Chronic stimulation of Nod2 mediates tolerance to bacterial products

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The Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (Nod) families of proteins are critical for bacterial recognition, and, acutely, this frequently leads to proinflammatory responses. Polymorphisms in Nod2 (CARD 15) are associated with an increased likelihood of developing Crohn's disease. However, it is not yet clear how Nod2 dysfunctions lead to defects in human intestinal immune homeostasis. Studies to date have focused on functions after acute, rather than chronic, Nod2 stimulation. However, the intestine is an environment of chronic bacterial product exposure with tolerance to luminal flora. We therefore hypothesized that long-term Nod2 stimulation contributes to down-regulation of inflammatory responses from innate immune receptors. We found that pretreatment with muramyl dipeptide (MDP), a ligand for Nod2, significantly decreased production of the proinflammatory cytokines TNF- α , IL-8, and IL-1 β upon Nod2, TLR4, and TLR2 restimulation in primary human monocyte-derived macrophages from a large cohort of individuals. Importantly, TNF- α -induced production of proinflammatory cytokines remained intact in these same cells. MDP-stimulated macrophages from Crohn's disease-relevant Leu1007insC Nod2 homozygote individuals were deficient in their ability to cross-tolerize to subsequent treatment with TLR2 and TLR4 ligands. We show that acute Nod2 stimulation induced IRAK-1 activation, and that chronic MDP treatment down-regulated IRAK-1 activation upon Nod2 or TLR4 restimulation. In a subset of individuals, chronic Nod2 stimulation induced expression of the IRAK-1 inhibitory protein IRAK-M. Significantly, intestinal macrophages exhibit tolerance to MDP per production of inflammatory cytokines. These results illustrate a role for chronic stimulation of Nod2 in mediating tolerance to bacterial products.

cytokines | human | inflammation | intestine | macrophages

Crohn's disease is an inflammatory bowel disorder associated with multiple genetic and environmental factors (1). Leu1007insC, one of the three major polymorphisms in the Nod2 gene, is associated with a 17-fold higher risk of Crohn's disease in homozygous carriers (2–4). Nod2 is a cytoplasmic protein expressed in various cell types, including monocyte-derived cells (5). The leucine-rich repeat (LRR) domain of Nod2 is required for signaling in response to muramyl dipeptide (MDP), the minimal component of bacterial peptidoglycan (PGN) able to specifically activate Nod2 (6). MDP stimulation of Nod2 acutely results in activation of NF- κ B and induction of proinflammatory cytokines, and these processes are severely impaired in Nod2 Leu1007insC homozygous individuals (2, 7, 8). Protein levels of Nod2 in individuals with the Leu1007insC mutation are, however, comparable to levels expressed by individuals with wild-type Nod2 (9). Hypothesized Nod2 functions in intestinal immune homeostasis include its role in (i) induction of proinflammatory cytokines necessary for intestinal defenses, in particular in combination with other pattern-recognition receptor (PRR) family members (8, 10); (ii) down-regulation of Th1 cytokines (11); and (iii) up-regulation of antimicrobial peptides (12, 13). However, it is still unclear whether defects in any, or all, of these proposed Nod2 functions lead to an increased susceptibility to Crohn's disease. Importantly, cytokine induction has been studied in the context of acute Nod2 stimulation, whereas

the intestine is an environment of chronic exposure to Nod2 ligands.

The intestinal immune system is able to maintain a controlled inflammatory environment despite the continual exposure to high levels of bacteria. One mechanism contributing to this is the relative tolerance to bacterial products exhibited by intestinal macrophages (14). It is unknown whether Nod2 contributes to the process of intestinal tolerance, an important question given the relevance of this protein to Crohn's disease, characterized by dysregulated intestinal immune homeostasis and loss of tolerance. In particular, although there are a number of studies reporting the effects of acute stimulation through Nod2 (3, 6–8, 10, 11, 13, 15–19), the consequences of chronic stimulation through Nod2 are not known.

To determine the consequences of chronic stimulation through Nod2, we stimulated primary human monocyte-derived macrophages for prolonged periods of time with MDP. We find that MDP pretreatment substantially reduces the production of TNF- α , IL-8, and IL-1 β after restimulation not only with MDP but also with Toll-like receptor (TLR) 2 and TLR4 ligands in macrophages from a large cohort of individuals. This down-regulation is not observed in macrophages harboring the Leu1007insC Nod2 variant, indicating that the intact wild-type LRR region is required for MDP-mediated tolerance.

