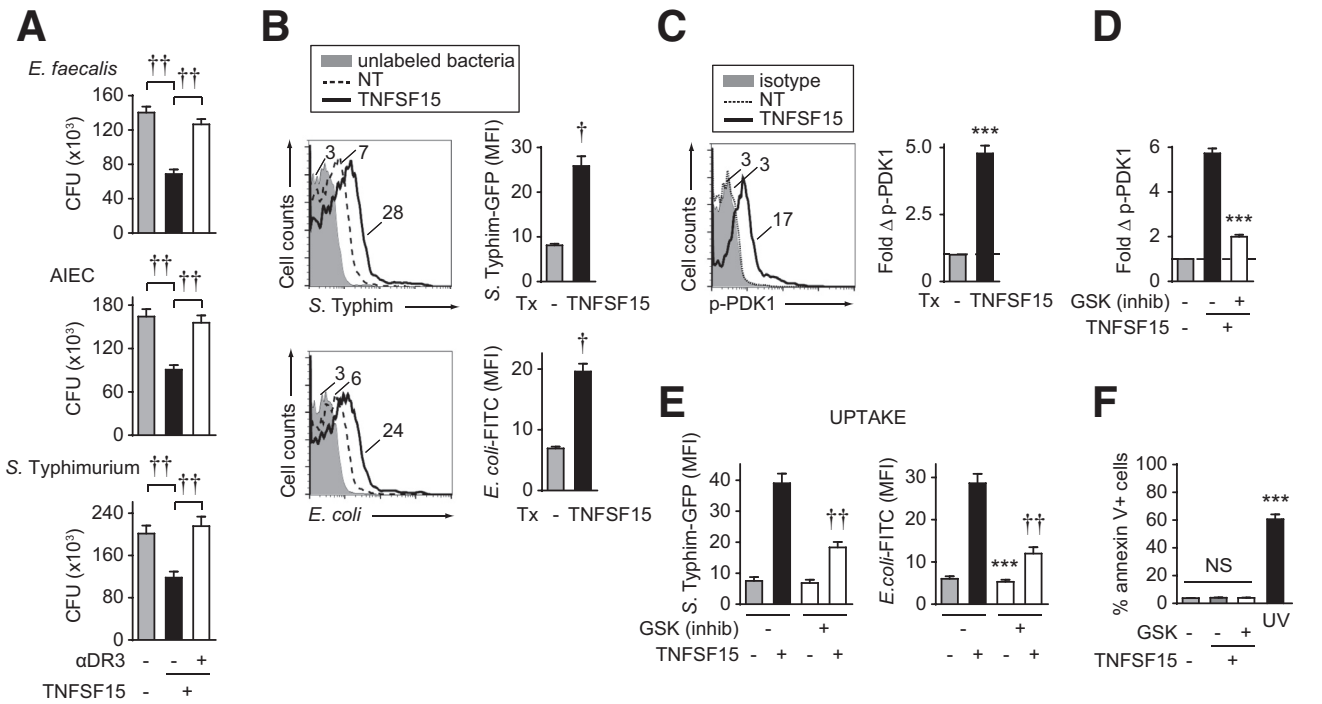

Figure 1. TNFSF15 treatment of human MDMs increases bacterial uptake and intracellular bacterial clearance. (*A*) MDMs were left untreated or treated with 10 ng/mL TNFSF15 for 48 hours ± pretreatment for 1 hour with neutralizing anti-DR3 antibodies (or isotype control) (n = 12 donors from 2 independent experiments, repeated in an additional 16 donors in the absence of neutralizing antibodies), and then co-cultured with *E faecalis*, AIEC, or *S* Typhimurium and assessed for intracellular bacterial clearance as per the Materials and Methods section. Mean colony forming units (CFU). (*B*) Human MDMs were left untreated or treated with 10 ng/mL TNFSF15 for 48 hours and then co-cultured with *S* Typhimurium-GFP or *E coli*–fluorescein isothiocyanate (FITC) bioparticles and uptake was assessed 20 minutes later by flow cytometry. *Left*: Representative flow cytometry with mean fluorescence intensity (MFI). *Right*: Summary graph of MFI (n = 8 donors from 2 independent experiments). (*C*) Human MDMs were left untreated or treated with 10 ng/mL TNFSF15 for 20 minutes. PDK1 activation was assessed by flow cytometry. *Left*: Representative flow cytometry. *Right*: Summary graph of fold PDK1 activation (n = 4 donors, similar results in an additional n = 4 over a time course). (*D–F*) Human MDMs were left untreated or treated with 10 ng/mL TNFSF15 ± GSK 233470 (PDK1 inhibitor; 1-hour pretreatment). (*D*) Fold PDK1 activation at 20 minutes (n = 4). (*E*) After 48 hours, cells were co-cultured with *S* Typhimurium–GFP or *E coli*–FITC bioparticles and uptake was assessed 20 minutes later (MFI) (n = 8 from 2 independent experiments). (*F*) Cell death was assessed at 48 hours by annexin V staining (n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control. Means + SEM. Significance comparison is between inhibitor to the vehicle control for the corresponding TNFSF15 treatment condition for panels *D–E*. ***$P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. NT, no treatment; Tx, treatment.

then cultured the cells with the resident intestinal bacteria *Enterococcus faecalis*. Intracellular levels of *E faecalis* were lower after prolonged TNFSF15 treatment (Figure 1*A*). To ensure that TNFSF15 interacted with DR3 on human MDMs to mediate this enhanced bacterial clearance, we used neutralizing anti-DR3 antibodies; TNFSF15 was unable to enhance bacterial clearance with DR3 blockade (Figure 1*A*). We saw similar outcomes upon infection with adherent invasive *Escherichia coli* (AIEC), which are enriched in the ilea of Crohn's disease patients,[34] and *S* Typhimurium, an invasive enteric pathogen (Figure 1*A*). Taken together, TNFSF15 interacts with DR3 on human MDMs to promote increased intracellular bacterial clearance.

TNFSF15 Promotes Pyruvate Dehydrogenase Kinase 1-Dependent Uptake of Microbes in Human MDMs

We next sought to address mechanisms for the TNFSF15-induced clearance of intracellular bacteria in macrophages. The initial step in bacterial clearance involves bacterial uptake. We therefore first assessed if TNFSF15 regulates bacterial uptake and if the lower levels of intracellular bacteria after prolonged TNFSF15 treatment were the result of lower levels of bacterial uptake. In fact, after prolonged TNFSF15 treatment, *S* Typhimurium-green fluorescent protein (GFP) uptake was increased in human MDMs (Figure 1*B*). We saw similar outcomes with fluorophore-labeled *E coli* bioparticles (Figure 1*B*).

We next assessed mechanisms through which TNFSF15 promotes bacterial uptake. The phosphoinositide 3-kinase (PI3K) pathway contributes to phagocytosis,[35] such that we asked if TNFSF15 regulation of PI3K might be a mechanism through which it regulates bacterial uptake. We assessed pyruvate dehydrogenase kinase 1 (PDK1) activation as a measure of PI3K pathway activation. TNFSF15 treatment of human MDMs led to PDK1 activation within 20 minutes (Figure 1*C*). Importantly, inhibiting PDK1 activation using a PDK1 pharmacologic inhibitor (Figure 1*D*) led to reduced live bacterial and bacterial particle uptake after

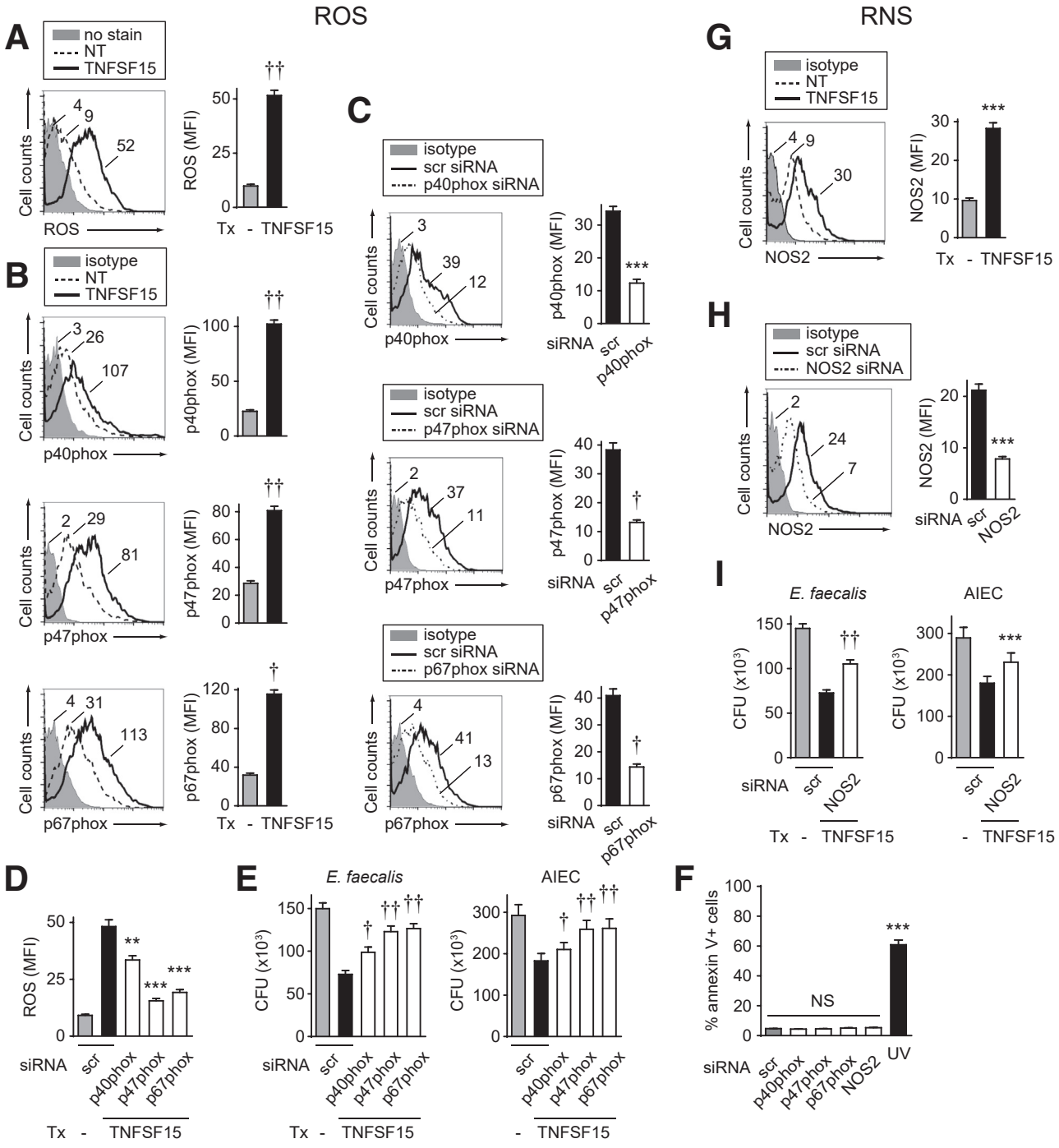

Figure 2. TNFSF15 promotes ROS and RNS pathways in MDMs. (*A*, *B*, and *G*) MDMs were treated with 10 ng/mL TNFSF15 for 48 hours. (*A*) ROS induction (n = 6; similar results in an additional n = 4). (*B*) NADPH oxidase members (n = 6; similar results additional n = 6). (*G*) NOS2 expression (n = 6; similar results in an additional n = 6). (*C–E*, *H*, and *I*) MDMs were transfected with scrambled or the indicated siRNAs, and then treated with 10 ng/mL TNFSF15 for 48 hours. (*C* and *H*) Expression of the indicated proteins by flow cytometry (n = 6). (*D*) ROS production (n = 6). (*E* and *I*) Intracellular bacterial clearance (n = 10 from 2 independent experiments). (*F*) MDMs were transfected with scrambled or the indicated siRNAs and cell death was assessed by annexin V staining (n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control for cell death. Means + SEM. (*A–C*, *G*, and *H*) Representative flow cytometry with MFI values is shown. (*D*, *E*, and *I*) Significance is between scrambled and the target siRNA for TNFSF15-treated cells. $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. MFI, mean fluorescence intensity; NT, no treatment; scr, scrambled; Tx, treatment.

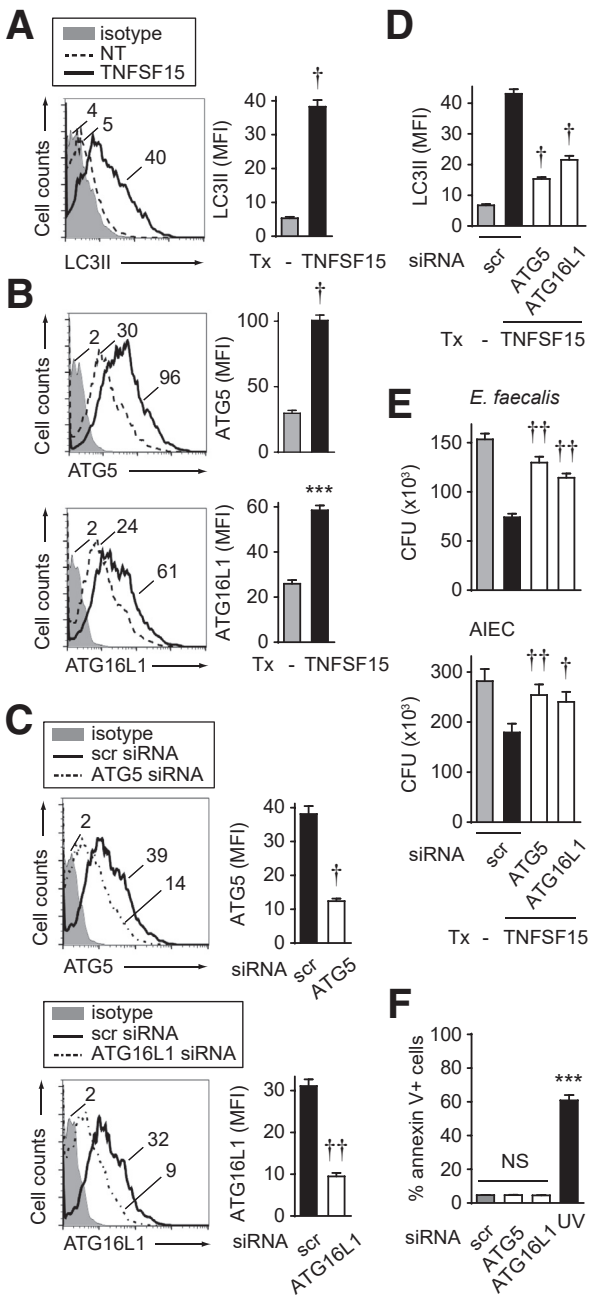

Figure 3. TNFSF15 promotes autophagy pathways in MDMs. (*A* and *B*) MDMs were treated with 10 ng/mL TNFSF15 for 48 hours. (*A*) LC3II expression (n = 6; similar results in an additional n = 4). (*B*) Autophagy molecule expression (n = 6; similar results in an additional n = 8 for ATG16L1 and n = 4 for ATG5). (*C–F*) MDMs were transfected with scrambled or the indicated siRNAs. (*C*) Expression of the indicated proteins by flow cytometry (n = 6). (*D* and *E*) Cells then were treated with 10 ng/mL TNFSF15 for 48 hours. (*D*) LC3II expression (n = 6). (*E*) Intracellular bacterial clearance (n = 10 from 2 independent experiments). (*F*) Cell death was assessed by annexin V staining (n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control. Means + SEM. (*A–C*) Representative flow cytometry with mean fluorescence intensity (MFI) values shown. Significance is between scrambled and the target siRNA for TNFSF15-treated cells for panels *D* and *E*. ***$P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. NT, no treatment; scr, scrambled; Tx, treatment.

prolonged TNFSF15 treatment (Figure 1*E*). Cell viability was unimpaired with PDK1 inhibition (Figure 1*F*). Therefore, the reduced intracellular bacteria at later time points after prolonged TNFSF15 treatment was in fact observed in the context of higher levels of PDK1-dependent bacterial uptake under these TNFSF15-treated conditions.

