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Nod2-Induced Autocrine Interleukin-1 Alters Signaling by ERK and p38 to Differentially Regulate Secretion of Inflammatory Cytokines

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Abstract

BACKGROUND&AIMS—Stimulation of nucleotide-binding oligomerization domain-containing (Nod)2 and other pattern recognition receptors (PRR) in human monocyte-derived macrophages induces interleukin (IL)-1, which increases mitogen-activated protein kinase (MAPK) activation and cytokine secretion. Activation of MAPK by different PRR has varied effects on inflammatory cytokine secretion. We investigated whether different levels of autocrine IL-1 mediate these varied effects.

METHODS—Macrophage responses to PRR ligands were analyzed by enzyme-linked immunosorbent assay and flow cytometry. We overexpressed or reduced MAPK levels (using small inhibitory RNA).

RESULTS—Nod2 and other PRR activated signaling via extracellular signal-related kinase (ERK) and p38 that inhibited inflammatory cytokine production by human monocyte-derived macrophages; autocrine IL-1 production prevented this inhibition. ERK and p38 inhibited inflammatory cytokine production by human macrophages that produce low levels of IL-1 (such as M2, endotoxin-tolerant, and intestinal macrophages); adding exogenous IL-1 caused ERK and p38 to stimulate production of inflammatory cytokines in these cells. In mouse macrophages, which do not produce IL-1 in response to PRR stimulation alone, addition of exogenous IL-1 reversed the ERK-mediated inhibition of IL-12p40. Increasing activation of c-Jun N-terminal kinase in Nod2-stimulated human monocyte-derived macrophages, in the absence of autocrine IL-1 signaling, caused ERK and p38 to stimulate inflammatory cytokines secretion. Infection of human intestinal macrophages with pathogens that induce IL-1 production reversed the inhibition of inflammatory cytokine production by ERK and p38.

CONCLUSIONS—In response to PRR stimulation of macrophages, the level of MAPK signaling is regulated by autocrine IL-1 and determines whether production of inflammatory cytokines is inhibited or stimulated. This mechanism could account for reported differences in MAPK regulation of inflammatory cytokines and propagate the inflammatory response to pathogens.

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Keywords

TLR; IBD; Salmonella

Introduction

Nucleotide-binding oligomerization domain 2 (*Nod2*) polymorphisms confer the strongest genetic risk toward Crohn's disease (CD), characterized by uncontrolled intestinal inflammation.¹ How *Nod2* polymorphisms mediate CD is incompletely understood. *Nod2* stimulation by the bacterial peptidoglycan component muramyl dipeptide (MDP) induces NF- κ B- and MAPK-mediated cytokine secretion in human and mouse myeloid-derived cells.^{1–6} Proper anti- versus pro-inflammatory cytokine regulation is critical for intestinal immune homeostasis; however, how specific MAPK regulate *Nod2*-mediated pro- and anti-inflammatory cytokine secretion is unclear. This regulation is the focus of the current study. *Nod2* stimulation induces the pro-inflammatory cytokine IL-1 β ;^{3,4,7–9} we recently found that autocrine IL-1 contributes to MAPK activation in MDM,⁸ which, subsequently amplifies PRR-induced cytokines.^{8,10} It is, however, unknown if IL-1 modifies the MAPK-mediated consequences of *Nod2* and other PRR by additional mechanisms, particularly with respect to pro- and anti-inflammatory cytokine secretion.

Cytokine regulation by TLR4-activated MAPK has been controversial.^{11–16} Some,^{11,14,16} but not all^{12,17} macrophage studies show that LPS-activated p38 induces specific pro-inflammatory cytokines. Unlike mouse macrophages where TLR4-induced ERK generally downregulates pro-inflammatory cytokines,^{12,16} ERK induces TNF- α and IL-1 β in human myeloid cells.^{11,13} Mouse macrophages do not secrete IL-1 upon PRR stimulation alone,¹⁸ whereas multiple studies show that human cells do.^{8,19,20} We therefore hypothesized that MAPK-mediated pro- and anti-inflammatory cytokine regulation in human and mouse is controversial partially due to autocrine IL-1 masking direct PRR effects.

Given the *Nod2* relevance to human disease, and differences between primary cells, cell lines and species, we utilized primary human MDM. Whereas MDP-activated JNK, ERK and p38 induced pro-inflammatory cytokines, we found that upon blocking autocrine IL-1 during *Nod2* stimulation, ERK and p38, in fact, downregulated pro-inflammatory cytokines. In contrast, protein kinase C (PKC) and phospholipase A (PLA) signaling upregulated *Nod2*-induced pro-inflammatory cytokines regardless of whether autocrine IL-1 was present or absent. Therefore, we identify autocrine IL-1 as a novel mechanism switching *Nod2*-activated ERK and p38 from inhibiting to stimulating pro-inflammatory cytokines. Consistently, in LPS-stimulated mouse bone-marrow-derived macrophages (BMDM), where IL-1 β is not secreted,¹⁸ ERK inhibits pro-inflammatory IL-12p40 secretion.¹⁶ Adding IL-1 β during LPS stimulation reverses ERK-mediated IL-12p40 inhibition, simulating human MDM regulation; this elucidates a novel mechanism regulating differences in mouse and human IL-12p40 induction. Furthermore, we define that autocrine IL-1 reverses ERK- and p38-mediated pro-inflammatory cytokine inhibition by increasing JNK strength of signaling. Autocrine IL-1 similarly masks *Nod1*-, TLR2- and TLR4-mediated pro-inflammatory cytokine regulation. Moreover, we find that in macrophage subsets with minimal PRR-induced IL-1 secretion, including M2 macrophages, endotoxin tolerant macrophages and intestinal myeloid cells, ERK and p38 inhibit pro-inflammatory cytokines. Exogenous IL-1 switches this regulation to stimulation of pro-inflammatory cytokines. Importantly, pathogens stimulating IL-1 secretion from intestinal macrophages switch ERK- and p38-mediated inhibition to induction of pro-inflammatory cytokines, thereby disrupting the 'tolerant' intestinal macrophage phenotype. We therefore elucidate novel mechanisms in PRR and IL-1R interactions and identify that the strength of MAPK signaling dramatically