Results

Prolonged Exposure of Primary Monocyte-Derived Macrophages to MDP Inhibits Proinflammatory Responses of Nod2 and TLR4 Pathways.

We sought to determine the consequences of chronic stimulation of Nod2 on subsequent activation of Nod2 and TLR pathways. To avoid unintended TLR ligand contaminants (8), we used synthetic Nod2 and TLR ligands. The synthetic Nod2 ligand MDP specifically activates Nod2 as demonstrated by a number of approaches (3, 6, 7, 11, 20). To determine the effects of chronic Nod2 stimulation on MDP-induced cytokine production, we treated macrophages from control individuals with MDP for times ranging from 3 to 48 h. TNF- α (Fig. 1A), IL-8, and IL-1 β [supporting information (SI) Fig. 6A] secretion was then measured 24 h after MDP retreatment. Consistent with previous reports (21), we observed variability in the absolute levels of cytokine secretion upon acute stimulation of macrophages among individuals. However, within 3 h, cells pretreated with MDP showed a decrease in proinflammatory cytokine production upon restimulation with MDP in the majority of individuals. This attenuation was significantly enhanced with longer preincubations in each of the individuals assessed (Fig. 1A).

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Fig. 1. Prolonged pretreatment with MDP induces self-tolerance and cross-tolerance to lipid A but not to TNF- α stimulation in primary monocyte-derived macrophages. Human primary macrophages from control individuals were left untreated or stimulated with 100 μ g/ml MDP for the indicated time periods, washed, and restimulated for an additional 24 h with 100 μ g/ml MDP (A) or 0.1 μ g/ml synthetic lipid A (B). Supernatants were assayed for TNF- α . Controls include 100 μ g/ml MDP pretreatment for each of the indicated time periods followed by washes alone. The average is shown by a black line. The data are representative of 4 of 4 (A) or 4 of 12 (B) individuals. (C) Macrophages from control individuals were left untreated or stimulated with 100 μ g/ml MDP for 48 h, washed, and restimulated for an additional 24 h with 10 ng/ml TNF- α . Supernatants were assayed for IL-8. The data are representative of 4 of 18 individuals. tx, treatment; M, MDP; L, lipid A; T, TNF- α .

Because bacteria are able to activate multiple PRRs (22), we determined whether chronic stimulation through Nod2 down-regulates responses from the receptor for LPS, TLR4. Synergy in cytokine production has been previously described after concomitant stimulation of Nod2 and other pathways, such as TLR4 (8, 11, 18), and we observed a similar synergistic/additive effect in human macrophages upon simultaneous Nod2 and TLR4 treatment (data not shown). To assess the consequences of long-term stimulation of Nod2 on subsequent TLR4 activation, we stimulated macrophages from control individuals with MDP for time periods ranging from 3 to 48 h and then restimulated the cells with synthetic lipid A, a TLR4 ligand. At early time points, in some individuals, we observed either an additive effect or a synergistic effect between MDP pretreatment and subsequent lipid A stimulation in the production of TNF- α (Fig. 1B), IL-8, or IL-1 β (SI Fig. 6B). However, after 24 h of MDP pretreatment, macrophages from all of the individuals demonstrated an attenuation of proinflammatory cytokines upon lipid A restimulation. This attenuation was even greater by 48 h of MDP pretreatment, and we therefore use this time point in our future studies. We will refer to the down-regulation of Nod2 and TLR-mediated responses upon chronic Nod2 stimulation as Nod2-mediated tolerance. The development of tolerance was not the effect of cell death as assessed by cell counts (data not shown). Importantly, MDP-pretreated macrophages were still responsive to TNF- α stimulation. Specifically, TNF- α -stimulated secretion of IL-8 was comparable whether or not macrophages were pretreated with MDP (Fig. 1C). Taken together, pretreatment of macrophages by MDP induced a time-dependent decrease in proinflammatory cytokine secretion upon subsequent MDP or lipid A stimulation.

