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A *TPL2* (*MAP3K8*) disease-risk polymorphism increases *TPL2* expression thereby leading to increased pattern recognition receptor-initiated caspase-1 and caspase-8 activation, signalling and cytokine secretion

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Abstract

Objective—IBD is characterised by dysregulated intestinal immune homeostasis and cytokine secretion. In the intestine, properly regulating pattern recognition receptor (PRR)-mediated signalling and cytokines is crucial given the ongoing host–microbial interactions. *TPL2* (*MAP3K8*, *COT*) contributes to PRR-initiated pathways, yet the mechanisms for *TPL2* signalling contributions in primary human myeloid cells are incompletely understood and its role in intestinal myeloid cells is poorly defined. Furthermore, functional consequences for the IBD-risk locus rs1042058 in *TPL2* are unknown.

Methods—We analysed protein, cytokine and RNA expression, and signalling in human monocyte-derived macrophages (MDMs) through western blot, ELISA, real-time PCR and flow cytometry.

Results—PRR-induced cytokine secretion was increased in MDMs from rs1042058 *TPL2* GG risk individuals. *TPL2* activation by the Crohn's disease-associated PRR nucleotide-oligomerisation domain (NOD)2 required PKC, and IKK β , IKK α and IKK γ signalling. *TPL2*, in turn, significantly enhanced NOD2-induced ERK, JNK and NF κ B signalling. We found that another major mechanism for the *TPL2* contribution to NOD2 signalling was through ERK-dependent and JNK-dependent caspase-1 and caspase-8 activation, which in turn, led to early autocrine interleukin (IL)-1 β and IL-18 secretion and amplification of long-term cytokines. Importantly, *Salmonella typhimurium*-induced cytokines from human intestinal myeloid-derived cells required *TPL2* as well as autocrine IL-1 β and IL-18. Finally, rs1042058 GG risk carrier MDMs from healthy individuals and patients with Crohn's disease had increased *TPL2* expression and NOD2-initiated *TPL2* phosphorylation, ERK, JNK and NF κ B activation, and early autocrine IL-1 β and IL-18 secretion.

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Conclusions—Taken together, the rs1042058 GG IBD-risk polymorphism in *TPL2* results in a gain-of-function by increasing TPL2 expression and signalling, thereby amplifying PRR-initiated outcomes.

INTRODUCTION

A hallmark of immune-mediated diseases is dysregulated cytokine secretion. In IBD, this dysregulation is often due to an altered host response to microbes, a response mediated through pattern recognition receptors (PRRs).^{1–3} More than 160 genetic loci are now associated with IBD.⁴ However, it is unclear how the majority of these risk loci contribute to IBD; understanding the functional consequences of disease-associated polymorphisms is essential for understanding IBD pathogenesis and implementing IBD therapy.

Polymorphisms in a region that includes the *TPL2* gene (also known as *MAP3K8*, *COT*) have been associated with IBD⁴ and ovarian cancer.⁵ TPL2 mediates both innate and T-cell responses.^{6, 7} Consistent with its role in innate myeloid-derived cells, TPL2 contributes to PRR-induced cytokine secretion^{8,9} and endotoxin shock in mice.⁸ Importantly, TPL2 contributes to colitis in mice.^{10, 11} TPL2 activity is regulated by phosphorylation on Thr290 via IKK β in mouse cells;^{12, 13} Thr290 phosphorylation is also observed in human monocytes.¹⁴ It is unknown if additional pathways contribute to this phosphorylation. TPL2 activation, in turn, leads to ERK activation in rodent cells.^{6–8, 15} TPL2-mediated activation of additional MAPK or NF κ B pathways can depend on the cell type, stimulus and species.^{16, 17} The vast majority of studies defining roles and mechanisms for TPL2 are conducted in rodent cells, with only a few human studies^{14, 16, 18} examining PRR-mediated activation of TPL2 and its downstream outcomes. However, such human studies are critical given the differences between the regulation and outcomes in human and mouse inflammatory pathways.¹⁹ Therefore, we sought to identify mechanisms regulating TPL2 activation and its outcomes in primary human myeloid cells, to establish the TPL2 contribution to human intestinal myeloid cell outcomes and to define the functional consequences of the IBD-associated rs1042058 polymorphism in the *TPL2* region.

In this study, we found that TPL2 was required for optimal nucleotide-oligomerisation domain (NOD)2-induced activation of ERK, JNK and NF κ B, and PRR-initiated cytokine secretion in primary human monocyte-derived macrophages (MDMs). NOD2 stimulation in MDMs increased TPL2 activation, which was dependent on IKK members and on classical and atypical PKC signalling. Moreover, TPL2 was required for NOD2-induced caspase-1 and caspase-8 activation, which regulated rapid autocrine interleukin (IL)-1 β and IL-18 secretion and amplification of additional NOD2-induced cytokines. This caspase activation depended on signalling by the TPL2 pathway intermediates ERK and JNK. Importantly, TPL2 activation was required for IL-1 β secretion and cytokine amplification in human intestinal myeloid cells upon stimulation with the pathogen *Salmonella typhimurium*. Finally, rs1042058 IBD-risk GG carrier MDMs from both healthy controls and patients with Crohn's disease demonstrate an increase in TPL2 expression, NOD2-mediated TPL2, ERK, JNK and NF κ B activation, early autocrine IL-1 β and IL-18 secretion and late PRR-induced secretion of additional cytokines. Taken together, we define that the rs1042058 IBD-risk polymorphism is a gain-of-function resulting in increased PRR-induced cytokine secretion

in MDMs and elucidate mechanisms through which TPL2 contributes to PRR-initiated inflammatory outcomes.