alters the direction of pro-inflammatory cytokine regulation, with important consequences in intestinal macrophages.

Materials and Methods

Recruitment and genotyping

Informed consent was obtained as approved by the Yale University institutional review board. We performed Nod2 genotyping by TaqMan (Applied Biosystems, Foster City, CA). We utilized cells from WT Nod2 healthy individuals, and WT or Leu1007insC Nod2 homozygote/compound heterozygote CD patients where indicated.

Mice

Experiments involving Balb/c and C57BL/6 mice (The Jackson Laboratory) were performed according to our institutional animal care and use committee and the NIH animal use guidelines.

Human and mouse cell culture

Human MDM and mouse BMDM were generated as in Hedl et al⁴ and Chi et al,¹⁵ respectively. MDM were stimulated for 24h with 100ng/ml LPS (Sigma-Aldrich, St. Louis, MO) and 20ng/ml IFN- γ (R&D Systems, Minneapolis, MI) (M1 polarization) or 20ng/ml IL-4 (R&D Systems) (M2 polarization). Mouse BMDM, RAW264.7 and J774 cells were treated as indicated. Myeloid cells (CD11c purity >75%) were isolated as in⁴ from colonic resection specimens from uninvolved intestine in 12 non-inflammatory bowel disease patients undergoing surgery for diverticular disease or colon cancer.

Cell stimulation

IL-1Ra (GenScript, Piscataway, NJ), apyrase (NEB Biolabs, Ipswich, MA), JNK inhibitor II, PD98059, SB202190 (Calbiochem), or DMSO control were added prior to treatments with MDP (Bachem, King of Prussia, PA), IL-1 β (eBioscience, San Diego, CA), lipid A (Peptides International, Louisville, KY), Pam₃Cys (Calbiochem, LaJolla, CA), TriDAP (Invivogen), ATP (Sigma-Aldrich) and *Salmonella enterica serovar Typhimurium* (*S. typhimurium*) or adherent invasive *E. coli* (AIEC) strain LF82 (generous gift from Dr. Emiko Mizoguchi), at multiplicity of infection (MOI) 1:1. Supernatants were assayed for human IL-1 β (Pierce Biotechnology, Rockford, IL), TNF- α , IL-8, IL-6 (BD Biosciences), IL-1Ra, IL-10 and IL-12p40 (R&D Systems) or for mouse TNF- α , IL-1 β , IL-10, IL-6 and IL-12p40 (eBioscience), MIP2 and IL-1Ra (R&D Systems) by ELISA.

MAPK activation

MAPK activation was determined by flow cytometry using Alexa 647-labeled phospho-c-Jun, phospho-ERK, phospho-MAPKAP2, phospho-JNK or phospho-p38 (Cell Signaling, Danvers, MA).

Transfection of small interfering RNAs (siRNAs) and JNK overexpressing plasmid

2.5nM scrambled or ON-TARGETplus SMARTpool siRNA against JNK1, ERK1, ERK2, or p38 α (Dharmacon, Lafayette, CO) (four pooled siRNAs for each gene) or 5 μ g pSR α -3HA-JNKK2-JNK1-WT (expressing constitutively active JNK) (generous gift from Dr. Ben Turk),²¹ or empty vector were transfected into MDM using Amaxa nucleofector technology (Amaxa, San Diego, CA).

mRNA expression

RNA isolation, reverse transcription and qPCR were performed as in². Samples were normalized to GAPDH. Primer sequences are available upon request.

Statistical analysis

Significance was assessed using a paired two-tailed Student t test. $p < 0.05$ was considered significant. In experiments involving multiple interventions we performed multiple comparison analysis utilizing the Holm-Bonferroni correction.

Results

JNK, ERK and p38 mediate cytokine induction upon stimulation with multiple PRR ligands in human MDM