TNFSF15 Induces Reactive Oxygen Species, Reactive Nitrogen Species, and Autophagy Pathways in Human MDMs

We next sought to assess the mechanisms through which TNFSF15 enhances the clearance of bacteria that have entered into human macrophages successfully. We first assessed reactive oxygen species (ROS) given the important role ROS production plays in mediating bacterial clearance.[1] TNFSF15 treatment induced ROS production in MDMs (Figure 2*A*). Polymorphisms in various genes in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex required for ROS production are associated with increased risk for both the common form of IBD[3] and early onset IBD.[36] We found that TNFSF15 induced the NADPH oxidase members p40phox, p47phox, and p67phox (Figure 2*B*). Through effective knockdown of these NADPH oxidase members (Figure 2*C*), we ensured that these NADPH oxidase members contributed to TNFSF15-induced ROS production (Figure 2*D*) and bacterial clearance (Figure 2*E*). Cell viability was intact under these knockdown conditions (Figure 2*F*).

Reactive nitrogen species (RNS) also can contribute to bacterial clearance, and a combination of ROS and RNS pathways is central in maintaining homeostasis in the intestinal mucosa.[37] TNFSF15 treatment induced nitric oxide synthase 2 (NOS2) expression (Figure 2*G*). Through effective knockdown of NOS2 (Figure 2*H*), we established that NOS2 was required for TNFSF15-induced bacterial clearance (Figure 2*I*). Cell viability was intact under these knockdown conditions (Figure 2*F*).

Autophagy is another important bacterial clearance mechanism[38] and polymorphisms in the autophagy-associated gene *ATG16L1* alter susceptibility to Crohn's disease.[3] TNFSF15 induced expression of the autophagy marker light chain 3-II (LC3II) (Figure 3*A*). Consistently, TNFSF15 promoted induction of the autophagy-associated proteins ATG5 and ATG16L1 (Figure 3*B*). Through effective ATG5 and ATG16L1 knockdown (Figure 3*C*), we established that these molecules were important for TNFSF15-induced autophagy (Figure 3*D*). We further established the importance of ATG5 and ATG16L1 in TNFSF15-induced bacterial clearance (Figure 3*E*). Cell viability was intact under these knockdown conditions (Figure 3*F*). Taken together, TNFSF15 promotes induction of ROS, RNS, and autophagy pathways, which, in turn, mediate TNFSF15-induced bacterial clearance.

ROS, RNS, and Autophagy Pathways Cooperate in Mediating TNFSF15-Induced Intracellular Bacterial Clearance

Because the ROS, RNS, and autophagy pathways each only partially contributed to the TNFSF15-induced bacterial clearance observed in human MDMs (Figures 2 and 3), we

assessed if these pathways cooperate to mediate TNFSF15-enhanced bacterial clearance. We used small interfering RNA (siRNA) to reduce expression of p47phox (as the NADPH oxidase subunit that most accounted for TNFSF15-induced ROS production) (Figure 2*D*), NOS2, and ATG5 (as the autophagy molecule that most contributed to TNFSF15-mediated autophagy) (Figure 3*D*). Combined reduction of these antimicrobial pathways led to the greatest impairment in TNFSF15-induced bacterial clearance (Figure 4*A*) (with intact cell viability in Figure 4*B*), thereby showing that these pathways cooperate to optimally mediate bacterial clearance upon TNFSF15 treatment.

TNF Receptor Associated Factor 2/Receptor-Interacting Protein Kinase 1/RIP3 Signaling Is Required for TNFSF15-Initiated Antimicrobial Pathways

We next sought to define the TNFSF15-initiated signaling pathways regulating the antimicrobial mechanisms we had defined. DR3 activation can induce distinct signaling pathways, including Fas-associated protein with death domain (FADD)/mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)/caspase-8 and TNF receptor associated factor 2 (TRAF2)/receptor-interacting protein kinase 1 (RIP1)/RIP3 pathways (Figure 5*A*). Tumor necrosis factor receptor 1-associated death domain (TRADD) is recruited to DR3 upon TNFSF15 treatment of T cells and likely is upstream of this pathway bifurcation[39] (Figure 5*A*). Upon TNFSF15 interactions with DR3, caspase-8 is activated through the FADD pathway in epithelial cell lines and fibroblasts,[40] which can induce apoptosis.[41] Although IL1β secretion classically is associated with caspase-1 activation, some studies have shown that caspase-8 can process IL1β downstream of select receptors.[42] Moreover, dectin-1–initiated caspase-8 activation and IL1β processing in human DC requires MALT1.[42] We previously found that TNFSF15 initiates FADD and MALT1 signaling to cleave caspase-8 and, in turn, rapid IL1β processing and early IL1β secretion (within 15 minutes).[14] We therefore effectively reduced the expression of each FADD, MALT1, and caspase-8 (Figure 5*B*). FADD, MALT1, and caspase-8 were not required for TNFSF15-induced PDK1 activation at 20 minutes (Figure 5*C*). Similar results were observed upon combined knockdown of these molecules (Figure 5*C*). We will use combined knockdown of the molecules in this pathway in the studies that follow. Consistent with the failure of the FADD/MALT1/caspase-8 pathway to regulate TNFSF15-induced PDK1 activation, this pathway was not required for TNFSF15-induced bacterial uptake (Figure 5*D*). We ensured effective functional outcomes with knockdown of the FADD/MALT1/caspase-8 pathway as assessed by reduced TNFSF15-induced early caspase-8 and IL1β processing (Figure 5*E*). Moreover, we confirmed our prior findings[14] that consistent with the role of autocrine/paracrine IL1β secretion, the TNFSF15-initiated FADD/MALT1/caspase-8 pathway was required for cytokine secretion (Figure 5*F*).

TNFSF15–DR3 signaling can proceed through another arm using TRAF2 to activate T cells[43] (Figure 5*A*). Signaling and outcomes via RIP1 and RIP3 downstream of TNF-superfamily receptors has been controversial and likely dependent on the stimulus and cell type.[44,45] As such, it is important to define the role that the TRAF2/RIP1/RIP3 signaling branch plays in TNFSF15-initiated outcomes, in particular antimicrobial clearance, in human macrophages. We previously found that RIP1 and RIP3 were required for TNFSF15-induced cytokine secretion in human MDMs, but not TNFSF15-induced rapid caspase-8 activation and early IL1β secretion[14]; we did not examine mechanisms through which RIP1/RIP3 may be regulating TNFSF15-dependent downstream outcomes in macrophages. In contrast to the FADD/MALT1/caspase-8 pathway, upon effective TRAF2, RIP1, and RIP3 knockdown (Figure 5*B*), each of these molecules was required for optimal levels of TNFSF15-induced PDK1 activation (Figure 5*C*). Combined knockdown of these molecules led to similar results (Figure 5*C*). Consistently, the TRAF2/RIP1/RIP3 pathways was required for TNFSF15-induced bacterial uptake (Figure 5*D*). In addition, in contrast to the FADD/MALT1/caspase 8 pathway, TRAF2/RIP1/RIP3 was not required for TNFSF15-induced caspase-8 and IL1β processing (Figure 5*E*). Of note is that effective TRADD knockdown (Figure 5*B*) led to reduced TNFSF15-induced caspase-8 and IL1β processing (Figure 5*E*), reduced TNFSF15-induced PDK1 activation and bacterial uptake (Figure 5*C* and *D*), and reduced cytokine secretion (Figure 5*F*), thereby supporting a model wherein TRADD is upstream of the 2 distinct TNFSF15-initiated signaling branches (Figure 5*A*).

We next assessed how these 2 distinct signaling pathways regulated TNFSF15-dependent intracellular bacterial clearance and the pathways contributing to this clearance. Although the FADD/MALT1/caspase-8 pathway contributed somewhat to TNFSF15-induced ROS, NOS2, and autophagy pathways (Figure 5*G*), and intracellular bacterial clearance (Figure 5*H*), the TRAF2/RIP1/RIP3 pathway contributed to a greater degree to these outcomes (Figure 5*G* and *H*). As expected, upstream TRADD similarly was required for optimal TNFSF15-induced ROS, NOS2, and autophagy pathways (Figure 5*G*), and intracellular bacterial clearance (Figure 5*H*). We ensured that cell viability was intact with knockdown of each of these molecules alone and in combination (Figure 5*I* and *J*). Taken together, TNFSF15-initiated TRAF2/RIP1/RIP3 signaling is required for bacterial uptake and intracellular bacterial clearance, while TNFSF15-initiated FADD/MALT1/caspase-8 contributes only to a mild degree to intracellular bacterial clearance, despite its important role in TNFSF15-induced cytokine secretion. These studies highlight distinct roles for TNFSF15-initiated FADD/MALT1/caspase-8 in microbial clearance and cytokine secretion.

The TRAF2/RIP1/RIP3 Pathway Is Required for Optimal TNFSF15-Induced Mitogen Activated Protein Kinase and Nuclear Factor-κB Signaling

As TNFSF15-initiated FADD/MALT1/caspase-8 activation leads to rapid post-translational processing of IL1β, we hypothesized that TNFSF15-induced TRAF2/RIP1/RIP3

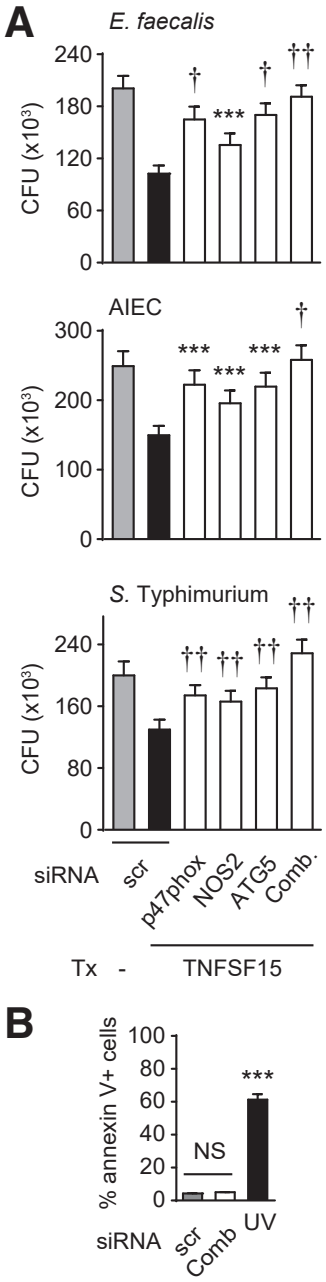

Figure 4. TNFSF15-induced ROS, RNS, and autophagy pathways cooperate to mediate intracellular bacterial clearance. MDMs were transfected with scrambled siRNA or the indicated siRNA, alone or in combination (comb). (*A*) Cells then were treated with TNFSF15 for 48 hours. Intracellular bacterial clearance was assessed (colony-forming units [CFU]) (n = 8 from 2 independent experiments). Significance is between scrambled and the target siRNA for TNFSF15-treated cells. (*B*) Cell death was assessed by annexin V staining (n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control. Mean + SEM. ***$P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. scr, scrambled; Tx, treatment.

leads to a distinct mechanism of signaling which then promotes antimicrobial pathways. In particular, TNFSF15–DR3 signaling can proceed through TRAF2 to activate nuclear factor-κB (NF-κB) signaling in T cells.[43] TNFSF15–DR3 signaling also can activate the mitogen activated protein kinase (MAPK) pathway in endothelial cells.[39] We therefore hypothesized that the TRAF2/RIP1/RIP3 pathway might lead to TNFSF15-initiated MAPK and NF-κB signaling in human MDMs, and, in turn, transcriptional regulation of downstream outcomes. We first confirmed that TNFSF15 induced activation of extracellular signal-regulated kinase (ERK), p38, Janus kinase (JNK), and NF-κB signaling pathways (Figure 6*A*). Moreover, through knockdown of MAPK and NF-κB pathways (Figure 6*B*), we established that MAPK and NF-κB signaling was required for TNFSF15-induced ROS, RNS, and autophagy pathways (Figure 6*C*), and intracellular bacterial clearance (Figure 6*D*). Furthermore, these pathways cooperated to regulate these outcomes (Figure 6*C* and *D*). Cell viability was intact under these conditions (Figure 6*E*). Consistent with the role of MAPK and NF-κB signaling in transcriptional regulation, the antimicrobial pathways we had identified to be up-regulated after prolonged TNFSF15 treatment were regulated by the MAPK and NF-κB pathways at a transcriptional level (Figure 7*A*). In contrast to the up-regulated antimicrobial pathways after prolonged TNFSF15 treatment, cytokine transcripts were down-regulated at this later time point (Figure 7*B*). We next assessed if 1 or both signaling arms initiated upon TNFSF15–DR3 activation was required for TNFSF15-induced MAPK and NF-κB signaling. The TNFSF15-induced early activation (15 min) of both MAPK and NF-κB signaling was reduced with knockdown of TRAF2/RIP1/RIP3, whereas these pathways remained mostly intact with FADD/MALT1/caspase-8 knockdown (Figure 7*C*). As expected given its upstream positioning, TRADD knockdown also led to reduced TNFSF15-initiated MAPK and NF-κB signaling (Figure 7*C*).