METHODS

Patient recruitment and genotyping

Informed consent was obtained per protocol approved by the institutional review board at Yale University. Healthy individuals had no personal or family history of autoimmune/inflammatory disease, including psoriasis, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, type 1 diabetes mellitus, Crohn's disease and UC, or a history of HIV. Given the limitation in peripheral cell numbers and the range of innate responses we sought to examine, two separate cohorts of 100 and 98 European ancestry individuals were recruited for NOD2/Toll-like receptor (TLR)2 dose-response studies in MDMs and MDDCs, and NOD2/TLR synergy studies in MDDCs, respectively. We further ascertained a separate cohort of 24 patients with Crohn's disease based on *TPL2* genotype (see online supplementary table S1). We genotyped polymorphisms by TaqMan (Applied Biosystems, Foster City, California, USA) or Sequenom platform (Sequenom, San Diego, California, USA).

Primary myeloid cell culture

Monocytes were purified from human peripheral blood mononuclear cells by positive CD14 selection (Miltenyi Biotec, Auburn, California, USA) or adhesion, tested for purity and cultured with macrophage colony-stimulating factor (M-CSF) (10 ng/mL) (Shenandoah Technology, Warwick, Pennsylvania, USA), or IL-4 (40 ng/mL) (R&D Systems, Minneapolis, Minnesota, USA) and granulocyte macrophage colony-stimulating factor (GM-CSF) (40 ng/mL) (Genscript, Piscataway, New Jersey, USA) for 7 days for MDM and MDDC differentiation, respectively. Myeloid cells (CD11c purity >75%) were isolated as in Ref. 20 from colonic resection specimens from uninvolved intestine in four non-IBD patients undergoing surgery for diverticular disease or colon cancer.

Myeloid cell stimulation

Myeloid-derived cells were treated with muramyl dipeptide (MDP) (Bachem, King of Prussia, Pennsylvania, USA), Pam₃Cys-Ser-(Lys)₄ (Calbiochem, La Jolla, California, USA), lipid A (Peptides International, Louisville, Kentucky, USA), flagellin, CL097, CpG, poly I:C, Tri-DAP (Invivogen, San Diego, California, USA) or *Salmonella enterica serovar Typhimurium* (*S. typhimurium*) at multiplicity of infection (MOI) 10:1. For antibody and inhibitor treatments, cells were incubated with IL-1Ra (IL-1R antagonist) (Genscript), anti-IL-18 receptor accessory protein (RAP) (R&D Systems), Ac-YVAD-Cho (YVAD), caspase-8 inhibitor II or TPL2 inhibitor (Calbiochem) 1 h prior to treatment. Supernatants were assayed for tumour necrosis factor (TNF)- α , IL-8, IL-10 (BD Biosciences), IL-23, IL-1 β and IL-18 (eBioscience) by ELISA.

Transfection of small interfering RNAs (siRNAs) and plasmids

Myeloid cells were transfected with 100 nM scrambled or ON-TARGETplus SMARTpool siRNA against TPL2, PKC α , PKC β , PKC ζ , IKK α , IKK β or IKK γ (Dharmacon, Lafayette,

Colorado, USA), or 5 µg pMCL-MKK1 (R4F) (constitutively active ERK kinase),²¹ pSRα-3HA-JNKK2-JNK1-WT (constitutively active JNK)²² (generous gifts from Dr Ben Turk) or empty vector using Amaxa nucleofector technology (Amaxa, San Diego, California, USA) for 48 h.

Protein expression analysis

Proteins were detected after cell permeabilisation by flow cytometry with Alexa Fluor 647-labelled, phycoerythrin-labelled or Alexa Fluor 488-labelled antibodies to phospho-ERK, phospho-p38, phospho-JNK, phospho-IκBα, phospho-TPL2 (Cell Signaling), phospho-IKKα, phospho-IKKβ, phospho-IKKγ or total TPL2 (Abcam, Boston, Massachusetts, USA). Western blot (as per²⁰) used anti-caspase-1, anti-caspase-8, anti-IL-1β (Cell Signaling), anti-IL-18 (Abcam) or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Calbiochem).

mRNA expression analysis

RNA was isolated, reverse transcribed and quantitative PCR performed as in Ref. 20. Samples were run in duplicate and normalised to GAPDH. Primer sequences are available upon request.

Statistical analysis

Significance was assessed using two-tailed t test. $p < 0.05$ was considered significant. A Bonferroni test was applied for multiple comparisons as appropriate. To maintain a consistent picogram per millilitre scale for cytokine concentration measures, the higher levels observed for IL-8 (nanogram per millilitre levels) are shown with a multiplication factor.

RESULTS

The *rs1042058* disease-risk polymorphism in the *TPL2* region dramatically increases PRR-induced cytokine secretion in primary human myeloid cells

As *TPL2* regulates pathways leading to PRR-induced cytokine secretion, and dysregulated cytokines and microbial responses are associated with IBD,¹ we asked if the *rs1042058* disease-risk polymorphism in the *TPL2* region regulates PRR-induced cytokine secretion in MDMs. *NOD2* is associated with Crohn's disease,¹ such that we investigated how primary human MDMs from 100 healthy individuals respond to treatment with MDP, the minimal component of bacterial cell-wall peptidoglycan that activates *NOD2*.^{23, 24} We measured *NOD2*-induced TNF-α protein secretion as this cytokine contributes to the inflammation in Crohn's disease.¹ We normalised *NOD2*-induced cytokine secretion to untreated cells and log₂ transformed the data. MDMs from *rs1042058* GG risk carriers secreted higher TNF-α levels than AA carriers, and GA carriers exhibited an intermediate phenotype (figure 1A).

Gram-positive bacteria contain the TLR2 ligand lipotechoic acid in addition to peptidoglycan; MDMs from *rs1042058* GG risk carriers secreted increased TLR2-induced TNF-α relative to GA and AA carriers (figure 1B). Both MDMs and dendritic cells (DCs) express PRRs, and in a separate cohort of 98 healthy controls we found that *rs1042058* GG

carrier MDDCs similarly showed increased NOD2-induced and TLR2-induced TNF- α secretion (figure 1C). Multiple PRRs are activated following microbial exposure, and NOD2 synergises with other PRRs;^{25, 26} MDDCs from rs1042058 GG risk carriers showed increased TNF- α secretion following stimulation of additional PRRs alone or in combination with NOD2 (figure 1C, D). IL-1 β is critical for NOD2-initiated cytokine amplification,²⁷ and IL-1 β secretion was regulated by rs1042058 genotype in a manner similar to TNF- α (figure 1E–H). Anti-inflammatory cytokines can be regulated differently than proinflammatory cytokines, but the rs1042058 genotype modulated IL-10 secretion similar to that observed for TNF- α and IL-1 β (data not shown). Taken together, the IBD-associated rs1042058 risk polymorphism in *TPL2* increases PRR-induced cytokine secretion in primary human myeloid cells.