Given the controversy surrounding ERK and p38-mediated regulation of pro- and anti-inflammatory cytokines,^{11,13,14,16} we investigated MAPK contributions to the PRR-induced pro-inflammatory cytokines TNF- α , IL-8 and IL-6 and the anti-inflammatory mediators IL-10 and IL-1Ra in primary human MDM. JNK1, ERK1 and p38 α isoforms are widely expressed and essential for cytokine induction.²² We therefore decreased their expression utilizing siRNA. Inhibition to 14%, 13%, and 9% of MDP-induced JNK, ERK, and p38 activation, respectively, was observed (Fig 1A). We used MDP concentrations approximating stool levels of muramic acid.²³ We find that decreased JNK1, ERK1 and p38 α expression attenuates both pro- and anti-inflammatory cytokines following MDP stimulation (Fig 1B&C). Nod1, TLR2 and TLR4 ligand stimulation had similar outcomes (Fig 1C). Paralleling the siRNA results, pharmacological JNK, ERK and p38 inhibitors decreased PRR-induced cytokines (Supplementary Fig 1A&B). We and others have commonly used these inhibitors at 1–50 μ M doses.^{8,11–16} In a dose response determination in MDM (data not shown), we found that 0.1 μ M of each inhibitor selectively and almost completely inhibited PRR-induced activation of the appropriate MAPK as assessed by the loss of phosphorylation of the downstream substrates c-Jun, ERK and MAPKAP2 (Supplementary Fig 2). We therefore utilized this low dose to minimize non-specific effects; viability was similar to untreated cells (per cell counts and annexin/propidium iodide staining). Unlike MDM from healthy controls (Fig 1B) and Nod2 WT CD patients (Fig 1D), MDP-stimulated Nod2 Leu1007insC carrier cells fail to produce pro- and anti-inflammatory cytokines,^{4,7,8,24} including IL-1 (Fig 1D). These cells, however, respond normally to TLR4 stimulation (Fig 1D). Therefore, JNK, ERK and p38 are required for pro- and anti-inflammatory cytokine secretion upon stimulation of multiple PRR in MDM.

Autocrine IL-1 masks ERK and p38-mediated differential cytokine regulation downstream of Nod2-initiated signaling

We did not detect differential MAPK regulation of pro- and anti-inflammatory cytokines in PRR-stimulated MDM, which has been described in some, but not all, studies upon TLR4 and TLR9 stimulation in various cell types.^{11–16} This might reflect species and cell type differences, or differential contributions of PRR-induced autocrine cytokines, including IL-1. Specifically, PRR-stimulated mouse macrophages require additional signals (e.g. ATP) to secrete IL-1.¹⁸ In contrast, PRR ligand stimulation alone induces IL-1 β in human MDM in multiple studies.^{8,19,20} Because we have identified autocrine IL-1 contributions to PRR-activated MAPK in primary MDM,⁸ we considered that autocrine IL-1 might modify differential pro- and anti-inflammatory cytokine regulation by specific MAPK. We therefore investigated how JNK, ERK and p38 regulate Nod2-induced cytokine secretion in the absence of autocrine IL-1. PRR-mediated IL-1Ra secretion (Fig 1B&C) naturally antagonizes IL-1R.²⁵ We therefore suppressed MAPK through independent pharmacological

and siRNA approaches, verified downregulation efficacy and specificity, and examined cytokine secretion upon: a) Nod2-initiated signaling (MDP stimulation), b) IL-1R signaling (IL-1 β stimulation) and c) Nod2 signaling in the absence of autocrine IL-1 (MDP stimulation upon IL-1Ra addition and termed IL-1R-independent Nod2 signaling).

We found that pharmacological inhibition (Fig 2A) or siRNA-mediated knockdown (Fig 1B&C, data not shown) of JNK, ERK or p38 decreased secretion of all cytokines assessed upon MDP and IL-1 stimulation. In the absence of autocrine IL-1, JNK inhibition (Fig 2A&B) or knockdown (Fig 3A) decreased both pro- and anti-inflammatory cytokine secretion. In contrast, inhibition (Fig 2A&B) or knockdown (Fig 3A) of either ERK or p38 decreased anti-inflammatory but increased pro-inflammatory cytokine secretion upon MDP stimulation in MDM in the absence of autocrine IL-1. Similar results were observed with alternative ERK and p38 inhibitors (Supplementary Fig 3). Furthermore, silencing of ERK2 (Fig 3B), which induces cytokines in myeloid cells,²² increased pro-inflammatory, but decreased anti-inflammatory cytokines following MDP stimulation in the absence of autocrine IL-1 (Fig 3C). Therefore, Nod2-induced autocrine IL-1 masks ERK and p38-mediated differential pro- and anti-inflammatory cytokine regulation.

Autocrine IL-1 masks differential anti- and pro-inflammatory cytokine regulation upon activation of multiple PRR

We previously found that autocrine IL-1 significantly contributes to not only Nod2-, but also Nod1-, TLR2- and TLR4-mediated cytokine secretion in MDM.⁸ We therefore questioned if autocrine IL-1 masked ERK- and p38-mediated differential pro- and anti-inflammatory cytokine regulation by these other PRR. Interestingly, similar to Nod2, autocrine IL-1 switched ERK and p38-signaling from inhibiting to stimulating pro-inflammatory cytokines downstream of Nod1-, TLR2- and TLR4-initiated signaling as assessed by both MAPK inhibitors (data not shown) and siRNA for ERK1 and p38 α (Supplementary Fig 4A). Also paralleling its role in Nod2-initiated signaling, JNK induced both pro- and anti-inflammatory cytokines downstream of these PRR (Supplementary Fig 4A). We then questioned if autocrine IL-1 also masks ERK1- and p38 α -mediated inhibition of pro-inflammatory cytokines in TLR2 and TLR4-stimulated MDM from Leu1007insC homozygotes/compound heterozygotes. These cells failed to induce cytokines following MDP stimulation in the absence of autocrine IL-1, and therefore showed no effect following MAPK inhibition under these conditions (Supplementary Fig 4B). However, similar to MDM from Nod2 WT healthy controls and Nod2 WT CD patients, autocrine IL-1 masked ERK1- and p38 α -mediated inhibition of IL-8 (Supplementary Fig 4B) and other pro-inflammatory cytokines (data not shown) upon TLR4 (Supplementary Fig 4B) and TLR2 (data not shown) stimulation in Leu1007insC homozygotes/compound heterozygotes. Taken together, autocrine IL-1 masks ERK- and p38-mediated inhibition of pro-inflammatory cytokines downstream of multiple PRR in human MDM.