Given the MAPK and NF-κB regulation of TNFSF15-induced intracellular bacterial clearance mechanisms, and the partial contribution of FADD/MALT1/caspase-8 to TNFSF15-induced intracellular bacterial clearance mechanisms, we considered that FADD/MALT1/caspase-8 might activate MAPK and NF-κB pathways, but that this activation might be occurring with different kinetics relative to the TNFSF15-initiated TRAF2/RIP1/RIP3 pathway. In particular, because the FADD/MALT1/caspase-8 pathway initiates post-translational processing of IL1β, which then feeds back to amplify TNFSF15-induced outcomes per our prior study,[14] we hypothesized that this pathway would lead to MAPK and NF-κB signaling at a later time consistent with the delay associated with autocrine/paracrine cytokine loops. We therefore examined MAPK and NF-κB signaling over a 2-hour time period. Although reducing the FADD/MALT1/caspase-8 pathway did not, for the most part, impair TNFSF15-induced early MAPK and NF-κB signaling (15 min), MAPK and NF-κB signaling was partially reduced by 30 minutes and this continued over the 2-hour time period examined (Figure 7*D*). Taken together, the TNFSF15-initiated TRAF2/RIP1/RIP3 pathway leads to early activation of MAPK and NF-κB pathways, which, in turn, are required for induction of TNFSF15-induced antimicrobial pathways and intracellular bacterial clearance in macrophages. In contrast, the

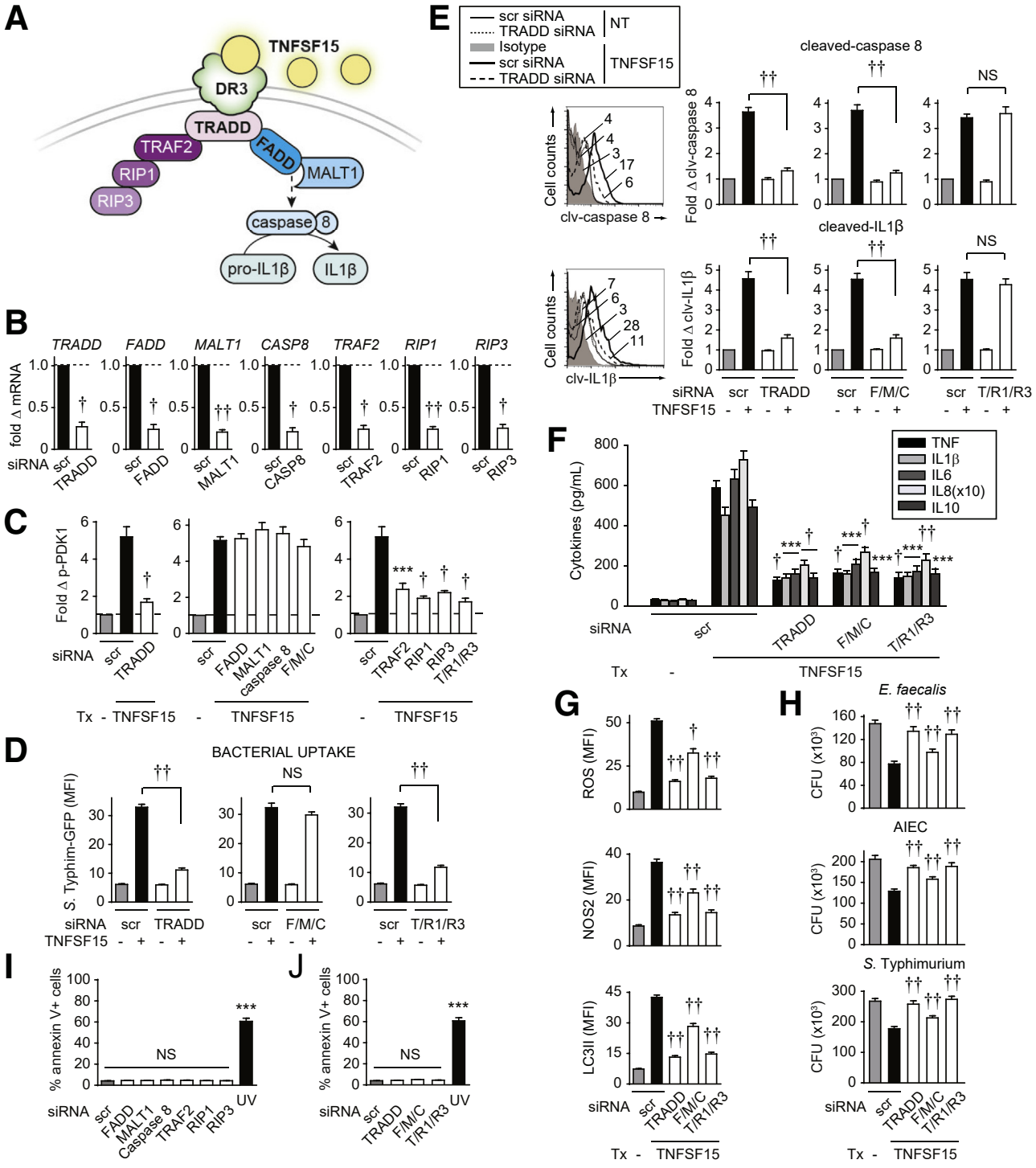

Figure 5. The TRAF2/RIP1/RIP3 pathway is required for TNFSF15-induced bacterial uptake and intracellular bacterial clearance pathways. (*A*) Model of proposed TNFSF15–DR3–initiated signaling in MDMs. (*B–J*) MDMs were transfected with siRNA to TRADD or to FADD, MALT1, caspase-8 alone or in combination (F/M/C), or to TRAF2, RIP1, or RIP3 alone or in combination (T/R1/R3). (*B*) Fold mRNA expression (n = 6). (*C–H*) Cells then were treated with 10 ng/mL TNFSF15. (*C*) Fold PDK1 activation at 20 minutes (n = 6 donors, similar results in n = 4 for single-siRNA–transfected groups). (*D*) Forty-eight hours later bacterial uptake was assessed (n = 8 from 2 independent experiments). (*E*) Fold cleaved (clv) caspase-8 or cleaved (clv) IL1β at 20 minutes. *Left*: Representative flow cytometry with MFI values. *Right*: Summary of fold cleaved caspase-8 or cleaved IL1β (n = 8 from 2 independent experiments). (*F*) Cytokine secretion at 24 hours (n = 6). (*G*) ROS, NOS2, or LC3II expression at 48 hours (n = 12 from 3 independent experiments). (*H*) After 48 hours cells were assessed for intracellular bacterial clearance (colony-forming units [CFU]) (n = 8 from 2 independent experiments). (*I* and *J*) Cell death was assessed by annexin V staining (n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control. Means + SEM. (*C–H*) Significance is between scrambled and the target siRNA for the corresponding TNFSF15-treated conditions or as indicated. $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. MFI, mean fluorescence intensity; NT, no treatment; scr, scrambled; Tx, treatment.

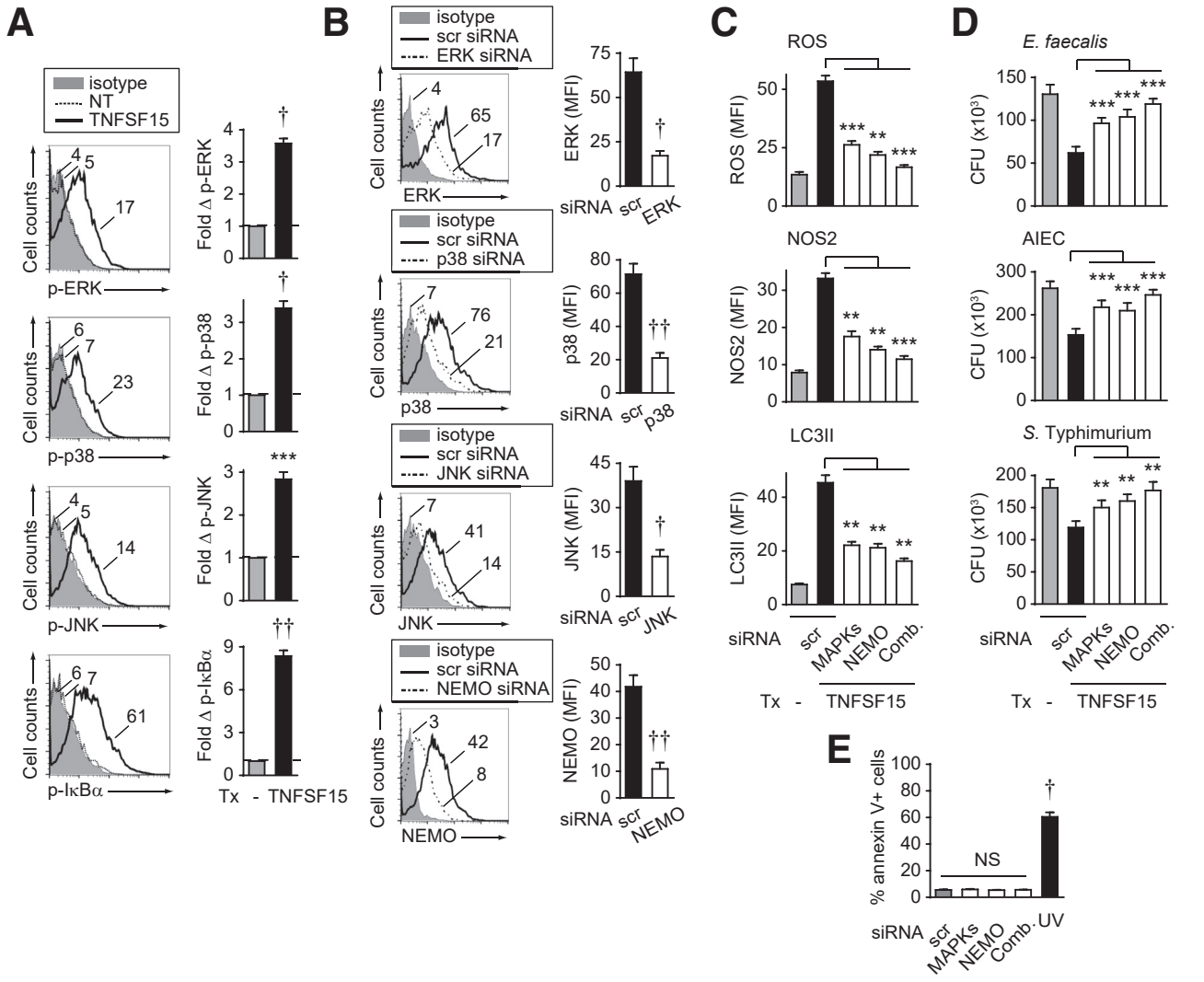

Figure 6. MAPK and NF-κB signaling is required for TNFSF15-induced microbial clearance. (*A*) MDMs were treated with 10 ng/mL TNFSF15 and assessed for activation of the indicated phospho-proteins at 15 minutes. *Left*: Representative flow cytometry with mean fluorescence intensity (MFI) values shown. *Right*: Summary graph of fold phospho-protein activation (n = 6). (*B–E*) MDMs were transfected with scrambled, ERK/p38/JNK (MAPK), or NF-κB essential modulator (NEMO) (NF-κB pathway), alone or in combination (comb). (*B*) Expression of the indicated proteins by flow cytometry (n = 6, similar results in an additional n = 4 for MAPKs). (*C* and *D*) Cells then were treated with 10 ng/mL TNFSF15 for 48 hours. (*C*) ROS, NOS2, or LC3II expression (n = 4, similar results in additional n = 8). (*D*) Intracellular bacterial clearance (colony-forming units [CFU]) (n = 4, similar results in an additional n = 8). (*E*) Cell death was assessed by annexin V staining (n = 4, similar results in an additional n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control. Means + SEM. $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. NT, no treatment; scr, scrambled; Tx, treatment.

TNFSF15-initiated FADD/MALT1/caspase-8 pathway contributes only to later MAPK and NF-κB pathway activation and minimally contributes to intracellular bacterial clearance.