TPL2 amplifies PRR-initiated cytokine secretion in MDMs

As the *TPL2* region polymorphism regulates PRR-induced cytokine secretion (figure 1), we next sought to establish if TPL2 regulates cytokine secretion following NOD2 and PRR stimulation in human MDMs. We therefore successfully knocked down TPL2 expression by siRNA (figure 2A); cell viability was unaffected (data not shown). TPL2 knockdown dramatically reduced NOD2-induced proinflammatory and anti-inflammatory cytokine secretion (figure 2B). We used 100 μ g/mL MDP as this dose range results in optimal cytokine secretion in human myeloid cells (figure 1A).^{20, 28} Similar results were obtained through an independent approach using a pharmacological TPL2 inhibitor (figure 2C). Moreover, TPL2 knockdown significantly attenuated cytokines induced by NOD1, TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 (figure 2D). Taken together, TPL2 signalling is critical for optimal cytokine secretion by multiple PRRs.

PRR stimulation regulates TPL2 expression

We next asked how PRR stimulation regulates TPL2 expression in primary human MDMs. Previous studies have shown that TLR4 stimulation in mouse BMDMs²⁹ and in overexpression systems¹³ decreases TPL2 expression at early time points; this decrease was due to proteasome-dependent and proteasome-independent mechanisms. We similarly found that TPL2 protein decreased following NOD2 stimulation, with the lowest expression observed 4 h after stimulation. At later time points TPL2 protein expression recovered, and in fact, increased (figure 3A, B). This increase corresponded with increased TPL2 mRNA, which peaked 2 h after MDP treatment (figure 3C). The regulation of TPL2 protein expression by TLR2, TLR4 and TLR5 ligands was similar to that observed following NOD2 stimulation (figure 3D). Taken together, TPL2 expression is dynamically regulated in MDMs upon PRR stimulation.

Optimal TPL2 phosphorylation requires PKC signalling

TLR4 stimulation of mouse macrophages results in TPL2 phosphorylation on Thr290.^{12, 13} In contrast, the one human monocyte study that to our knowledge examines TPL2 phosphorylation showed that Thr290 phosphorylation was constitutive rather than TLR4 dependent.¹⁴ In both situations, however, Thr290 phosphorylation was required for TPL2-mediated ERK activation.^{12, 14} In mouse macrophages Thr290 phosphorylation leads to TPL2 dissociation from a larger protein complex, activation of TPL2 kinase activity and a

subsequent decrease in TPL2 protein.^{13, 29} This decrease is closely linked to TPL2 activation²⁹ and is proteasome dependent.^{13, 14} We therefore first questioned how NOD2 stimulation of MDMs regulates TPL2 phosphorylation on Thr290 and found that this phosphorylation was induced within 10 min of MDP treatment and peaked at 30 min (figure 4A). Increased TPL2 Thr290 phosphorylation occurred despite the decrease in total TPL2 levels, such that the ratio of phospho-TPL2 to total TPL2 levels showed an even more pronounced effect (figure 4B). In mouse RAW264.7 macrophages IKK β was required for TPL2 phosphorylation on Thr290.¹² Using IKK β knockdown (figure 4C), we confirmed that this was so in human MDMs (figure 4D). IKK β associates with IKK α and IKK γ , and IKK β substrates can occasionally be phosphorylated by IKK α .³⁰ We therefore questioned if these molecules also regulated TPL2 phosphorylation. Interestingly, knockdown of each IKK α and IKK γ decreased NOD2-induced TPL2 activation (figure 4D). Of note is that IKK α , IKK β and IKK γ knockdown led to decreased NOD2-induced activation of each other to varying degrees (figure 4E). This IKK family member cross-regulation occurred at the level of activation, and not at the level of transcriptional expression (figure 4C). Therefore, IKK α , IKK β and IKK γ are each necessary for optimal NOD2-mediated TPL2 activation in human MDMs.

We next examined additional mechanisms regulating TPL2 activation. Through *in silico* analysis we identified that Thr290 is located in a motif that could serve as a PKC phosphorylation site. PKCs consist of conventional PKCs (eg, PKC α and β) and atypical PKCs (eg, PKC ζ), and we previously found that both PKC types contribute to NOD2-initiated cytokine secretion.³¹ Upon successful knockdown of PKC α , β and ζ (figure 4F), we found that each kinase was required for TPL2 phosphorylation on Thr290 (figure 4G). Knockdown of MAPKs, which have not been reported to affect upstream TPL2 signalling, did not affect TPL2 phosphorylation (data not shown). Consistent with the Thr290 phosphorylation-dependent decrease in TPL2 expression upon activation, upon PKC knockdown, TPL2 expression failed to decrease after NOD2 stimulation (figure 4G). PKCs can regulate IKK activation,³² such that we questioned if PKC signalling was required for NOD2-mediated IKK β activation, which could, in turn, contribute to the TPL2 phosphorylation observed. We found that PKC α and PKC β , but not PKC ζ , partially contributed to IKK β activation (figure 4H). Taken together, we now uncover that in addition to the requirement for IKK β for TPL2 phosphorylation, IKK α and IKK γ as well as classical and atypical PKCs are required for optimal NOD2-induced TPL2 phosphorylation.