MDP-initiated PKC and PLA2 pathways regulate both pro- and anti-inflammatory cytokines

We next asked if MDP-induced autocrine IL-1 masks differential regulation of pro- and anti-inflammatory cytokine secretion downstream of additional signaling pathways. Atypical PKCs, conventional PKCs and PLA2²⁶ mediate IL-1R-induced cytokine secretion. Moreover, Nod2 activates conventional and atypical PKC pathways in mouse macrophages;²⁷ it is unknown if this is the case in human MDM. Furthermore, to our knowledge, Nod2-mediated PLA2 signaling has not been examined. We find that inhibiting conventional PKC, PKC ζ and the PLA2 pathways decreases Nod2-induced pro- and anti-inflammatory cytokines in MDM regardless of whether autocrine IL-1 is present or absent (Supplementary Fig 5). Therefore, we find that Nod2 signaling in human MDM utilizes conventional PKCs, PKC ζ and PLA2 pathways and that, in contrast to ERK and p38, these

pathways induce both pro- and anti-inflammatory cytokines by Nod2 signaling in the absence of autocrine IL-1.

The strength of MAPK signaling determines pro-inflammatory cytokine regulation

The strength and kinetics of MAPK signaling can profoundly influence cellular outcomes and cytokine secretion.^{28,29} We hypothesized that the Nod2-induced autocrine IL-1-mediated modulation of ERK and p38 requirements for pro-inflammatory cytokines may be due to altered magnitude of MAPK activation; we previously identified that Nod2-induced autocrine IL-1 increases MAPK activation.⁸ Therefore, we asked if selective MAPK activation during MDP stimulation in the absence of autocrine IL-1 is sufficient to switch ERK-signaling outcomes from inhibiting to stimulating pro-inflammatory cytokines. To address this, we utilized a plasmid overexpressing constitutively active JNK;²¹ we confirm that it specifically activates the downstream substrate, c-Jun, to physiological levels (Fig 4A&B). Constitutively active JNK alone did not significantly induce cytokines in MDM (Fig 4C). As we previously demonstrated, ERK1 inhibits pro-inflammatory cytokines during MDP stimulation in the absence of autocrine IL-1; as such, ERK1 knockdown under these conditions increases pro-inflammatory cytokines (Fig 4D, *top*). In contrast, upon ERK1 knockdown during MDP stimulation without autocrine IL-1, but with increased JNK signaling, pro-inflammatory cytokines are now decreased (Fig 4D, *bottom*). The decrease was observed both relative to conditions without ERK1 inhibition (Fig 4D, *bottom*), and relative to conditions without JNK activation but with ERK1 inhibition (Fig 4D, *top*). Constitutively active JNK similarly switched Nod2-initiated, p38-mediated inhibition to stimulation of pro-inflammatory cytokines (data not shown). To independently confirm these findings, we added anisomycin, which activates both JNK and p38,¹⁴ to MDP-stimulated MDM in the absence of autocrine IL-1; anisomycin also reversed ERK signaling from inhibiting to stimulating pro-inflammatory cytokines (Supplementary Fig 6). Therefore, the ERK-mediated pro-inflammatory cytokine regulation upon MDP stimulation of MDMs can be reproduced in the absence of autocrine IL-1 by increasing the strength of JNK signaling.

Differential IL-1 production by human and mouse macrophages contributes to their distinct ERK-mediated IL-12p40 regulation

Mouse macrophage studies have frequently identified that unlike human MDM, TLR4- and other PRR-induced ERK signaling inhibits pro-inflammatory IL-12p40;^{11–13,16} the reasons for this discrepancy have been unclear. Unlike human MDM, which secrete IL-1 following LPS stimulation (Fig 5A *left*), mouse macrophages secrete IL-1 only if accompanied by a second inflammasome-activating stimulus such as ATP (Fig 5B *left*).^{8,18–20} We hypothesized that this absence of autocrine IL-1 in mouse macrophages might explain species differences in ERK-mediated IL-12p40 regulation upon PRR stimulation. We first confirmed that LPS-mediated ERK activation inhibits IL-12p40 in primary Balb/c BMDM (Fig 5B *right*). We see similar ERK-mediated IL-12p40 inhibition in LPS-stimulated primary human MDM in the absence of autocrine IL-1 (Fig 5A, *right*), paralleling our findings in MDP-stimulated human MDM (Fig 2&3). However, upon IL-1 addition to LPS-stimulated Balb/c BMDM to mimic autocrine IL-1 secretion by human MDM, IL-12p40 secretion was decreased upon ERK inhibition (Fig 5B, *right*). We observe similar IL-12p40 regulation in human MDM in the presence of autocrine IL-1 (Fig 5A, *right*). These findings indicate that exogenous IL-1 masks the LPS-initiated, ERK-mediated inhibition of IL-12p40 in mouse BMDM, so that they behave like human MDM.