Autocrine/Paracrine Soluble TNFSF15 Promotes Nucleotide-Binding Oligomerization Domain 2–Induced Intracellular Bacterial Clearance

Because PRRs are activated by microbial products to initiate antimicrobial responses, and we previously found that autocrine/paracrine TNFSF15 dramatically amplifies PRR-induced cytokines, we asked if autocrine/paracrine TNFSF15 is required for optimal PRR-induced antimicrobial pathways. We[46] and others[47,48] have found that chronic PRR stimulation enhances microbial clearance pathways and this prolonged stimulation simulates environments of ongoing microbial exposure such as the intestine. We examined the PRR nucleotide-binding oligomerization domain 2 (NOD2) given its association with Crohn's disease[2]; muramyl dipeptide (MDP) is the minimal ligand that stimulates NOD2. As expected, intracellular clearance of *E faecalis* was more effective after prolonged NOD2 stimulation (48 h), and both baseline and NOD2-enhanced bacterial clearance were impaired with neutralization of DR3 (Figure 8*A*). We observed similar

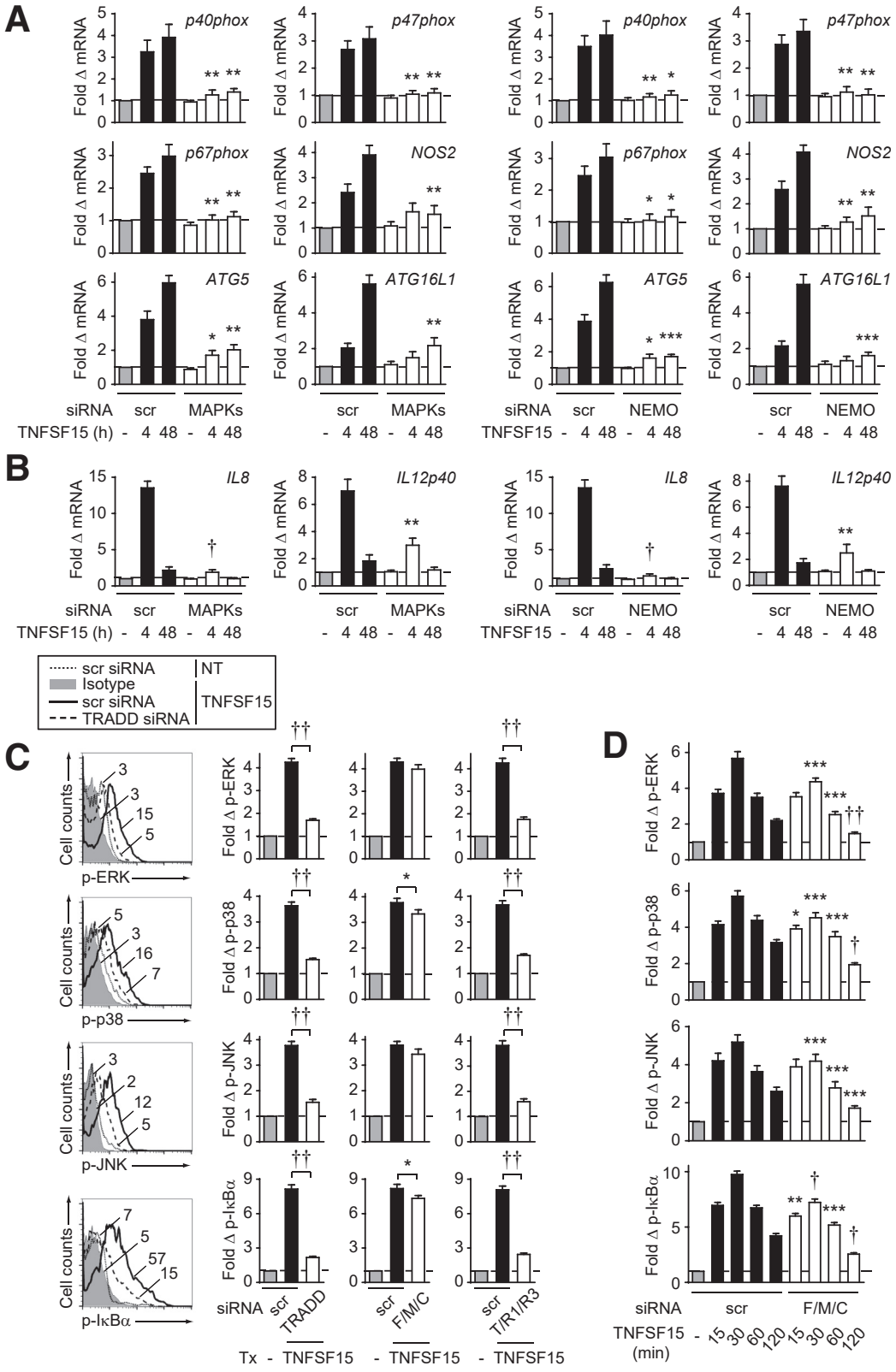

Figure 7. The TRAF2/RIP1/RIP3 pathway is required for optimal TNFSF15-induced MAPK and NF-κB signaling. (*A* and *B*) MDMs were transfected with scrambled, a combination of ERK/p38/JNK (MAPKs), or NEMO siRNA, and then treated with 10 ng/mL TNFSF15. Fold mRNA expression at 4 and 48 hours (n = 6, similar results in an additional n = 6). (*C* and *D*) MDMs were transfected with scrambled, TRADD, combined FADD/MALT1/caspase-8 (F/M/C), or combined TRAF2/RIP1/RIP3 (T/R1/R3) siRNA, and then treated with 10 ng/mL TNFSF15. (*C*) Fold phospho-protein expression at 15 minutes (n = 8 from 2 independent experiments). (*D*) Fold phospho-protein expression at the indicated times (n = 8 from 2 independent experiments). Means + SEM. Significance is between scrambled and the target siRNA for the corresponding TNFSF15-treated cells or as indicated. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. Scr, scrambled; Tx, treatment.

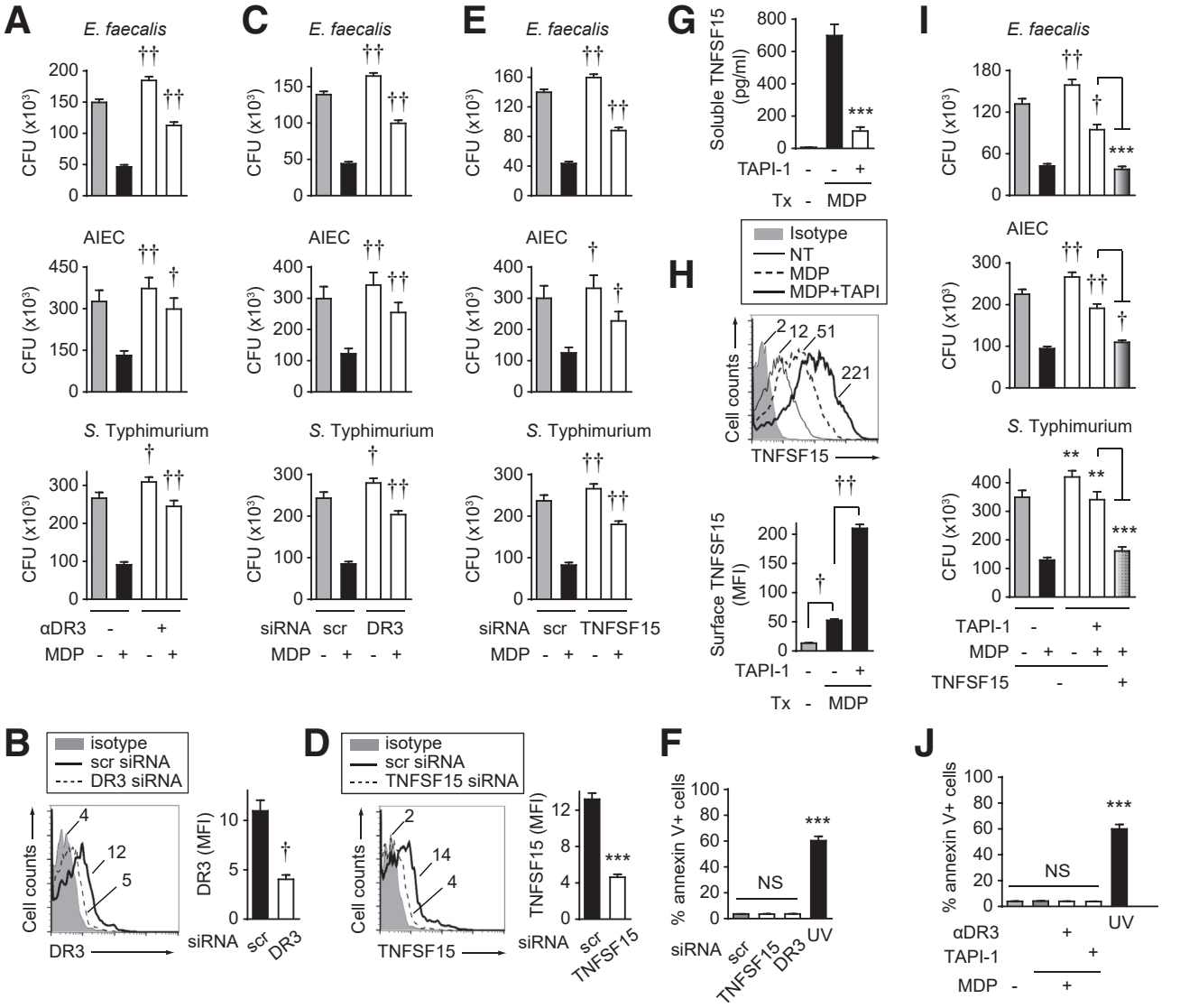

Figure 8. Autocrine/paracrine TNFSF15 is required for optimal levels of NOD2-induced intracellular bacterial clearance. (*A*) MDMs were treated with 100 μg/mL MDP for 48 hours ± neutralizing αDR3 antibodies (or isotype control) (1 hour pretreatment). Intracellular bacterial clearance was assessed (colony-forming units [CFU]) (n = 12 from 2 independent experiments; similar results in an additional n = 4). (*B–F*) MDMs were transfected with scrambled or (*B*, *C*, and *F*) DR3 siRNA or (*D–F*) TNFSF15 siRNA. (*B* and *D*) Expression of the indicated proteins by flow cytometry (n = 8 from 2 independent experiments for DR3; n = 4 for TNFSF15). (*C* and *E*) Cells then were treated with 100 μg/mL MDP for 48 hours and intracellular bacterial clearance was assessed (n = 12 from 2 independent experiments). (*F*) Cell death was assessed by annexin V staining (n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control. (*G–J*) MDMs were treated with 100 μg/mL MDP for 48 hours ± TAPI-1 (inhibits TACE; 1 hour pretreatment) ± 10 ng/mL TNFSF15. (*G*) TNFSF15 secretion (n = 4). (*H*) Cell surface TNFSF15 by flow cytometry with representative flow cytometry and summary graph of mean fluorescence intensity (MFI) (n = 6; similar results in an additional n = 4). (*I*) Intracellular bacterial clearance was assessed (CFU) (n = 8 from 2 independent experiments for *E faecalis* and AIEC with similar results in an additional n = 4; n = 4 for *S* Typhimurium with similar results for an additional n = 4). (*J*) Cell death was assessed by annexin V staining (n = 4). UV stimulation at 50–100 J/m^2 was used as a positive control. Means + SEM. Significance is between isotype and the indicated antibody, scrambled and the target siRNA, or vehicle and inhibitor for the corresponding MDP-treated condition or as indicated. $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. NT, no treatment; scr, scrambled; Tx, treatment.

results upon infection with AIEC and *S* Typhimurium (Figure 8*A*). We confirmed these results through an independent approach with knockdown of DR3 (Figure 8*B* and *C*). We further confirmed these results with knockdown of TNFSF15 (Figure 8*D* and *E*). Cell viability was intact with knockdown of these molecules (Figure 8*F*). Finally, we assessed the role of soluble vs membrane-bound TNFSF15 in mediating NOD2-enhanced microbial clearance. We previously found that NOD2 stimulation activates tumor necrosis factor converting enzyme (TACE), which then cleaves transmembrane TNFSF15 to release soluble TNFSF15.[14] We confirmed that with inhibition of TACE

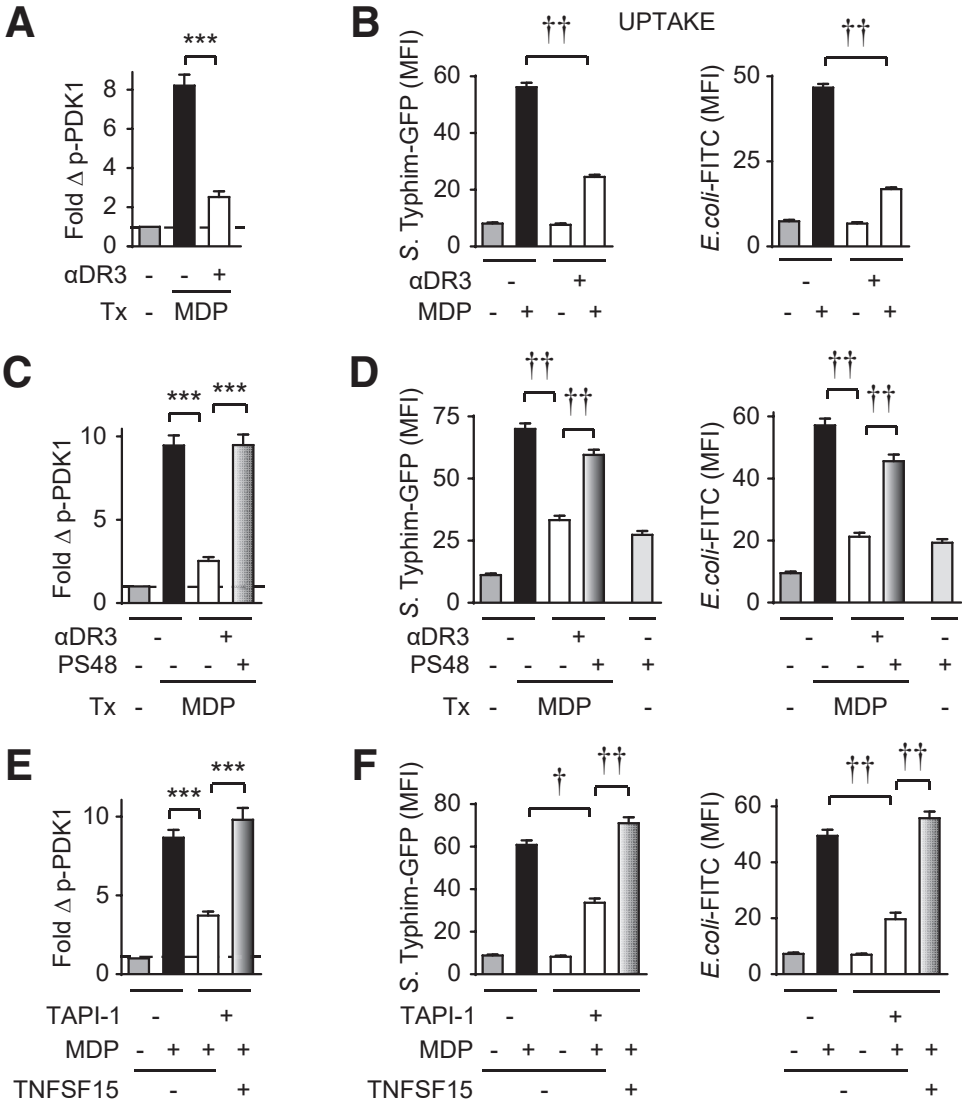

Figure 9. Autocrine/paracrine TNFSF15 promotes NOD2-induced bacterial uptake. (*A* and *B*) MDMs were treated with 100 μg/mL MDP ± neutralizing αDR3 antibodies (or isotype control). (*A*) Fold PDK1 activation at 20 minutes (n = 4). (*B*) After 48 hours, bacterial uptake was assessed (n = 8 from 2 independent experiments). (*C* and *D*) MDMs were treated with 100 μg/mL MDP ± neutralizing αDR3 antibodies ± PS48 (activates PDK1). (*C*) Fold PDK1 activation at 20 minutes (n = 6; similar results in an additional n = 4). (*D*) After 48 hours bacterial uptake was assessed (n = 10 from 2 independent experiments). PS48 alone is shown as a control. (*E* and *F*) MDMs were treated with 100 μg/mL MDP ± TACE ± 10 ng/mL TNFSF15. (*E*) Fold PDK1 activation at 20 minutes (n = 6). (*F*) After 48 hours bacterial uptake was assessed (n = 8 from 2 independent experiments). Means + SEM. ***$P < .001$; †$P < 1 \times 10^{-4}$; ††$P < 1 \times 10^{-5}$. FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; Tx, treatment.

using TAPI-1, NOD2-induced soluble TNFSF15 was reduced (Figure 8*G*), and, consistently, surface TNFSF15 was increased (Figure 8*H*). TACE inhibition led to less-effective intracellular bacterial clearance in baseline and particularly chronic NOD2-stimulated MDMs (Figure 8*I*). Because cell surface TNFSF15 is actually increased under these conditions, these data establish an important role for soluble TNFSF15 in mediating NOD2-induced bacterial clearance. Importantly, complementing soluble TNFSF15 in TAPI-1–treated, NOD2-stimulated MDMs restored intracellular bacterial clearance (Figure 8*I*). Cell viability was intact with TAPI-1 (Figure 8*J*). For the studies that follow in human MDMs, we will focus on blocking autocrine/paracrine TNFSF15 using neutralizing anti-DR3 antibodies or prevent the release of soluble TNFSF15 through TAPI-1–mediated inhibition of TACE. Taken together, these data show that autocrine/paracrine soluble TNFSF15 is required for optimal levels of NOD2-induced bacterial clearance in human MDMs.