TPL2 is required for optimal NOD2-initiated ERK, JNK and NF κ B pathway activation

We next asked how TPL2 regulates NOD2-induced signalling pathways involved in cytokine secretion in human MDMs. These include the MAPKs ERK, p38 and JNK and the NF κ B pathway.^{31, 33–35} Consistent with the role for TPL2 in activation of ERK in rodent cells,^{6–8, 15} TPL2 knockdown dramatically attenuated ERK activation following NOD2 stimulation in primary human MDMs (figure 5A). How TPL2 activates additional signalling pathways leading to cytokine secretion is more controversial, and is likely dependent on cell subset, species and stimulus. TPL2 had no effect on JNK, p38 or NF κ B signalling in TLR4-stimulated mouse macrophages⁸ or human monocytes,¹⁶ but was required for JNK and NF κ B, but not p38 activation in TNF-stimulated mouse fibroblasts.¹⁷ We found that TPL2

knockdown in human MDMs partially decreased NOD2-induced JNK and NF κ B activation (figure 5A, B), but had no significant effect on NOD2-induced p38 activation (figure 5A). Taken together, in addition to its essential role in PRR-induced ERK activation, TPL2 also contributes to JNK and NF κ B pathway activation in human MDMs.

TPL2 is required for NOD2-induced rapid caspase-1 and caspase-8 activation and subsequent early autocrine IL-1 β and IL-18 secretion

Given the dramatic decrease in NOD2-induced cytokine secretion in TPL2-deficient MDMs (figure 2B) and our previous findings that early autocrine IL-1 β and IL-18 are critical for optimal NOD2-induced cytokines,^{27, 36, 37} we questioned if TPL2 contributes to NOD2-induced early autocrine IL-1 β and IL-18. A role for TPL2 in the rapid post-translational pathways leading to these early autocrine loops has not yet been reported. We prevented early (15 min) autocrine IL-1 β and IL-18 consumption through IL-1R and IL-18RAP blockade, respectively, so as to enable detection of these cytokines in supernatants at this early time. Early IL-1 β and IL-18 secretion was abolished in NOD2-stimulated MDMs upon TPL2 knockdown (figure 6A) or following TPL2 inhibition (figure 6B). Consistently, TPL2 knockdown eliminated intracellular levels of mature IL-1 β and IL-18 (figure 6C). Caspase-1 activation commonly contributes to IL-1 β and IL-18 maturation,³⁸ and we have found it is required for these outcomes upon NOD2 stimulation in human MDMs.^{27,37} In addition to caspase-1, we recently found that caspase-8 also contributes to NOD2-mediated IL-1 β secretion.³⁹ We further found that caspase-8 was required for optimal early autocrine IL-18 secretion (figure 6D). We therefore examined TPL2 regulation of these caspases. TPL2 was required for NOD2-induced activation of both caspase-1 and caspase-8 (figure 6E). NOD2-induced ERK and JNK activation are completely and partially dependent on TPL2, respectively (figure 5). Further, ERK has been recently shown in TLR4-stimulated human myeloid cells to contribute to caspase-1 activation.⁴⁰ We found that upon either ERK or JNK inhibition, early IL-1 β and IL-18 cleavage and secretion was partially attenuated (figure 6B, C). Importantly, combined ERK and JNK inhibition completely abolished early IL-1 β and IL-18 cleavage and secretion, similar to what was seen following TPL2 inhibition (figure 6B, C). Consistently, inhibition of ERK or JNK alone partially inhibited, whereas combined inhibition of ERK and JNK completely eliminated both caspase-1 and caspase-8 activation (figure 6E). Inhibition of TPL2, ERK or JNK did not affect cell death (data not shown). As NOD2-induced autocrine IL-1 β and IL-18 secretion amplifies secretion of additional cytokines,^{27, 37} we asked if rescuing ERK and JNK activation in TPL2-deficient MDMs (figure 6F) was sufficient to restore early autocrine (figure 6G) and long-term cytokine (figure 6H) secretion following NOD2 stimulation. We found that this was the case (figure 6G, H). Taken together, NOD2-induced TPL2 activation and TPL2-dependent ERK and JNK activation are required for caspase-1 and caspase-8 activation which leads to early autocrine IL-1 β and IL-18 and long-term cytokine secretion.

TPL2 is required for pathogen-induced IL-1 β and IL-18 autocrine loops and cytokine amplification in primary human intestinal myeloid cells

To our knowledge, TPL2 contributions to human intestinal myeloid cell responses have not been defined. Given the *TPL2* genetic association to IBD, we questioned if TPL2 is required for intestinal myeloid cell cytokine secretion. Intestinal macrophages secrete minimal

cytokines upon PRR stimulation, yet they secrete IL-1 β following exposure to select pathogens, such as *S. typhimurium*.^{36, 41} This autocrine IL-1 β can then amplify secretion of additional cytokines, such as IL-8 (figure 7A).³⁶ To our knowledge, whether autocrine IL-18 secretion contributes to pathogen-induced cytokine secretion in intestinal macrophages has not been reported. We found that IL-18R blockade decreased *S. typhimurium*-induced IL-1 β and IL-8 secretion in human intestinal myeloid cells (figure 7A), thereby highlighting an important role for both autocrine IL-1 β and IL-18 in mediating *S. typhimurium*-induced outcomes (figure 7A). A similar critical role for autocrine IL-1 β and IL-18 in *S. typhimurium*-induced cytokines was observed in peripheral MDMs (figure 7B). Moreover, TPL2 activation was required for *S. typhimurium*-induced cytokine secretion in human intestinal myeloid cells (figure 7A), paralleling the results observed in peripheral MDMs (figure 7B). Taken together, TPL2 is required for cytokine secretion from human intestinal myeloid cells upon exposure to pathogenic bacteria.

rs1042058/rs2907 G risk carrier MDMs from healthy individuals and patients with Crohn's disease show increased TPL2 expression, increased NOD2-induced TPL2, ERK, JNK and I κ B α activation and early autocrine IL-1 β and IL-18 relative to AA carriers