As expected, mouse BMDM produce autocrine IL-1 upon PRR stimulation followed by ATP (Fig 5B, *left*); consistently, ATP addition following LPS treatment switches ERK-mediated inhibition of IL-12p40 to stimulation (Fig 5C). One reason proposed to mediate

inflammasome activation and IL-1 secretion upon PRR stimulation alone in human MDM, is that PRR-stimulated MDM secrete ATP, causing autocrine/paracrine effects.³⁰ We thus asked if apyrase-mediated autocrine ATP degradation would mimic IL-1R blockade resulting in ERK inhibition of IL-12p40 secretion in human MDM, similar to mouse BMDM. We find this to be the case (Fig 5D). We found that other pro-inflammatory cytokines are regulated similarly to IL-12p40 in PRR-ligand-stimulated human MDM (Fig 2&5D). However, PRR stimulation of mouse macrophages induces mechanisms that differentially regulate IL-12p40 relative to other pro-inflammatory cytokines;³¹ we now observe that in mouse BMDM, LPS-induced ERK inhibits IL-12p40, but not TNF- α , MIP2 (a human IL-8 analogue), and IL-6 (Fig 5E). The anti-inflammatory cytokines in mouse BMDM are regulated similarly to human MDM (Fig 5E). To verify that this distinct regulation of IL-12p40 compared to other pro-inflammatory cytokines is not unique to Balb/c BMDM, we examined C57BL/6 BMDM, and J774 and RAW264.7 mouse macrophage lines. As with Balb/c BMDM, LPS-induced ERK inhibits IL-12p40, while exogenous IL-1 switches ERK to stimulate IL-12p40 (Fig 5F). Also similar to Balb/c BMDM, TNF- α , MIP2 and IL-6 are inhibited upon LPS stimulation in the presence or absence of IL-1 in other mouse macrophages (data not shown), further highlighting that human MDM and mouse macrophages regulate these cytokines differently. Therefore, the controversy between mouse and human cells regarding ERK regulation of IL-12p40 secretion is partially due to distinct autocrine IL-1 secretion, which, in turn, masks ERK-mediated inhibition of PRR-induced pro-inflammatory cytokines.

ERK and p38 inhibit PRR-induced pro-inflammatory cytokines in low IL-1-secreting M2 and 'tolerant' human MDM

Given the importance of autocrine IL-1 in ERK- and p38-mediated regulation of pro-inflammatory cytokines in human MDM, we hypothesized that macrophage subsets secreting either high or low IL-1 upon PRR stimulation would demonstrate distinctly different ERK- and p38-mediated cytokine regulation. We therefore examined M1 and M2 macrophages. MDP-stimulated human M1 macrophages produce significantly more IL-1 and other pro-inflammatory cytokines than M2 macrophages (Fig 6A). We further verified macrophage polarization by measuring human M1 and M2 marker expression (Fig 6B).³² We find that in MDP-treated M1 macrophages, ERK and p38 induced pro-inflammatory cytokines (Fig 6C), and asked if this is due to high autocrine IL-1 production (Fig 6A). Increasing IL-1Ra from 0.5 to 1 μ g/ml to parallel the similar 2-fold IL-1 β increase in M1 macrophages compared to undifferentiated MDM (Fig 6A) demonstrated that IL-1 masks ERK- and p38-mediated inhibition of Nod2-induced pro-inflammatory cytokines (Fig 6C). Conversely, in MDP-treated M2 macrophages where IL-1 secretion is low, ERK and p38 inhibited pro-inflammatory cytokines even without IL-1 blockade (Fig 6D); IL-1 addition reversed this regulation (Fig 6D). Therefore, under M1 conditions, presumably reflecting an inflammatory state, autocrine IL-1 promotes PRR-mediated inflammation by activating ERK and p38 to increase pro-inflammatory cytokines. The opposite regulation is observed in anti-inflammatory M2 macrophages.

ERK and p38 inhibit pro-inflammatory cytokines in tolerant MDM and intestinal macrophages

MDM chronically stimulated through PRR, such as Nod2, undergo 'endotoxin tolerance', i.e. downregulated pro-inflammatory cytokines, including IL-1, following PRR restimulation.^{3,4,7} We find that in such Nod2-induced tolerant MDM secreting reduced IL-1, ERK and p38 activation upon MDP re-treatment inhibits pro-inflammatory cytokines; exogenous IL-1 switches this inhibition to stimulation (Fig 6E). However, ERK and p38 induce anti-inflammatory cytokines irrespective of IL-1 contributions in these cells.

PRR-ligand-stimulated intestinal macrophages secrete minimal pro-inflammatory cytokines, including IL-1.³³ This likely reflects multiple intestinal mechanisms, including ongoing PRR stimulation and anti-inflammatory cytokine exposure.³⁴ Similar to previous reports,³³ we detected low pro-inflammatory IL-8 secretion in LPS-stimulated human intestinal myeloid cells (Fig 7D), but did not detect IL-1 secretion (Fig 7B). Consistent with the lack of IL-1, we find that in LPS-stimulated intestinal myeloid cells ERK and p38 downregulate IL-8; exogenous IL-1 switches this downregulation to stimulation (Fig 7A). Taken together, PRR-mediated stimulation of ERK and p38 decreases pro-inflammatory cytokine secretion in human intestinal myeloid cells, thereby contributing to their tolerant phenotype.