Autocrine/Paracrine TNFSF15 Promotes NOD2-Induced Antimicrobial Pathways

We next assessed the contribution of autocrine/paracrine TNFSF15 to NOD2-enhanced antimicrobial pathways, with a focus on the pathways we had found to be induced upon direct TNFSF15 treatment (Figures 1–7). We therefore first examined PDK1-dependent bacterial uptake. Upon blocking autocrine/paracrine TNFSF15 with neutralizing DR3 antibodies, NOD2-induced PDK1 activation (Figure 9*A*) and bacterial uptake (Figure 9*B*) were reduced. Restoration of PDK1 activation with PS48 under these conditions (Figure 9*C*) rescued NOD2-induced bacterial uptake (Figure 9*D*). Moreover, TAPI-1–treated, NOD2-stimulated MDMs showed a similar reduction in PDK1 activation and bacterial uptake, which was restored upon complementation of soluble TNFSF15 (Figure 9*E* and *F*).

We next assessed if the TNFSF15-induced pathways mediating intracellular bacterial clearance were regulated by autocrine/paracrine TNFSF15 upon NOD2 stimulation. Preventing NOD2-induced autocrine/paracrine TNFSF15 by

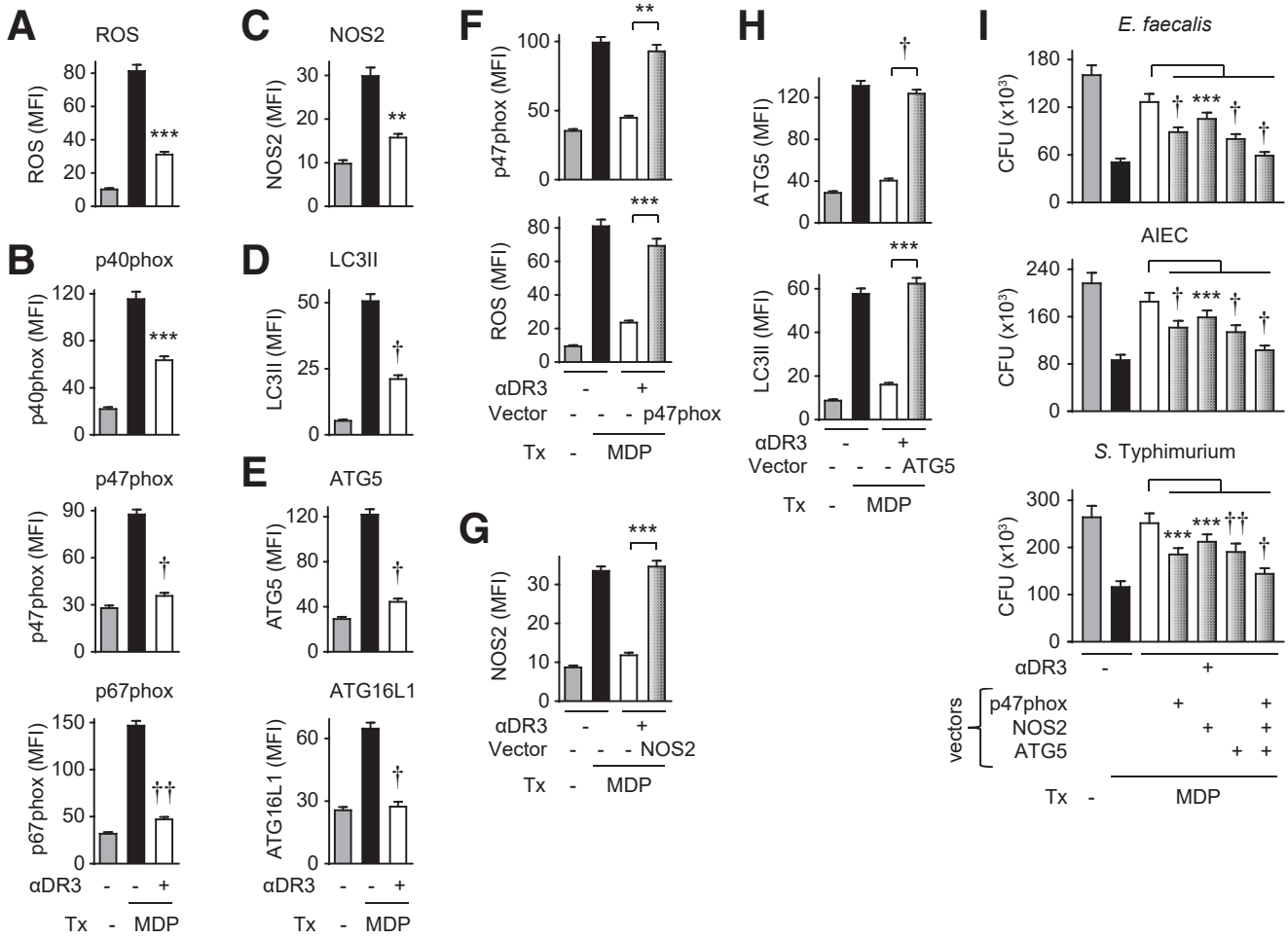

Figure 10. Autocrine/paracrine TNFSF15 promotes NOD2-induced antimicrobial pathways. (*A–E*) MDMs were treated with 100 μg/mL MDP for 48 hours ± neutralizing αDR3 antibodies (or isotype control). (*A*) ROS induction (n = 6; similar results in an additional n = 4). (*B–E*) Expression of the indicated proteins (n = 6; similar results in an additional n = 6). (*F–I*) MDMs were transfected with empty vector or vectors expressing p47phox, NOS2, ATG5, alone or in combination, and treated with 100 μg/mL MDP for 48 hours ± neutralizing αDR3 antibodies (1 hour pretreatment). (*F*) p47phox expression and ROS production. (*G*) NOS2 expression. (*H*) ATG5 and LC3II expression (n = 4, similar results in an additional n = 4). (*I*) Intracellular bacterial clearance (n = 8 from 2 independent experiments). Means + SEM. (*A–E*) Significance is between isotype control and neutralizing αDR3 antibodies for MDP-treated cells. $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. CFU, colony-forming unit; MFI, mean fluorescence intensity; Tx, treatment.

blocking DR3 reduced induction of ROS (Figure 10*A*), NADPH oxidase complex members (Figure 10*B*), NOS2 (Figure 10*C*), autophagy (Figure 10*D*), and autophagy-associated molecules (Figure 10*E*). To clearly establish that each of these TNFSF15-dependent pathways contributed to NOD2-induced intracellular bacterial clearance, we restored each of these pathways in DR3-blocked, NOD2-stimulated cells to the levels observed in the absence of DR3 blockade. In particular, we transfected cells with a vector expressing p47phox to restore ROS production (Figure 10*F*), a vector expressing NOS2 (Figure 10*G*), and a vector expressing ATG5 to restore autophagy (Figure 10*H*). Restoration of each of these pathways partially restored NOD2-enhanced bacterial clearance (Figure 10*I*), and restoration of these pathways in combination restored bacterial clearance even further (Figure 10*I*). Therefore, autocrine/paracrine TNFSF15 is required for NOD2-enhanced antimicrobial pathways and bacterial clearance in human MDMs.

Autocrine/Paracrine TNFSF15-Initiated MAPK and NF-κB Pathways Are Required for NOD2-Induced Antimicrobial Pathways

We had established that TNFSF15 activated MAPK and NF-κB pathways, and that these signaling pathways were required for TNFSF15-dependent intracellular bacterial clearance mechanisms (Figures 6, 7). We confirmed that autocrine/paracrine TNFSF15 was required for NOD2-induced MAPK (Figure 11*A*) and NF-κB (Figure 11*B*) pathway activation. Importantly, restoring ERK, p38, and JNK activation through transfection of vectors expressing constitutively active constructs for each of these molecules in DR3-blocked, NOD2-stimulated MDMs (Figure 11*C*), partially restored ROS, NOS2, and LC3II induction

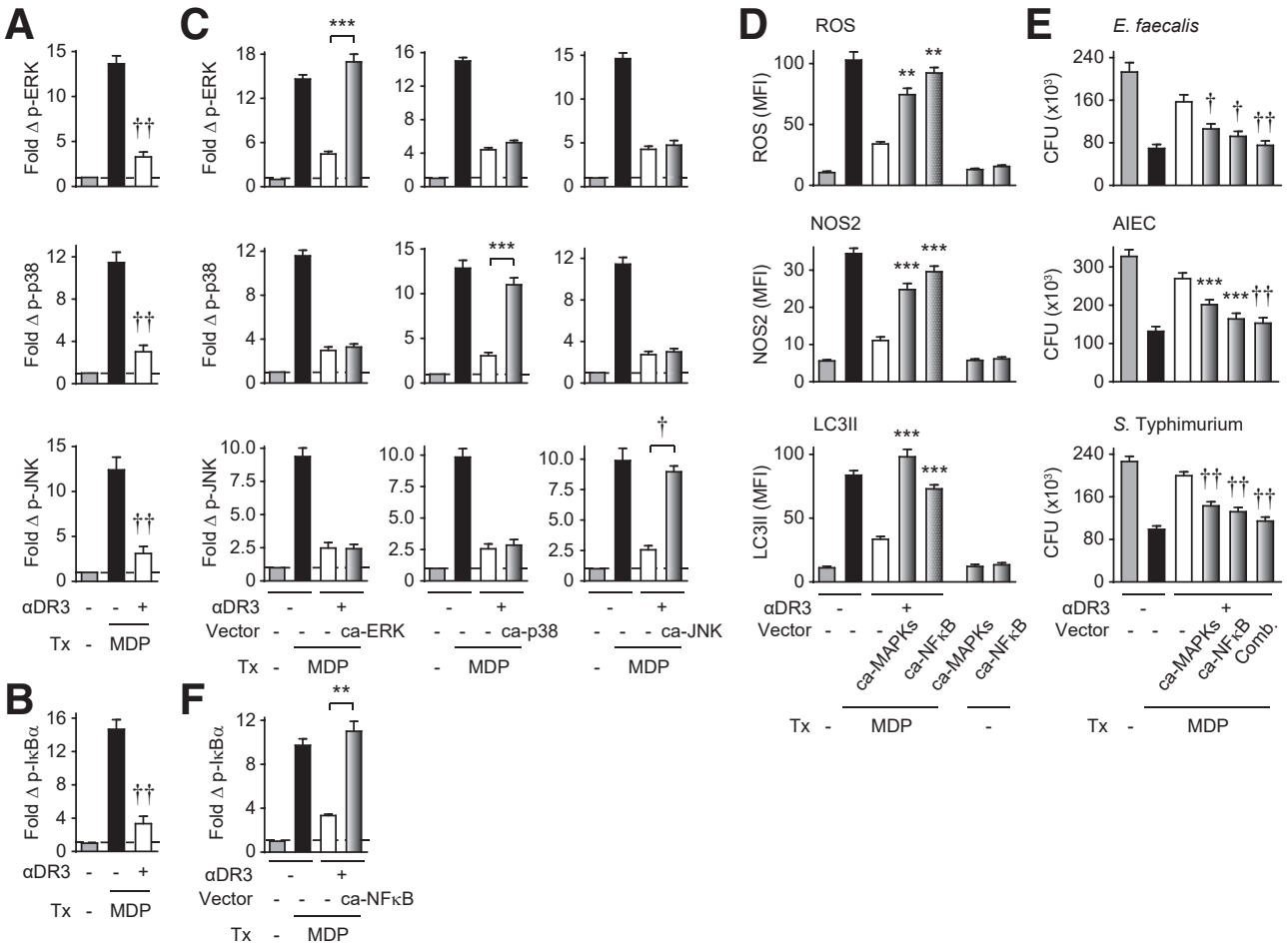

Figure 11. Autocrine/paracrine TNFSF15-initiated MAPK and NF-κB activation promotes NOD2-induced antimicrobial pathways. (*A* and *B*) MDMs were treated with 100 μg/mL MDP ± neutralizing αDR3 antibodies (1 hour pretreatment) (or isotype control). Fold phospho-protein expression (n = 8 from 2 independent experiments). (*C–F*) MDMs were transfected with empty vector or vectors leading to constitutively active ERK, p38, or JNK (ca-MAPK when in combination) or NF-κB (ca-NF-κB), alone or in combination (comb), and treated with 100 μg/mL MDP for 48 hours ± neutralizing αDR3 antibodies. (*C* and *F*) Fold phospho-protein expression (n = 4). (*D*) ROS production and NOS2 or LC3II expression (mean fluorescence intensity [MFI]) (n = 4, similar results in an additional n = 4). (*E*) Intracellular bacterial clearance (colony-forming unit [CFU]) (n = 8 from 2 independent experiments for *E faecalis* and *S* Typhimurium; n = 4 with similar results in an additional n = 4 for AIEC). Means + SEM. (*A* and *B*) Significance is between isotype control to neutralizing αDR3 antibodies for MDP-treated cells or (*C–F*) empty vector–transfected cells to target vector-transfected cells for αDR3-blocked, MDP-treated cells. $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. Tx, treatment.