As MDMs from rs1042058 GG carriers showed increased PRR-induced cytokine expression (figure 1), we next questioned the mechanism for this genotype-dependent regulation. The rs1042058 polymorphism results in a synonymous mutation, which is in linkage disequilibrium with *TPL2* intronic polymorphisms, in particular rs2907, which could putatively affect TPL2 mRNA expression. Consistent with the cytokine secretion results in figure 1, we found that rs1042058/rs2907 GG MDMs from healthy controls showed increased TPL2 mRNA (figure 8A) and protein (figure 8B) expression compared with AA carrier cells, with the heterozygote carriers showing an intermediate expression level. Furthermore, rs1042058/rs2907 GG carrier MDMs exhibited increased NOD2-induced TPL2 activation compared with AA carriers (figure 8C). Moreover, compared with rs1042058/rs2907 AA carrier MDMs, cells from GG carriers showed higher NOD2-induced activation of ERK, JNK and NF κ B pathways (figure 8D), consistent with the ability of TPL2 to regulate these signalling pathways in human MDMs (figure 5). Finally, given the involvement of TPL2 in PRR-mediated secretion of early autocrine IL-1 β and IL-18 (figures 6 and 7), we assessed the genotype-dependent regulation of these early cytokines in NOD2-stimulated MDMs. rs1042058/rs2907 GG carrier MDMs secreted higher levels of early IL-1 β and IL-18 compared with AA carrier cells (figure 8E). We next examined if the rs1042058/rs2907 polymorphism regulates the above outcomes in MDMs from patients with Crohn's disease (see online supplementary table S1 for patient information). Similar to healthy controls, rs1042058/rs2907 G-allele carrying MDMs from patients with Crohn's disease showed increased TPL2 mRNA (figure 9A) and protein (figure 9B) expression, increased NOD2-induced ERK, JNK and NF κ B pathway activation (figure 9C), early autocrine IL-1 β and IL-18 secretion (figure 9D) and long-term secretion of additional cytokines (figure 9E). Taken together, rs1042058/rs2907 GG risk carrier MDMs from healthy individuals and patients with Crohn's disease show increased TPL2 expression and PRR-induced TPL2 activation, along with increased activation of downstream signalling pathways and early autocrine IL-1 β and IL-18 secretion.

DISCUSSION

Intestinal immune homeostasis is dependent on proper responses to microbial products.^{1–3} In this study using myeloid cells from a large cohort of individuals, we show that IBD-risk rs1042058 GG carriers in *TPL2* secrete significantly higher cytokines upon stimulation of multiple PRRs. We further define mechanisms regulating pathways both upstream and downstream of TPL2 in primary human MDMs which contribute to TPL2-mediated outcomes. We find that PKCs, IKK α , IKK β and IKK γ are necessary for optimal TPL2 activation in human MDMs. We further establish that upon NOD2 stimulation, TPL2, in turn, is critical for ERK and for optimal JNK and NF κ B activation. We uncover a previously undefined role for TPL2 in activating caspase-1 and caspase-8 upon NOD2 stimulation. This activation results in early autocrine IL-1 β and IL-18 secretion, which then amplifies additional NOD2-induced cytokines in MDMs and human intestinal myeloid cells. Combined ERK and JNK activation (activated downstream of TPL2) were similarly required for NOD2-induced caspase-1 and caspase-8 activation; rescuing ERK and JNK activation in TPL2-deficient MDMs restored early IL-1 β and IL-18 secretion and long-term secretion of additional cytokines. Importantly, we identify that upon pathogenic infection of intestinal myeloid-derived cells, TPL2 is required for autocrine IL-1 β , which in turn amplifies secretion of other cytokines, thereby elucidating a previously undefined role for TPL2 in intestinal myeloid-derived cell responses. Finally, MDMs from rs1042058 IBD-risk *TPL2* carriers from both healthy individuals and patients with Crohn's disease express higher TPL2 mRNA and protein and demonstrate increased NOD2-induced activation of TPL2, ERK, JNK and NF κ B, and early autocrine IL-1 β and IL-18 secretion. Taken together, we identify mechanisms through which TPL2 contributes to PRR-initiated outcomes and determine that the rs1042058 IBD-risk polymorphism in *TPL2* is a gain-of-function resulting in increased PRR signalling and cytokine secretion (figure 9F).

Previous studies have implicated IKK β as contributing to activating TPL2 phosphorylation on Thr290;¹² we find this to be the case in primary human MDMs (figure 4D). In addition, we now demonstrate that IKK α and IKK γ are similarly required for TPL2 activation (figure 4D). These three kinases are present in a complex, and we find that in most cases the lack of each kinase leads to suboptimal phosphorylation of the other two upon PRR stimulation (figure 4E), indicating that coregulation of these IKK family members may ultimately contribute to optimal TPL2 phosphorylation. A role for PKCs in TPL2 activation has to our knowledge not been reported, and we now determine that conventional PKCs and the atypical PKC ζ are required for TPL2 phosphorylation (figure 4G). These results are consistent with the Thr290 on TPL2 being within a PKC consensus motif. As the classical PKCs partially regulated IKK β activation in MDMs, it is possible that they also mediate TPL2 activation indirectly. In contrast, PKC ζ did not regulate IKK β activation (figure 4H). Given that TPL2 activation is central for the proper regulation of multiple inflammatory pathways, elucidating how TPL2 is regulated is of importance in elucidating inflammatory outcomes.

The role of MAPKs in regulating proinflammatory caspases has not been well explored. A recent study found that TLR4-mediated ERK activation in human monocytes results in NLRP3 inflammasome activation and IL-18 processing; reactive oxygen species were

proposed to mediate this regulation.⁴⁰ Another study identified cross-talk between the inflammasome component apoptosis-associated speck-like protein (ASC) and ERK in mouse macrophages,⁴² and p38 has been implicated in activating caspase-1 in THP-1 cells.⁴³ To our knowledge, we identify a previously undefined role for TPL2 and JNK in also contributing to PRR-mediated caspase-1 as well as to caspase-8 activation, thereby leading to early autocrine IL-1 β and IL-18 secretion. Optimal TPL2-mediated IL-1 β and IL-18 secretion depends on the combined activation of ERK and JNK (figure 6). We previously found that this early IL-1 β and IL-18 secretion can then further dramatically amplify PRR-induced MAPK signalling and cytokine secretion.^{27, 37} As a result, these combined TPL2-associated processes lead to a feedforward loop. Therefore, we uncover that in addition to ERK, TPL2 and JNK mediate PRR-induced early IL-1 β and IL-18 processing and secretion through caspase-1 and caspase-8 activation; these cytokines, in turn, contribute to long-term cytokine secretion.