S. typhimurium-induced autocrine IL-1 switches ERK and p38 from inhibiting to stimulating pro-inflammatory cytokine induction in intestinal myeloid cells

Mouse intestinal macrophages were recently shown to secrete IL-1 following exposure to specific flagellin-containing pathogens including *S. typhimurium*.³⁵ We therefore questioned if *S. typhimurium* might similarly induce IL-1 secretion in human intestinal myeloid-derived cells, and if this, in turn, could switch ERK and p38 signaling from inhibiting to stimulating IL-8 secretion. We find this to be the case, as upon *S. typhimurium* co-culture, human intestinal myeloid cells secreted IL-1 (Fig 7B) and activated ERK and p38 to induce IL-8 (Fig 7C). Furthermore, IL-1R blockade during *S. typhimurium* stimulation reversed this outcome, with ERK and p38 signaling now inhibiting IL-8 secretion. As a control we used AIEC, an *E. coli* strain colonizing the ileum of CD patients,³⁶ which did not induce IL-1 β (Fig 7B). AIEC-induced ERK and p38 signaling inhibited IL-8; IL-1 addition switched this regulation to stimulation (Fig 7C). Therefore, while PRR-stimulated intestinal macrophages downregulate pro-inflammatory cytokine secretion partially through ERK- and p38-mediated inhibition, we find that specific intestinal pathogens that induce IL-1 can switch ERK and p38 signaling in these cells to induce pro-inflammatory cytokines, thereby disrupting ‘tolerance’ and promoting intestinal inflammation through increased cytokine secretion (Fig 7D).

Discussion

Pro- and anti-inflammatory cytokine modulation is central to intestinal homeostasis. We identify through both knockdown and pharmacologic approaches a novel mechanism altering PRR-mediated cytokine regulation: autocrine IL-1 switches ERK- and p38-signaling from inhibiting to stimulating pro-inflammatory cytokines in primary human MDM (Supplementary Fig 7). In contrast, ERK and p38 induce anti-inflammatory cytokines regardless of whether autocrine IL-1 is present or absent. Moreover, JNK induced both pro- and anti-inflammatory cytokines by Nod2 signaling in the absence of autocrine IL-1, as did conventional and atypical PKCs and PLA2, thereby demonstrating distinct regulation by specific signaling pathways. Autocrine IL-1 also masks pro-inflammatory cytokine regulation by Nod1-, TLR2- and TLR4-activated ERK and p38. We further find that ERK- and p38-mediated pro-inflammatory cytokine inhibition during Nod2 stimulation in the absence of autocrine IL-1 signaling is reversed by increasing JNK activation, thereby identifying that the strength of MAPK signaling is a mechanism modifying the distinct pro-inflammatory cytokine regulation in MDM. Controversial PRR-induced MAPK-signaling outcomes in mouse and human cells might partially reflect varying autocrine IL-1 secretion in cells from these species. Consistently, we find that in mouse BMDM and mouse macrophage cell lines, which do not secrete IL-1 following PRR stimulation, ERK-mediated IL-12p40 inhibition is reversed upon IL-1 addition. Importantly, in human macrophage subsets secreting low IL-1, such as M2 macrophages, endotoxin tolerant MDM and intestinal myeloid cells, ERK and p38 downregulate pro-inflammatory cytokines; exogenous IL-1 switches this downregulation to stimulation. Significantly, pathogenic bacteria inducing

IL-1 secretion in intestinal myeloid cells switch ERK and p38 signaling to increase inflammation, thereby highlighting a mechanism disrupting intestinal macrophage ‘tolerance’. We therefore identify that IL-1-regulated strength of MAPK signaling dramatically affects whether PRR-initiated ERK and p38 signaling inhibits or stimulates pro-inflammatory cytokines, resulting in distinct pro-inflammatory outcomes in different macrophage subsets.

How PRR-induced MAPK regulate IL-1Ra relative to pro-inflammatory cytokines has to our knowledge not been investigated. We now identify that PRR-mediated MAPK activation regulates IL-1Ra similar to IL-10, and distinct to pro-inflammatory cytokines. This parallels our findings that Nod2-activated mTOR induces IL-10 and IL-1Ra while downregulating pro-inflammatory cytokines.⁷ Furthermore, IL-1Ra naturally antagonizes IL-1R^{7,25} and is secreted following PRR stimulation (Fig 1) and in vivo during inflammation, including in colitis models.³⁷ Besides blocking autocrine IL-1-induced inflammatory effects, the IL-1Ra-mediated decreased strength of MAPK signaling could, in turn, switch PRR-initiated ERK and p38 signaling to inhibit pro-inflammatory cytokines. These combined effects may partially account for IL-1Ra-mediated amelioration of inflammation in both animal models³⁷ and human inflammatory diseases,³⁷ and for consequences in individuals carrying autoimmune disease-associated IL-1Ra polymorphisms.³⁸

Interestingly, unlike in human MDM, regulation of IL-12p40 is distinct from regulation of other pro-inflammatory cytokines in mice;³¹ in mouse BMDM IL-1 modulates ERK-mediated regulation of IL-12p40, but not of TNF- α , IL-6 and MIP2 (Fig 5). Given that IL-12p40 is a shared subunit in both IL-12 and IL-23, such distinct regulation could affect both Th1 and Th17 differentiation outcomes.