(Figure 11*D*), and intracellular bacterial clearance (Figure 11*E*). Furthermore, restoring NF-κB activation through transfection of a construct leading to constitutive activation of the NF-κB pathway in DR3-blocked, NOD2-stimulated MDMs (Figure 11*F*) also partially restored each of the antimicrobial pathways (Figure 11*D*) and intracellular bacterial clearance (Figure 11*E*). Restoring MAPK and NF-κB signaling pathways in combination in DR3-blocked, NOD2-stimulated MDMs led to somewhat more effective rescue of intracellular bacterial clearance (Figure 11*E*). Taken together, autocrine/paracrine TNFSF15 promotes NOD2-induced MAPK and NF-κB activation in NOD2-stimulated MDMs, which is turn is required for subsequent induction of the ROS, RNS, and autophagy pathways that mediate NOD2-enhanced intracellular bacterial clearance.

TNFSF15 Can Enhance Intracellular Bacterial Clearance After Prior PRR Stimulation, and Autocrine/Paracrine TNFSF15 Contributes to the Enhanced Bacterial Clearance Observed With Multiple PRRs

Intestinal myeloid cells are exposed to microbial products on an ongoing basis, such that we asked if exposure to TNFSF15 after PRR stimulation, as might occur during an acute response to invasive pathogens, could further enhance intracellular bacterial clearance. We therefore first treated MDMs with MDP and then 24 hours later treated these cells with TNFSF15 (see timeline in Figure 12*A*). In MDP-pretreated MDMs, subsequent treatment with TNFSF15 was able to enhance intracellular

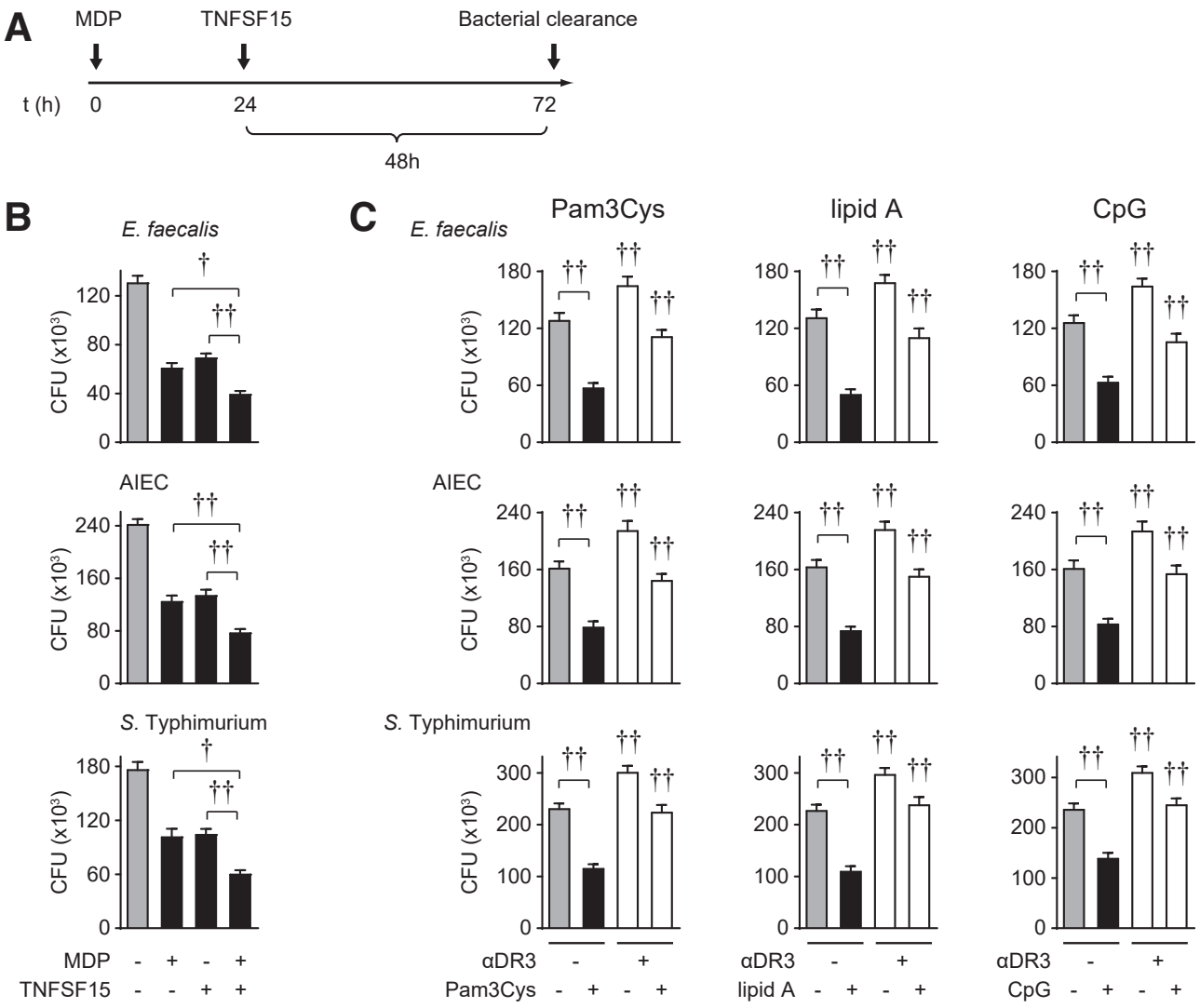

Figure 12. TNFSF15 signaling potentiates NOD2-mediated intracellular bacterial clearance and autocrine/paracrine TNFSF15 promotes bacterial clearance upon stimulation of multiple PRRs. (*A* and *B*) MDMs were pretreated with 100 μg/mL MDP and 24 hours later treated with 10 ng/mL TNFSF15 for an additional 48 hours. (*A*) Timeline schematic for treatment strategy. (*B*) Intracellular bacterial clearance was assessed (colony-forming units [CFU]) (n = 12 from 2 independent experiments). (*C*) MDMs were treated with 10 μg/mL Pam3Cys, 0.1 μg/mL lipid A, or 10 μg/mL CpG for 48 hours ± neutralizing αDR3 antibodies (1 hour pretreatment) (or isotype control), then co-cultured with the indicated bacteria, and intracellular bacterial clearance was assessed (colony-forming units [CFU]) (n = 12 from 2 independent experiments). Significance is between isotype control to neutralizing αDR3 antibodies for the corresponding treatment condition or as indicated. Means + SEM. $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$.

bacterial clearance compared with that observed with either MDP or TNFSF15 treatment alone (Figure 12*B*).

Because intestinal myeloid cells are exposed to multiple PRR ligands, we next assessed if autocrine/paracrine TNFSF15 was required for enhancing bacterial clearance after stimulation of various PRRs. Autocrine/paracrine TNFSF15 promoted the enhanced clearance of *E faecalis*, AIEC, and *S* Typhimurium after chronic stimulation of Toll-like receptor (TLR)2, TLR4, and TLR9 (Figure 12*C*). Therefore, autocrine/paracrine TNFSF15 promotes the enhanced bacterial clearance observed upon chronic stimulation of a range of PRRs and subsequent TNFSF15 exposure further enhances the efficacy of intracellular bacterial clearance of MDMs previously stimulated through PRRs.

Autocrine/Paracrine TNFSF15 Promotes Intracellular Bacterial Clearance in Intestinal Myeloid Cells

To assess if autocrine/paracrine TNFSF15 promotes intracellular bacterial clearance in intestinal myeloid cells, we used mouse systems. We first confirmed that TNFSF15 treatment of mouse bone marrow–derived macrophages (BMDMs) induced secretion of cytokines (Figure 13*A*), and that this was blocked effectively by a neutralizing

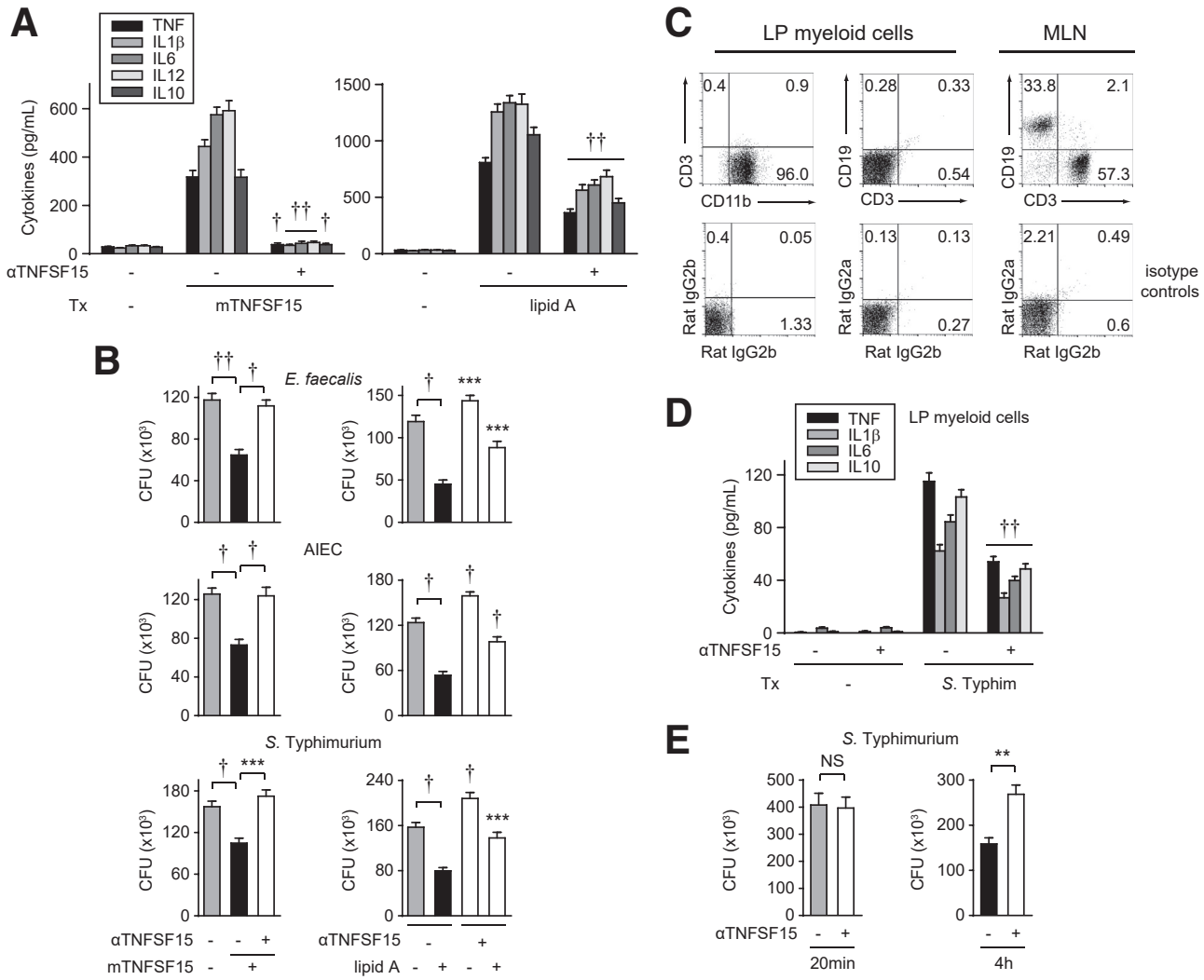

Figure 13. Autocrine/paracrine TNFSF15 promotes intracellular bacterial clearance in mouse BMDMs and intestinal myeloid cells. (*A* and *B*) Mouse BMDMs were treated with 10 ng/mL TNFSF15 or 0.1 μg/mL lipid A ± neutralizing αTNFSF15 antibodies (30 minutes pretreatment) (or isotype control). (*A*) Cytokine secretion after 24 hours (8 replicates, representative of 2 independent experiments). (*B*) Forty-eight hours later intracellular bacterial clearance was assessed (colony-forming units [CFU]) (10 replicates from 2 independent experiments). (*C–E*) Colonic lamina propria (LP) CD11b$^+$ myeloid cells were isolated. (*C*) Representative flow cytometry showing purity of intestinal myeloid cells (percentages indicated). CD19$^+$ and CD3$^+$ cells are minimal, with mesenteric lymph node (MLN) shown as a positive control for these markers. (*D* and *E*) Cells were co-cultured with *S* Typhimurium ± αTNFSF15 antibodies (30 minutes pretreatment) (or isotype control) (n = 7 from 2 independent experiments). (*D*) Cytokine secretion at 12 hours. (*E*) *Left*: CFU were assessed after 20 minutes. *Right*: Gentamicin was added and cells were cultured for an additional 220 minutes (total of 4 hours) (CFU). Significance is between isotype control to neutralizing αTNFSF15 antibodies for the corresponding treatment condition or as indicated. Means + SEM. $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. Tx, treatment.