To our knowledge we have identified a previously undefined role for TPL2 in intestinal myeloid cells. We find that pathogen-induced TPL2 activation is required for autocrine IL-1 β , which leads to subsequent cytokine amplification in human intestinal myeloid-derived cells. We further find that autocrine IL-18 also amplifies subsequent cytokine secretion in these cells. Consistent with this, TPL2 activation contributes to experimental colitis in mice.^{10, 11} This contribution has been attributed to a TPL2-mediated increase in both innate and adaptive immunity. Corroborating these results, we find that the IBD-associated polymorphism in *TPL2* leads to increased TPL2 activation in myeloid-derived cells. In contrast, TPL2 in myofibroblasts protects from epithelial injury-induced colitis.⁴⁴ Therefore, given the distinct role of TPL2 in different cell types during various forms of intestinal inflammation, it will be important to clearly dissect the outcomes of TPL2 signalling in a variety of IBD-relevant cell types.

While intermediates in the TPL2 signalling pathway have undergone clinical trials for immune-mediated diseases,⁴⁵ direct TPL2 inhibitors have not been reported thus far. However, pre-clinical trials in animal models show that TPL2 contributes to inflammatory diseases,^{46–48} including experimental colitis in mouse models.¹⁰ Given our results showing that IBD-risk polymorphisms in *TPL2* result in increased TPL2 expression and activation, thereby increasing PRR-induced inflammatory outcomes, and our findings that TPL2 regulates inflammatory outcomes in human intestinal myeloid cells, this molecule could prove to be a promising target in IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance of this study

What is already known about this subject?

- ▶ The rs1042058 IBD-risk polymorphism is in a region that includes the *TPL2* gene.
- ▶ TPL2 regulates ERK signalling in pattern recognition receptor (PRR)-activated mouse macrophages.
- ▶ IKK β can phosphorylate TPL2.
- ▶ TPL2 activation contributes to inflammation in mouse models of colitis.

What are the new findings?

- ▶ TPL2 phosphorylation requires PKC, as well as IKK β , IKK α and IKK γ signalling.
- ▶ TPL2 is required for nucleotide-oligomerisation domain (NOD)2-initiated ERK, JNK and NF κ B signalling in human macrophages.
- ▶ NOD2-induced TPL2 activation leads to rapid ERK/JNK-dependent caspase-1 and caspase-8 activation, resulting in early autocrine interleukin (IL)-1 β and IL-18 secretion, and amplification of additional cytokines in human macrophages.
- ▶ TPL2 is required for *Salmonella typhimurium*-induced cytokine secretion in human intestinal myeloid cells.
- ▶ Monocyte-derived macrophages (MDMs) from rs1042058 GG carriers express increased TPL2, and demonstrate increased NOD2-initiated TPL2 phosphorylation, ERK, JNK and NF κ B activation, and early IL-1 β and IL-18 secretion, and ultimately dramatically increased PRR-induced cytokine secretion.

How might it impact on clinical practice in the foreseeable future?

- ▶ This study identifies an IBD-associated *TPL2* risk polymorphism as a gain-of-function leading to increased PRR-induced cytokines, thereby highlighting its potential utility as a therapeutic target in IBD.

Figure 1.

Primary human myeloid cells from IBD-associated rs1042058 GG carriers in *TPL2* demonstrate increased cytokine secretion upon PRR stimulation. Human MDMs (n=100) were treated for 24 h with (A and E) 1, 10 or 100 μg/mL MDP, or (B and F) 1, 10 or 100 μg/mL Pam₃Cys. Human MDDCs (n=98) were treated for 24 h with 1 μg/mL MDP (NOD2 ligand) and 1 μg/mL Pam₃Cys (TLR2 ligand), 0.1 μg/mL polyI:C (TLR3 ligand), 0.01 μg/mL lipid A (TLR4 ligand), 0.5 ng/mL flagellin (TLR5 ligand), 0.1 μg/mL CL097 (TLR7 ligand) or 0.1 μg/mL CpG DNA (TLR9 ligand) for 24 h alone (C and G) or in combination (D and H). Fold TNF-α (A–D) or IL-1β (E–H) induction (log₂ transformed) upon PRR stimulation stratified on rs1042058 genotype+SEM. *p<0.05; **p<0.01; ***p<0.001; †p<1×10⁻⁴; ††p<1×10⁻⁵. IL, interleukin; MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; NOD, nucleotide-oligomerisation domain; PRR, pattern-recognition receptor; TNF, tumour necrosis factor.

Figure 2.

TPL2 signalling is required for optimal PRR-mediated cytokine secretion. (A) MDMs (n=4) were transfected with scrambled (solid line) or TPL2 (dotted line) siRNA. Representative flow cytometry with TPL2 mean fluorescence intensity (MFI) values. Isotype control (grey shading). Summarised data of TPL2 MFI+SEM. (B) MDMs (n=4) were transfected with scrambled or TPL2 siRNA for 48 h, then treated with 100 μ g/mL MDP for 24 h. (C) MDMs (n=4) were preincubated with 50 nM TPL2 inhibitor, then treated with 100 μ g/mL MDP for 24 h. (D) MDMs (n=4) were transfected with scrambled or TPL2 siRNA for 48 h, then treated with 100 μ g/mL Tri-DAP, 10 μ g/mL Pam₃Cys, 100 μ g/mL poly I:C, 0.1 μ g/mL lipid A, 5 ng/mL flagellin, 1 μ g/mL CL097 or 10 μ g/mL CpG DNA for 24 h. (B–D) Shown is cytokine secretion +SEM. *p<0.05; **p<0.01; ***p<0.001; †p<1 $\times$ 10⁻⁴; ††p<1 $\times$ 10⁻⁵. IL, interleukin; MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; PRR, pattern-recognition receptor; scr, scrambled; TNF, tumour necrosis factor; Tx, treatment.