Nod2 and IL-1R are critical for intestinal homeostasis.¹ Furthermore, both *Nod2*¹ and *NALP3* polymorphisms³⁹ are associated to CD. It is therefore important to dissect the distinct Nod2 and IL-1R signaling regulation to understand how they jointly maintain intestinal homeostasis. Furthermore, one must understand the regulation of these pathways in human cells given species differences in IL-1 production. MAPK inhibitors are being assessed therapeutically in inflammatory/autoimmune diseases, including IBD.⁴⁰ However, our findings may ultimately have implications for differential efficacy of these agents; inhibiting ERK and p38 pathways may cause adverse consequences in intestinal immunoregulation through Nod2 and other PRRs. Our findings highlight the importance of dissecting PRR signaling outcomes independently from PRR-induced autocrine loops. Moreover, we further elucidate how the IL-1R modifies Nod2-mediated regulation of pro- and anti-inflammatory cytokines, and how modulating strength of MAPK signaling by IL-1 can dramatically alter the direction of pro-inflammatory cytokine regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Nod nucleotide-binding oligomerization domain

MDP	muramyl dipeptide
CD	Crohn's disease
MDM	monocyte-derived macrophage
IL-1R	IL-1R receptor
IL-1Ra	IL-1 receptor antagonist
PRR	pattern-recognition receptor
MDP	muramyl dipeptide
IBD	inflammatory bowel disease

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Figure 1. PRR-activated JNK1, ERK1 and p38α induce pro- and anti-inflammatory cytokines
(A) Human MDM transfected with scrambled, JNK1, ERK1, or p38α siRNAs were analyzed for silencing efficacy and specificity by confirming inhibition of MDP-induced phosphorylation of the respective kinase or downstream substrate by phospho-flow. *Left:* Representative histograms with indicated mean fluorescence intensity (MFI). MDP-stimulated cells with isotype controls are shown. *Right:* Data (n=6–8) are represented as fold phosphoprotein induction normalized to untreated cells+SEM. **(B,C)** MDM (n=8) were transfected with scrambled, JNK1, ERK1 or p38α siRNAs. 48h later, MDM were stimulated with 100μg/ml MDP, 100μg/ml TriDAP, 10μg/ml Pam₃Cys or 0.1μg/ml lipid A for 24h. **(B)**

Supernatants were assayed for cytokines. **(C)** The percent cytokine secretion normalized to cells treated by the respective stimulus in scrambled siRNA-transfected cells+SEM. Significance is to cytokine concentrations with scrambled siRNA. **(D)** MDM from Nod2 WT CD patients (n =2, solid symbols) or Nod2 Leu1007insC homozygote/compound heterozygote CD patients (n =2, open symbols) were stimulated with 100µg/ml MDP or 0.1µg/ml lipid A. Supernatants were assayed for cytokines. **, p<0.01; ††, p<1×10⁻⁵. Lines over adjacent bars indicate identical P values.

Figure 2. Autocrine IL-1 masks the differential pro- and anti-inflammatory cytokine regulation by ERK and p38 in MDP-stimulated MDM

MDM (n=8) were incubated with JNK inhibitor II, PD98059 or SB202190 each at 0.1μM and then stimulated with 100μg/ml MDP (Nod2-initiated signaling), 10ng/ml IL-1β (IL-1R-initiated signaling) or 100μg/ml MDP+0.5μg/ml IL-1Ra (Nod2 signaling in the absence of autocrine IL-1, i.e. IL-1R-independent Nod2 signaling). Data (n=8) are represented as (A) the percent cytokine secretion normalized to cells treated by the respective stimulus without MAPK inhibitors+SEM, or (B) absolute cytokine secretion upon Nod2 signaling in the absence of autocrine IL-1+/-MAPK inhibition in a representative 4 of 8 individuals. We confirmed JNK, ERK and p38 inhibition effects upon MDP stimulation without autocrine

IL-1 in another n=12. Significance is to cytokine concentrations without MAPK inhibition. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; †, $p < 1 \times 10^{-4}$; ††, $p < 1 \times 10^{-5}$. Lines over adjacent bars indicate identical p-values. Tx, treatment.

Figure 3. MDP-activated ERK1, ERK2 and p38 α inhibit pro-inflammatory cytokine secretion in the absence of autocrine IL-1 in MDM

(A) MDM transfected with scrambled, JNK1, ERK1 or p38 α siRNAs (n=8) were stimulated with 100 μ g/ml MDP+0.5 μ g/ml IL-1Ra for 24h. Data are represented as percent cytokine secretion normalized to scrambled siRNA-transfected cells stimulated with MDP in the absence of autocrine IL-1+SEM. Significance is to cytokine concentrations with scrambled siRNA. We confirmed ERK1 and p38 α siRNA results in an additional n=12. (B,C) MDM were transfected with scrambled or ERK2 siRNAs. (B) Efficacy and specificity of silencing as confirmed by inhibition of MDP-induced phosphorylation of the kinase by phospho-flow. *Left*: Representative flow cytometry with indicated MFI. MDP-stimulated cells with isotype controls are shown. *Right*: Data (n=6) are represented as the fold phospho-protein induction normalized to untreated cells+SEM. (C) Cells were stimulated with 100 μ g/ml MDP+ 0.5 μ g/ml IL-1Ra for 24h. Data are represented as the percent cytokine normalized to siRNA-transfected cells stimulated with MDP in the absence of autocrine IL-1+SEM. Significance is to cytokine concentrations with scrambled siRNA. *, p<0.05; **, p<0.01; ***, p<0.001; †, p<1 $\times$ 10⁻⁴; ††, p<1 $\times$ 10⁻⁵. Lines over adjacent bars indicate identical p-values. Tx, treatment.