TNFSF15 antibody (Figure 13*A*). We further confirmed that autocrine/paracrine TNFSF15 promoted the secretion of additional cytokines upon TLR4 stimulation of BMDMs (Figure 13*A*); we examined TLR4 in mouse cells because of the very low level of cytokines observed in mouse cells with NOD2 stimulation. Similar to our observations in human MDMs (Figure 1*A*), prolonged TNFSF15 treatment enhanced intracellular bacterial clearance in mouse BMDMs, and this was blocked effectively by anti-TNFSF15 (Figure 13*B*). Autocrine/paracrine TNFSF15 also promoted the enhanced intracellular bacterial clearance observed after prolonged TLR4 stimulation (Figure 13*B*). We next isolated lamina propria CD11b+ myeloid cells from wild-type mice and confirmed purity (Figure 13*C*). Upon co-culture of intestinal myeloid cells with *S* Typhimurium, autocrine/paracrine TNFSF15 was required for optimal secretion of cytokines (Figure 13*D*), similar to our prior observations in human intestinal myeloid cells.[14] Finally, although uptake of *S* Typhimurium at 20 minutes was not altered, clearance of intracellular bacteria at later times was less effective in intestinal myeloid cells treated with neutralizing anti-TNFSF15 antibodies (Figure 13*E*). Therefore, autocrine/paracrine TNFSF15

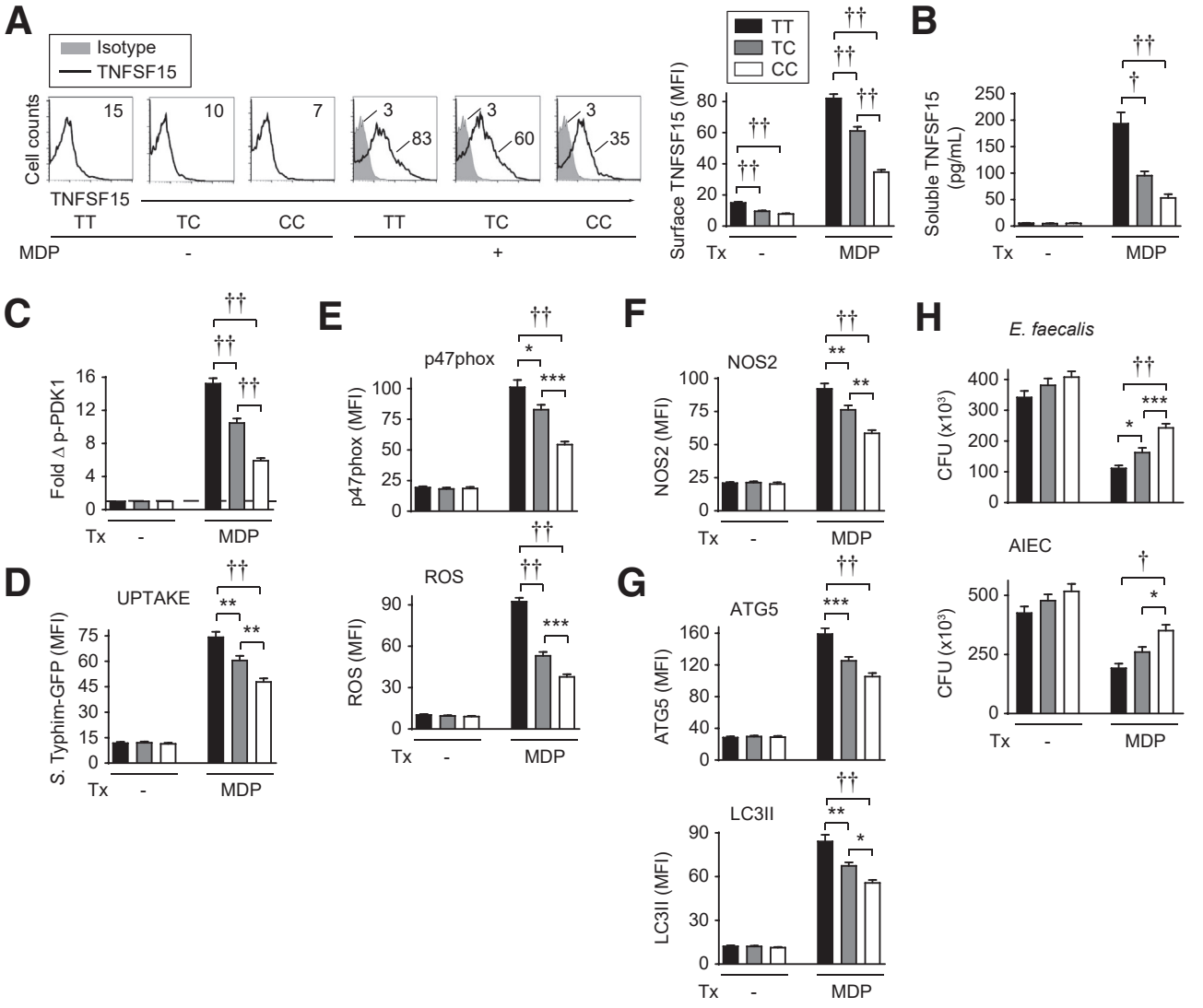

Figure 14. MDMs from high TNFSF15-expressing rs6478108 TT IBD risk carriers show increased NOD2-initiated antimicrobial pathways. MDMs from rs6478108 TT, TC, or CC carriers were left untreated or treated with 100 μg/mL MDP. (*A*) TNFSF15 surface expression by flow cytometry at 24 hours (n = 10 donors/genotype). (*B*) TNFSF15 secretion at 24 hours (n = 10/genotype). (*C*) Fold PDK1 activation at 20 minutes (n = 10/genotype). (*D*) Bacterial uptake at 20 minutes (n = 10/genotype). (*E*) p47phox expression and ROS production at 48 hours (n = 10/genotype). (*F*) NOS2 expression at 48 hours (n = 10/genotype). (*G*) ATG5 and LC3II expression at 48 hours (n = 10/genotype). (*H*) After 48 hours intracellular bacterial clearance was assessed (colony-forming units [CFU]) (n = 10/genotype). Means + SEM. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. MFI, mean fluorescence intensity; Tx, treatment.

promotes cytokines and clearance of intracellular bacteria in mouse BMDMs and intestinal myeloid cells.

MDMs From High TNFSF15-Expressing rs6478108 TT IBD Risk Carriers Show Increased NOD2- and DR3-Initiated Antimicrobial Pathways

Given the ability of TNFSF15 to promote antimicrobial pathways in human MDMs, and the requirement for autocrine/paracrine TNFSF15 in NOD2-enhanced antimicrobial pathways, we next assessed if the various TNFSF15-dependent mechanisms we had identified were regulated by *TNFSF15* IBD risk variants. Rs6478108 is located in an intronic region of *TNFSF15*, and we previously found that myeloid cells from rs6478108 TT risk carriers expressed higher levels of surface and soluble TNFSF15 compared with CC carriers.[14] We confirmed these results here, and these *TNFSF15* genotype differences are particularly pronounced with MDP treatment (Figure 14*A* and *B*). Upon NOD2 stimulation, we observed increased PDK1 activation (Figure 14*C*) and bacterial uptake (Figure 14*D*), increased induction of p47phox and ROS (Figure 14*E*), NOS2 (Figure 14*F*), and ATG5 and autophagy (Figure 14*G*), and increased intracellular bacterial clearance (Figure 14*H*). We observed a similar

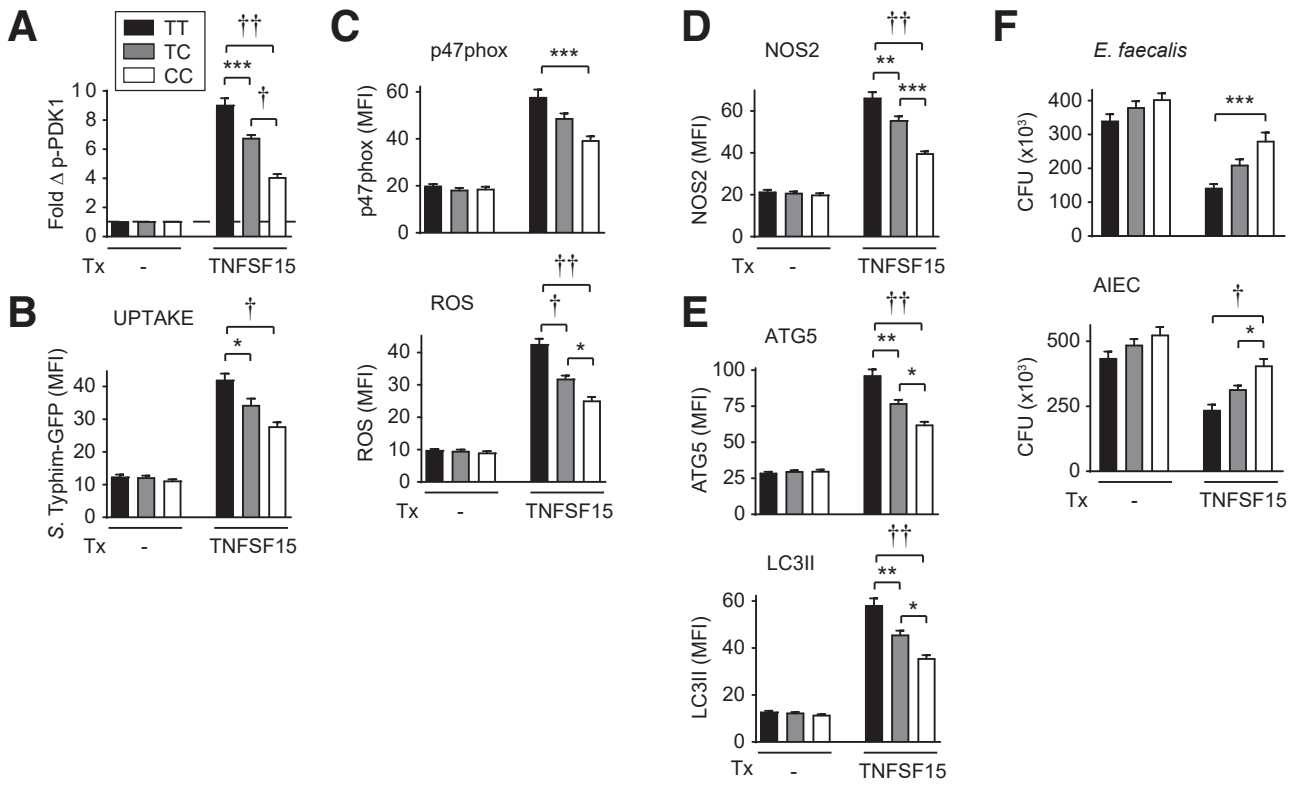

Figure 15. MDMs from high TNFSF15-expressing rs6478108 TT IBD risk carriers show increased DR3-initiated antimicrobial pathways. MDMs from rs6478108 TT, TC, or CC carriers were left untreated or treated with 10 ng/mL TNFSF15. (*A*) Fold PDK1 activation at 20 minutes (n = 10 donors/genotype). (*B*) Bacterial uptake at 20 minutes (n = 10/genotype). (*C*) p47 expression and ROS production at 48 hours (n = 10/genotype). (*D*) NOS2 expression at 48 hours (n = 10/genotype). (*E*) ATG5 and LC3II expression at 48 hours (n = 10/genotype). (*F*) After 48 hours intracellular bacterial clearance was assessed (colony-forming units [CFU]) (n = 10/genotype). Means + SEM. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{\dagger}P < 1 \times 10^{-4}$; $^{\dagger\dagger}P < 1 \times 10^{-5}$. Tx, treatment.

pattern in these antimicrobial mechanisms and outcomes upon TNFSF15 treatment of rs6478108 TT risk carriers compared with CC carriers (Figure 15*A*–*F*). Therefore, MDMs from IBD risk rs6478108 TT carriers show an increase in antimicrobial mechanisms and intracellular bacterial clearance relative to CC carriers.

Discussion

Cytokines can both promote inflammatory diseases and drive pathways to clear microbial challenges. In this study, through independent knockdown and antibody blockade approaches, we show that the disease-associated inflammatory cytokine TNFSF15 promotes antimicrobial pathways in human MDMs. Moreover, TNFSF15–DR3 interactions are required for the enhanced microbial clearance observed with prolonged PRR stimulation, thereby simulating conditions within intestinal tissues. Furthermore, in myeloid cells already conditioned through PRRs, TNFSF15 can further enhance their ability to clear bacteria. TACE cleaves transmembrane TNFSF15 to soluble TNFSF15; the soluble form of TNFSF15 is required for promoting antimicrobial pathways in macrophages. TNFSF15–DR3 interactions require TRAF2/RIP1/RIP3 to initiate PDK1-dependent bacterial uptake and MAPK- and NF-κB–dependent induction of ROS (p40phox, p47phox, p67phox), RNS, and autophagy pathways (ATG5, ATG16L1), which, in turn, regulate intracellular bacterial clearance in MDMs. In contrast, the TNFSF15-initiated FADD/MALT1/caspase-8 pathway is not required for bacterial uptake, and only partially contributes to intracellular microbial clearance mechanisms, despite the key role for this pathway in cytokine secretion. Human myeloid-derived cells carrying the *TNFSF15* rs6478108 TT risk variant express increased TNFSF15 and show increased TNFSF15- and PRR-induced bacterial uptake and intracellular bacterial clearance, along with the TNFSF15-dependent pathways leading to these outcomes, compared with CC carrier MDMs. These studies highlight that although high-expressing TNFSF15 rs6478108 T carriers are at increased risk for IBD, they may be able to clear bacteria more effectively, and, conversely, that although low-expressing *TNFSF15* carriers are relatively protected from IBD, they may be at increased risk for infection (Figure 16).