Figure 3.

NOD2 stimulation regulates TPL2 expression in human MDMs. (A and B) MDMs (n=8) were treated for the indicated time points with 100 μ g/mL MDP. (A) Representative flow cytometry plots with TPL2 MFI values. Isotype control (grey shading). (B) Summary TPL2 MFI+SEM. (C) MDMs (n=8) were treated for the indicated time points with 100 μ g/mL MDP. Fold TPL2 mRNA induction normalised to untreated cells (represented by the dotted line at 1)+SEM. (D) MDMs (n=8) were treated for the indicated time points with 100 μ g/mL Pam₃Cys, 0.1 μ g/mL lipid A or 5 ng/mL flagellin. TPL2 MFI+SEM. *p<0.05; **p<0.01; ***p<0.001; †p<1 $\times$ 10⁻⁴; ††p<1 $\times$ 10⁻⁵. MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; NOD, nucleotide-oligomerisation domain; Tx, treatment.

Figure 4.

PKC and IKK signalling are required for optimal TPL2 activation. (A) MDMs (n=4) were treated for the indicated time points with 100 μ g/mL MDP. *Left*: Representative flow cytometry plots with phospho-TPL2 and TPL2 MFI values. Isotype control (grey shading). *Right*: Summary graphs of phospho-TPL2 and TPL2 MFI+SEM. Similar results were observed in an additional n=4. (B) Summarised data showing the ratio of fold phospho-TPL2 induction to total TPL2 normalised to untreated levels (dotted line at 1)+SEM. (C–E) MDMs were transfected with scrambled, IKK α , IKK β or IKK γ siRNA for 48 h. (C) Change

in mRNA expression compared with cells transfected with scrambled siRNA (represented by the dotted line at 1)+SEM (n=4). (D–E) Cells were treated for 30 min with 100 µg/mL MDP. (E, *Left*): Representative flow cytometry plots with phospho-IKKα, IKKβ or IKKγ values. Isotype control (grey shading). (D–E, *Right*): Summary graph of phospho-TPL2, IKKα, IKKβ or IKKγ MFI+SEM (n=4 for each). (F–H) MDMs were transfected with scrambled, PKCα, PKCβ or PKCγ siRNA for 48 h. (F) Change in mRNA expression compared with cells transfected with scrambled siRNA (represented by the dotted line at 1)+SEM (n=4). (G) MDMs were treated for 30 min with 100 µg/mL MDP. Summary graphs of phospho-TPL2 and TPL2 MFI+SEM (n=4). (H) MDMs were treated for 30 min with 100 µg/mL MDP. *Left*: Representative flow cytometry plots with phospho-IKKβ values. Isotype control (grey shading). *Right*: Summary graphs of phospho-IKKβ MFI+SEM (n=4). *p<0.05; **p<0.01; ***p<0.001; †p<1×10⁻⁴; ††p<1×10⁻⁵. MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; scr, scrambled; Tx, treatment.

Figure 5.

TPL2 is required for optimal NOD2-initiated ERK, JNK and NFκB signalling in human MDMs. MDMs (n=8) were transfected with scrambled or TPL2 siRNA for 48 h. Cells were then treated with 100 μg/mL MDP for 15 min. *Left:* Representative flow cytometry plots with MFI values for (A) phospho-ERK, phospho-p38, phospho-JNK, or (B) phospho-IκBα. MDP-stimulated isotype controls (grey shading). *Right:* Summarised data showing the fold phospho-protein induction normalised to untreated cells (dotted line at 1)+SEM. Similar results were observed in an additional n=8 for phospho-ERK and phospho-JNK.

***p<0.001; †p<1×10⁻⁴. MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; NOD, nucleotide-oligomerisation domain; Scr, scrambled; Tx, treatment.

Figure 6.

TPL2-dependent ERK and JNK activation is required for NOD2-induced caspase-1 and caspase-8 activation and early autocrine IL-1 β and IL-18 secretion. (A) MDMs (n=4) were transfected with scrambled or TPL2 siRNA for 48 h. Cells were then preincubated with 0.5 μ M IL-1Ra (prevents early IL-1 β consumption) or 300 ng/mL anti-IL-18RAP (prevents early IL-18 consumption) or the appropriate isotype control and treated with 100 μ M MDP for 15 min. Shown is cytokine secretion+SEM. (B) MDMs (n=4) were preincubated for 1 h with 0.5 μ M IL-1Ra or 300 ng/mL anti-IL-18RAP to prevent consumption of IL-1 β or IL-18, respectively, and 50 nM TPL2 inhibitor, 0.1 μ M PD98059 (inhibits ERK activation) and/or 0.1 μ M JNK inhibitor II, and then treated with 100 μ M MDP for 15 min. Shown is cytokine secretion+SEM. Similar results were seen in an additional n=4. (C and E) MDMs were preincubated with 50 nM TPL2 inhibitor, 0.1 μ M PD98059 and/or 0.1 μ M JNK inhibitor II, then treated with 100 μ M MDP for 15 min. (C) Mature IL-1 β and

IL-18 expression from two donors, or (E) cleaved caspase-1 and caspase-8 expression from two donors by western blot. GAPDH was assessed on the same blot. (D) MDMs (n=4) were preincubated with 300 ng/mL anti-IL-18RAP±50 µM caspase-8 inhibitor, then treated with 100 µg/mL MDP for 15 min. Shown is IL-18 secretion+SEM. (F) MDMs (n=8) were transfected with scrambled or TPL2 siRNA±4 µg pMCL-MKK1 (R4F) (constitutively active ERK kinase, ca-ERK) and pSRα-3HA-JNKK2-JNK1-WT (constitutively active JNK, ca-JNK) or EV for 48 h, and then treated with 100 µg/mL MDP for 15 min and analysed by flow cytometry for phosphokinase induction. (*Left*) Representative histograms with indicated MFI and isotype controls (solid grey histogram). (*Right*) Fold MAPK activation normalised to EV, scrambled siRNA-transfected cells (represented by the dotted line at 1)+SEM. (G and H) MDMs were transfected as in (F), and (G) preincubated with 0.5 µg/mL IL-1Ra or 300 ng/mL anti-IL-18RAP to prevent IL-1β or IL-18 consumption, respectively, and treated with 100 µg/mL MDP for 15 min (n=8), or (H) treated with 100 µg/mL MDP for 24 h (n=8). Cytokine secretion+SEM. **p<0.01; ***p<0.001; ††p<1×10⁻⁵. EV, empty vector; IL, interleukin; MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; NOD, nucleotide-oligomerisation domain; Tx, treatment.