Figure 4. JNK activation reverses ERK-mediated inhibition of pro-inflammatory cytokines during IL-1R-independent Nod2 signaling in MDM

(A,B) MDM (n=7–8) were transfected with 5 μ g pSR α -3HA-JNKK2-JNK1 WT (constitutively active JNK, caJNK) or empty vector (EV) and 48h later analyzed by phospho-flow for phospho-kinase induction. (A) Representative histograms with indicated MFI and isotype controls. (B) Data are represented as the fold phospho-kinase induction normalized to EV-transfected cells+SEM. (C) MDM (n=8) were transfected with EV and stimulated with 100 μ g/ml MDP for 24 hours or transfected with caJNK and left untreated. Data are represented as the percent cytokine normalized to 100 μ g/ml MDP-stimulated, EV-transfected cells+SEM. (D) MDM (n=8) were transfected with EV or caJNK, then scrambled siRNA or ERK1 siRNA and subsequently stimulated with 100 μ g/ml MDP

+0.5µg/ml IL-1Ra for 24h. (*left*) Data are represented as the percent cytokines normalized to scrambled siRNA-transfected cells stimulated with (*top*) MDP+IL-1Ra with EV and (*bottom*) MDP+IL-1Ra with caJNK +SEM. (*right*) Absolute cytokine secretion in a representative 4 of 8 individuals. **, $p < 0.01$; †, $p < 1 \times 10^{-4}$; ††, $p < 1 \times 10^{-5}$. Tx, treatment; p-, phospho-.

Figure 5. Autocrine IL-1 masks similar IL-12p40 regulation by ERK in mouse BMDM and human MDM

(A) Human MDM (n=8) were incubated with the ERK inhibitor PD98059 where indicated and stimulated with 0.1 μ g/ml LPS or 0.1 μ g/ml LPS+0.5 μ g/ml IL-1Ra for 24h. (B) Balb/c BMDM were incubated with PD98059 and stimulated with (left) 0.1 μ g/ml LPS for 24h+/-5mM ATP for 30 min or (right) 0.1 μ g/ml LPS or combined 0.1 μ g/ml LPS+10ng/ml IL-1 β for 24h. (C) Balb/c BMDM were incubated with PD98059 and stimulated with 0.1 μ g/ml LPS for 24h+/-5mM ATP for 30 min. (D) MDM (n=8) were incubated with PD98059 and stimulated with 0.1 μ g/ml LPS+/-2.5 units/ml apyrase for 24h. Data are represented as the percent cytokines normalized to LPS+apyrase-stimulated cells without PD98059+SEM.

Significance compared to cytokine concentrations without PD98059 is shown. Balb/c BMDM (**E**) or C57BL/6 BMDM, J774 and RAW 264.7 mouse macrophages (**F**) were treated as in (**B**). (**A–F**) Supernatants were assayed for indicated cytokines. Mouse macrophage experiments were conducted in triplicate and reflect two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ††, $p < 1 \times 10^{-5}$. Lines over adjacent bars indicate identical p-values. Tx, treatment.

Figure 6. Differential IL-1 secretion in M1, M2 and tolerant macrophages distinctly regulates ERK-induced pro-inflammatory cytokines

(A) MDM (n=8) from the same individuals were differentiated into M1 or M2 macrophages and stimulated with 100 μ g/ml MDP for 24h. Supernatants were assayed for cytokines. (B) mRNA expression of M1 and M2 markers was assessed by RT-PCR. Data are represented as the fold gene expression normalized to M1 cells+SEM. (C) M1 or (D) M2 MDM transfected with scrambled, JNK1, ERK1 or p38 α siRNAs (n=8) were stimulated with (C) 100 μ g/ml MDP (left) or MDP+1 μ g/ml IL-1Ra (right) for 24h or (D) 100 μ g/ml MDP (left), or MDP +10ng/ml IL-1 β (right) for 24h. (E) MDM (n=8) were tolerized by treating with 100 μ g/ml MDP for 48h, then transfected with scrambled, JNK1, ERK1 or p38 α siRNAs, washed and restimulated with 100 μ g/ml MDP (left) or MDP+10ng/ml IL-1 β (right) for an additional 24h. (C–E) Data are represented as the percent cytokine secretion normalized to scrambled

siRNA-transfected cells treated with the respective stimulus+SEM. Significance is to cytokine concentrations with scrambled siRNA. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; †, $p<1\times 10^{-4}$; ††, $p<1\times 10^{-5}$. Lines over adjacent bars indicate identical p-values. Tx, treatment; scr, scrambled.

Figure 7. IL-1-inducing pathogens switch ERK and p38-activation from inhibiting to stimulating pro-inflammatory cytokine secretion in intestinal myeloid cells

(A) Human intestinal myeloid cells (n=4) were incubated with JNK inhibitor II, PD98059 or SB202190 each at 0.1 μ M and then stimulated with 0.1 μ g/ml LPS or 0.1 μ g/ml LPS+10ng/ml IL-1 β . Data are represented as the percent IL-8 secretion normalized to cells treated by the respective stimulus without MAPK inhibitors+SEM. (B) Human intestinal myeloid cells (n=8) were stimulated with LPS, *S. typhimurium* or AIEC for 12h. Supernatants were measured for IL-1 β . (C) Intestinal myeloid cells (n=8) were incubated with MAPK inhibitors as in (A) and then stimulated with *S. typhimurium*+/-0.5 μ g/ml IL-1Ra or AIEC+/-10ng/ml IL-1 β . Data are represented as the percent IL-8 secretion normalized to cells treated by the respective stimulus without MAPK inhibitors+SEM. (D) Intestinal myeloid cells (n=8) were stimulated as in (B). Supernatants were measured for IL-8. **, p<0.01; ***, p<0.001; †, p<1 $\times$ 10⁻⁴; ††, p<1 $\times$ 10⁻⁵.