Despite the important role for TNFSF15–DR3 interactions in regulating inflammatory outcomes, roles for this pathway in microbial clearance have not been well defined. A study showed that $DR3^{-/-}$ mice were unimpaired in their ability to mediate expulsion of intestinal helminths.[49] However, $DR3^{-/-}$ mice were less effective at

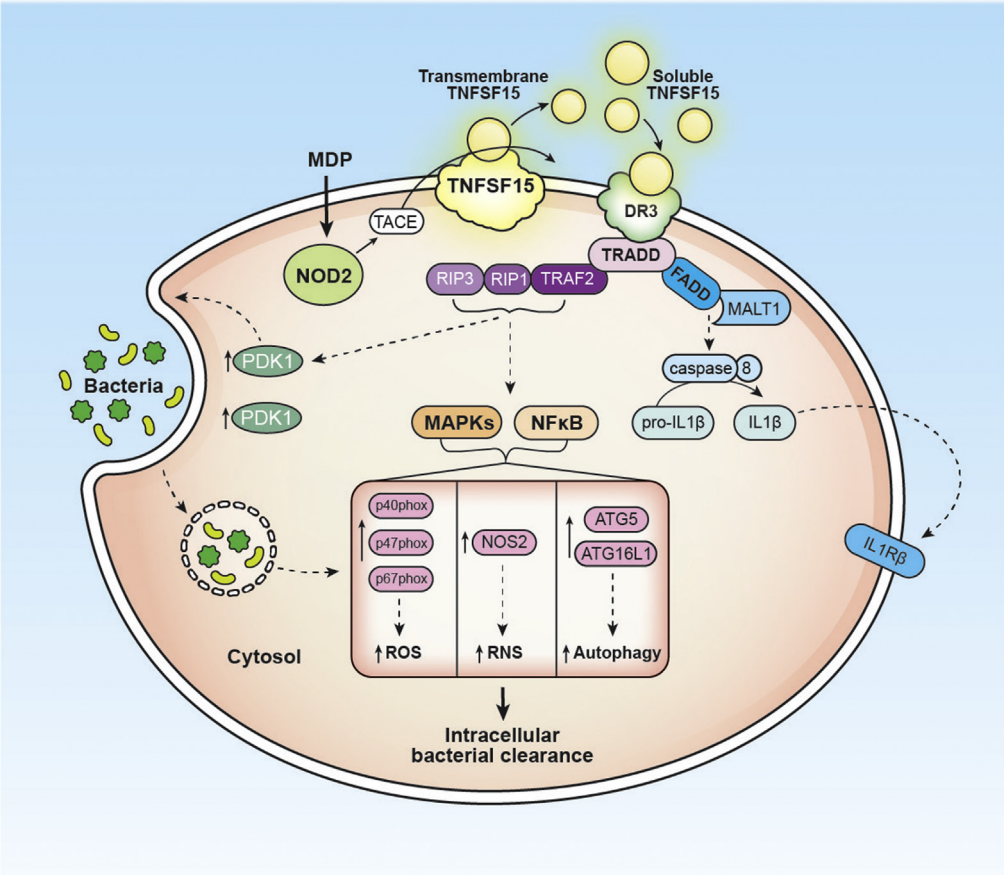

Figure 16. Model of TNFSF15 regulation of antimicrobial pathways. Upon PRR stimulation of macrophages, TACE is activated and leads to the release of soluble TNFSF15. Soluble TNFSF15 then feeds back to interact with DR3 on macrophages to initiate TRAF2/RIP1/RIP3 and FADD/MALT1/caspase-8 signaling pathways. TRAF2/RIP1/RIP3 pathway activation leads to PDK1-dependent bacterial uptake and MAPK- and NF-κB–dependent up-regulation of ROS (p40phox, p47phox, p67phox), RNS, and autophagy (ATG5, ATG16L1) pathways, which promote intracellular bacterial clearance. MDMs from high TNFSF15-expressing rs6478108 TT IBD risk carriers in the *TNFSF15* region show increased efficacy in inducing these antimicrobial pathways, and, conversely, MDMs from low TNFSF15-expressing rs6478108 CC IBD risk carriers clear bacteria less effectively.

regulating viral[50] and bacterial[32,33] infection, with the focus of DR3 being on its role on T cells in reducing bacterial burden.[32] We now show a previously undefined role for TNFSF15 in promoting antimicrobial pathways in human macrophages. Interestingly, the 2 signaling pathways initiated upon TNFSF15 interactions with DR3 show distinct roles in this process. TNFSF15-initiated activation of TRAF2/RIP1/RIP3 results in rapid PDK1 activation, which leads to bacterial uptake, and early MAPK and NF-κB activation, which leads to multiple antimicrobial pathways, including ROS, RNS, and autophagy pathways. These antimicrobial pathways cooperate to mediate intracellular bacterial clearance. In contrast, although TNFSF15-initiated activation of the FADD/MALT1/caspase-8 pathway leads to caspase-8 processing, subsequent IL1β processing, and early IL1β secretion, which functions in an autocrine/paracrine manner to promote secretion of additional cytokines, we now find this pathway arm does not play a role in bacterial uptake and only minimally contributes to intracellular bacterial clearance. This may in part be owing to the delayed activation of the MAPK and NF-κB pathways associated with the time required for autocrine/paracrine IL1β to initiate these antimicrobial pathways relative to what is observed downstream of the TRAF2/RIP1/RIP3 pathway.

The rs6478108 T allele is the major allele, such that the majority of individuals are disease risk carriers. The increased levels of inflammatory cytokines secreted in high TNFSF15-expressing IBD risk carriers upon encounter with microbial products likely is 1 mechanism contributing to the increased susceptibility to IBD. At the same time, this more robust response in macrophages from *TNFSF15* region IBD risk carriers simultaneously results in more effective intracellular bacterial clearance. Conversely, this would imply that the *TNFSF15* variants with reduced risk for immune-mediated diseases may be disadvantageous in select infectious diseases. Whether common genetic variants confer a slight increase in susceptibility to transient bacterial infections can be difficult to assess and track[51] and therefore is not as well studied as how these variants affect chronic immune-mediated diseases or chronic infectious diseases. Targeting of tumor necrosis factor (TNF) family members has

been a mainstay in immune-mediated diseases, and modulating TNFSF15–DR3 interactions is currently in clinical trials for select inflammatory diseases, including IBD. Our studies highlight that therapeutic blockade of TNFSF15 may increase the risk for infection and that rs6478108 C carriers with reduced TNFSF15 expression are at reduced risk for IBD but may be at increased risk for infection.

Materials and Methods

Donor Recruitment and Genotyping

Human cell studies were conducted as approved by the Yale University Institutional Review Board. Genotyping for polymorphisms was conducted by TaqMan (Life Technologies, Grand Island, NY).

Mouse Studies

C57BL/6 mice were maintained in a specific pathogen–free facility and used at approximately 3 months of age. Experiments were performed in accordance with Yale University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Table 1. Primer Sequences

Gene	Primer sequences
TRADD	5'-CGTGGGGCTGGGTAGTTGTCTCCA-3' 5'-CAGGCCACCCCTTCCTCTCCACAT-3'
FADD	5'-CGCAGCCCCGGCCGCTTGCAGACC-3' 5'-GGGAGGCGCTGGAGGCCAACCG-3'
MALT1	5'-GCCTCAGTTGCCTAGAC-3' 5'-CCAAGACTGCCTTTGAC-3'
Caspase 8	5'-TGCTGAGCACGTGGAGTTAG-3' 5'-GCAGGAGAATATAATCCGCTCCA-3'
TRAF2	5'-GGAGGCATCCACCTACGATG-3' 5'-GGGAGAAGATGGCGGGTATG-3'
RIP1	5'-AGTCCTGGTTTGCTCCTTCCC-3' 5'-GCGTCTCCTTTCCTCCTCTCTG-3'
RIP3	5'-CTCTCTGCGAAAGGACCAAG-3' 5'-TCGTAGCCCCACTTCCTATG-3'
p40phox	5'-ATGGCTGTGGCCCAGCAGCT-3' 5'-CTCCTGTTTCACACCCACGTAG-3'
p47phox	5'-AGTACCGCGACAGACATCAC-3' 5'-CGCTCTCGCTCTTCTCTACG-3'
p67phox	5'-TGCCAAAAGGTGGGGACATT-3' 5'-CAAGAGAGCTGCCAGGAGAC-3'
NOS2	5'-CGCAGAGAACTCAGCCTCAT-3' 5'-TGCCTTGAGAACTTCGGGAC-3'
ATG5	5'-TTTGCATCACCTCTGCTTTC-3' 5'-TAGGCCAAAGGTTTCAGCTT-3'
ATG16L1	5'-GCATGACGTACCAAACAGGC-3' 5'-AGTTGAGCTAACTCCCCACG-3'
IL8	5'-ATGACTTTCAAGCTGGCCGTGGCT-3' 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'
IL12p40	5'-AGGGACATCATCAAACCTGACC-3' 5'-GCTGAGGTCTTGTCCGTGAA-3'
GAPDH	5'-TGCACCACCAACTGCTTAGC-3' 5'-GGCATGGACTGTGGTCATGAG-3'

Primary Human Myeloid Cell Culture

Human peripheral blood mononuclear cells were isolated from peripheral blood using Ficoll-Paque (GE Dharmacon, Lafayette, CO). Monocytes were purified from peripheral blood mononuclear cells by adhesion and differentiated to MDMs for 7 days with 10 ng/mL macrophage colony-stimulating factor (Shenandoah Biotechnology, Warwick, PA) as described by Hedl et al.[52]

Messenger RNA Expression

Total RNA was isolated, reverse transcribed, and quantitative polymerase chain reaction was performed with normalization to glyceraldehyde-3-phosphate dehydrogenase. Primers are listed in Table 1.

Myeloid Cell Stimulation

Human MDMs were treated with MDP (Bachem, Torrance, CA), Pam3Cys (MilliporeSigma, Burlington, MA), lipid A (Peptides International, Louisville, KY), CpG DNA (InvivoGen, San Diego, CA), or TNFSF15 (Peprotech, Rocky Hill, NJ). In some cases, cells were given neutralizing anti-DR3 antibodies (1 μg/mL; JD3; ThermoFisher Scientific, Waltham, MA) for 1 hour before treatments. In other cases, cells were given 10 μmol/L TAPI-1 (MilliporeSigma) for 1 hour before treatments. Supernatants were assayed for cytokine secretion per the manufacturer's instructions using antibodies to the following proteins: TNFSF15 (Peprotech), TNF (Mab1 and Mab11), IL6 (MQ2-13A5 and MQ2-39C3), IL8 (G265-5 and G265-8), IL10 (JES3-9D7 and JES3-12G8) (BD Biosciences, San Jose, CA), or IL1β (CRM56 and CRM57) (eBioscience, San Diego, CA).

Flow Cytometry

Intracellular proteins were determined by flow cytometry using antibodies against phospho-PDK1 (C49H2), phospho-ERK (197G2), phospho-p38 (28B10), phospho-JNK (G9), ERK1/2 (137F5), ATG16L1 (D6D5), cleaved caspase-8 (11G10), cleaved IL1β (D3A3Z) (Cell Signaling Technology, Danvers, MA), or phospho-IκBα (B-9), p38 (A-12), JNK (D-2), NEMO (F-10), ATG5 (C-1), LC3B (G-9), p40phox (D-8), p47phox (A-7), p67phox (D-6), and NOS2 (C-11) (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies for cell surface expression in human MDMs included TNFSF15 (Tandys1a) or DR3 (JD3) (ThermoFisher Scientific), and in mouse cells included CD3 (17A2), CD19 (1D3/CD19) (Biolegend, San Diego, CA), and CD11b (M1/70) (eBioscience).

Transfection of siRNAs and Plasmids

Primary human MDMs were transfected with 100 nmol/L scrambled or ON-TARGETplus or siGENOME SMARTpool siRNA against DR3, TNFSF15, p40phox, p47phox, p67phox, NOS2, ATG5, ATG16L1, ERK, p38, JNK, NEMO, TRADD, FADD, MALT1, caspase-8, TRAF2, RIP1, RIP3 (Dharmacon) (4 pooled siRNAs for each gene) for 48 hours and studies then were conducted. MDMs were transfected with the following vectors: 2 μg vectors expressing ATG5 (Addgene plasmid 24922; kindly deposited by Toren Finkel[53]), NOS2

(a generous gift from Tony Eissa[54]), p47phox (generous gift from Celine DerMardirossian[55]), 4 μg pMCL-MKK1 (R4F) (constitutively active ERK kinase),[56] 4 μg pSRα-3HA-JNKK2-JNK1-WT (constitutively active JNK)[57] (generous gifts from Dr Ben Turk), 4 μg pCDNA3-Flag MKK6(glu) (constitutively active p38 kinase)[58] (Addgene plasmid 13518; kindly deposited by Roger Davis), 2 μg IKK-2 S177E S181E (constitutively active NF-κB) (Addgene plasmid 11105; kindly deposited by Anjana Rao[59]), or empty vector (a control vector that does not express genes) using Amaxa nucleofector technology (Lonza, Walkersville, MD).

Intracellular ROS Measurement

Intracellular ROS production was measured by flow cytometry using 10 μmol/L cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (ThermoFisher Scientific).

Bacterial Entry

Macrophages were co-cultured with 2.5 $\times$ 10^7/mL *E coli*–fluorescein isothiocyanate (FITC) bioparticles (ThermoFisher Scientific) or at 10:1 multiplicity of infection with live bacteria *S* Typhimurium-GFP (kindly provided by Jorge E. Galan) for 20 minutes. Cell surface fluorescence was quenched with 0.25 mg/mL trypan blue for 1 minute, and after 4% paraformaldehyde fixation cells were analyzed by flow cytometry. In some cases, a PDK1 agonist (12 μmol/L PS 48; Santa Cruz Biotechnology) or a PDK1 inhibitor (3 μmol/L GSK 233470; Tocris, Briston, United Kingdom) were added.

Intracellular Bacterial Clearance

Human MDMs were infected with *E faecalis*, AIEC (a generous gift from Dr E. Mizoguchi), or *S* Typhimurium at 10:1 multiplicity of infection for 20 minutes washed with phosphate-buffered saline, and incubated in Hank's balanced salt solution with 20 μg/mL gentamicin for a total of 2 hours. Cells were washed, lysed with 1% Triton X-100 (MilliporeSigma), and plated on MacConkey or Luria-Bertani agar.

Generation of Mouse Bone Marrow–Derived Macrophages and Stimulation

Bone marrow cells were cultured in Dulbecco's modified Eagle medium containing 10% L929-conditioned medium and used at 6–8 days. Cells were treated with 10 ng/mL recombinant TNFSF15 (Biolegend) or lipid A (Peptides International). In some cases, cells were treated first with neutralizing anti-TNFSF15 antibodies (2 μg/mL, 5G4.6; Bio X Cell, Lebanon, NH) for 30 minutes. Supernatants were assayed for cytokines as follows: TNF (6B8 and MP6-XT22), IL6 (MP5-20F3 and MP5-32C11), IL12/p40 (C15.6 and C17.8), IL10 (JES5-2A5 and JES5-16E3), IL1β (B122) (Biolegend), and biotin-IL1β (eBioscience).

Intestinal Lamina Propria Cell Isolation

Colonic lamina propria cells were isolated as previously described[60] and myeloid cells then were purified with CD11b beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Statistical Analyses

Significance was assessed using a 2-tailed Student *t* test along with a Bonferroni–Holm correction for multiple comparisons where appropriate. A 1-way analysis of variance with a Tukey post-test was used for comparing means across genotypes. A *P* value less than .05 was considered significant.

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