Figure 7.

TPL2 is required for IL-1 β secretion and amplification of cytokine secretion in *Salmonella typhimurium*-treated human intestinal myeloid cells. (A) Human intestinal myeloid cells (n=4) or (B) Peripheral MDMs (n=4) were preincubated with 50 nM TPL2 inhibitor (TPL2 inh), 0.5 μ g/mL IL-1Ra or 300 ng/mL anti-IL-18RAP, and then treated with *S. typhimurium* (*S. typh*) at MOI 10:1 for 24 h. Shown is cytokine secretion+SEM. [†]p<1 $\times$ 10⁻⁴; ^{††}p<1 $\times$ 10⁻⁵. IL, interleukin; Inh, inhibitors; MDMs, monocyte-derived macrophages; MOI, multiplicity of infection; Tx, treatment.

Figure 8.

MDMs from healthy control rs1042058/rs2907 G risk carriers express increased TPL2 mRNA and protein and show increased NOD2-induced TPL2, ERK, JNK and NFκB pathway activation and early autocrine IL-1β and IL-18 secretion relative to AA carriers. MDMs from healthy control rs1042058/rs2907 carriers (n=8/genotype) were assessed as follows: (A) TPL2 mRNA expression (change in CT values normalised to GAPDH and represented as a linear scale)+SEM. (B) Representative flow cytometry plots with MFI values and summarised data for TPL2 protein expression+SEM. (C) MDMs were left

untreated or treated with 100 µg/mL MDP for 30 min. Representative flow cytometry plots with MFI values and summarised data for phospho-TPL2 MFI+SEM. (D) MDMs were left untreated or treated with 100 µg/mL MDP for 15 min. Representative flow cytometry plots with MFI values and summarised data for fold phospho-ERK, -JNK or -IκBα induction normalised to untreated cells+SEM. (E) MDMs were preincubated with 0.5 µg/mL IL-1Ra (prevents early IL-1β consumption) or 300 ng/mL anti-IL-18RAP (prevents early IL-18 consumption) and then treated with 100µg/mL MDP for 15 min. Shown is cytokine secretion +SEM. *p<0.05; **p<0.01; ***p<0.001; †p<1×10⁻⁴; ††p<1×10⁻⁵. IL, interleukin; MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; NOD, nucleotide-oligomerisation domain; Tx, treatment.

Figure 9.

MDMs from rs1042058/rs2907 G risk patients with Crohn's disease express increased TPL2 mRNA and protein and show increased NOD2-induced ERK, JNK and NFκB pathway activation, early autocrine IL-1β and IL-18 secretion and late secretion of additional cytokines relative to AA carriers. MDMs from rs1042058/rs2907 GG, GA or AA carrier patients with Crohn's disease (n=8/genotype) were assessed as follows: (A) TPL2 mRNA expression (change in CT values normalised to GAPDH and represented as a linear scale) +SEM. (B) Summarised flow cytometry data for TPL2 protein expression+SEM. (C) Cells were left untreated or treated with 100 µg/mL MDP for 15 min. Summarised flow cytometry data for fold phospho-ERK, -JNK or -IκBα induction normalised to untreated cells+SEM. (D) Cells were preincubated with 0.5 µg/mL IL-1Ra (prevents early IL-1β consumption) or 300 ng/mL anti-IL-18RAP (prevents early IL-18 consumption) and then treated with 100 µg/mL MDP for 15 min. Cytokine secretion +SEM. (E) Cells were treated with 100 µg/mL MDP for 24 h. Cytokine secretion+SEM. *p<0.05; **p<0.01; ***p<0.001; †p<1×10⁻⁴; ††p<1×10⁻⁵. (F) A model for the PRR-mediated activation of TPL2 and the resulting downstream responses. MDP stimulation of NOD2 in MDMs activates PKCs, and PKCα,

PKC β and PKC ζ are each required for optimal TPL2 phosphorylation on Thr290 (required for TPL2 activation). NOD2-induced activation of IKK α , IKK β and IKK γ is also required for optimal TPL2 Thr290 phosphorylation. TPL2 phosphorylation is accompanied by the degradation of total protein, such that TPL2 expression is decreased early after stimulation of NOD2 and other PRRs. Following phosphorylation, TPL2 activates the ERK, JNK and NF κ B pathways downstream of multiple PRR. ERK and JNK signalling, in turn, contributes to the activation of caspase-1 and caspase-8, and the subsequent rapid post-translational processing and secretion of early autocrine IL-1 β and IL-18 (within 15 min of NOD2 activation). Autocrine IL-1 β and IL-18 signalling, in turn, amplifies PRR-initiated signalling and secretion of additional cytokines. Relative to A carriers, rs1042058 G disease risk carriers from both healthy individuals and patients with Crohn's disease express increased TPL2 and demonstrate increased PRR-induced TPL2 phosphorylation, ERK, JNK and NF κ B signalling, early IL-1 β and IL-18 secretion, and long-term cytokine secretion. IL, interleukin; MDMs, monocyte-derived macrophages; MDP, muramyl dipeptide; NOD, nucleotide-oligomerisation domain; PRR, pattern-recognition receptor; Tx, treatment.