We next established that whereas a 48-h pretreatment at the lowest dose of MDP assessed (1 μ g/ml) completely abrogated the subsequent MDP-induced TNF- α , IL-8, and IL-1 β production (data not shown), responses to lipid A after MDP pretreatment showed a dose-dependent effect in TNF- α (SI Fig. 7), IL-8, and IL-1 β production (data not shown). We selected a dose of 100 μ g/ml MDP for our subsequent tolerance experiments because this dose effectively induced tolerance, has been used in other studies (11, 23), and approximates the levels of muramic acid seen in colon (24).

Macrophages Pretreated with MDP Exhibit Tolerance to TLR2 or TLR4 Restimulation, Whereas Macrophages from Leu1007insC Nod2 Homozygotes Fail to Exhibit This Tolerance. The above results indicate that MDP mediates both self-tolerizing effects and cross-

tolerizing effects to TLR4 stimulation. MDP is a breakdown product of bacterial peptidoglycan (PGN), an abundant Gram-positive cell wall component that also contains lipoteichoic acid, a TLR2 ligand. We therefore asked whether MDP pretreatment of macrophages suppresses proinflammatory cytokine production via TLR2 stimulation. In cells from each of the individuals assessed, in addition to inhibiting Nod2- and TLR4-induced proinflammatory cytokine responses, prolonged MDP stimulation significantly reduced the production of TNF- α (Fig. 2A), IL-8, and IL-1 β (data not shown) after TLR2 stimulation by its synthetic ligand, Pam₃Cys-Ser-(Lys)₄ hydrochloride (Pam₃Cys). We show the data for TNF- α because of the important role of this cytokine in Crohn's disease. In addition, we find that the chemokines MCP-1 and MIP-1 β undergo similar MDP-mediated self- and cross-tolerance (data not shown).

We further questioned whether MDP-pretreatment can cross-tolerize to higher doses of Pam₃Cys and lipid A. We find that MDP-pretreatment down-regulated Pam₃Cys and lipid A cytokine responses even at treatment levels as high as 1000 μ g/ml Pam₃Cys and 10 μ g/ml lipid A (SI Fig. 8).

Because Leu1007insC Nod2 homozygous individuals have an increased susceptibility to Crohn's disease, we next questioned whether cells from Leu1007insC homozygous individuals exhibit a defect in MDP-induced cross-tolerance to TLR2 and TLR4 pathways. Primary cells from Leu1007insC Nod2 mutants fail to secrete proinflammatory cytokines upon MDP stimulation (Fig. 2B), consistent with our previous results (7), and thus subsequent tolerance of proinflammatory cytokine secretion to MDP restimulation is not measurable. Secretion of TNF- α (Fig. 2B), IL-1 β , and IL-8 (data not shown) from Leu1007insC-derived macrophages upon stimulation with lipid A or Pam₃Cys is comparable to that of control macrophages. Significantly, Leu1007insC homozygote macrophages do not decrease proinflammatory cytokine levels upon lipid A or Pam₃Cys stimulation after MDP pretreatment, demonstrating that Leu1007insC macrophages have impaired tolerance responses (Fig. 2B). Fig. 2C summarizes the MDP-mediated tolerance in monocyte-derived macrophages from the large number of control individuals compared with Leu1007insC homozygous individuals. The TNF- α response of MDP pretreated cells was attenuated to 8.35% ($n \geq 50$), 21.9% ($n \geq 30$), and 20.3% ($n \geq 50$) upon subsequent restimulation with MDP, Pam₃Cys, and lipid A, respectively, compared with non-MDP-pretreated cells. IL-8 production was similarly attenuated to 21.6%, 23.3%, and 30.8% after MDP, Pam₃Cys, and lipid A retreatments, respectively, whereas IL-1 β levels decreased to 7.68%, 9.75%, and 23.5% for

MDP Pretreatment Reduces IRAK-1 Activation. IRAK-1 is a signaling molecule involved in TLR (22) and likely Nod2 pathways (20), and it contributes to induction of proinflammatory cytokines. The ability of Nod2 stimulation to directly induce IRAK-1 kinase activity has not been previously reported. We now find that acute MDP stimulation of primary macrophages induces IRAK-1 kinase activation with slightly varying kinetics across individuals but is optimal at 40 min after stimulation (SI Fig. 9A). In light of the down-regulation of proinflammatory cytokine secretion upon chronic Nod2 stimulation, we next questioned whether chronic Nod2 stimulation decreases IRAK-1 kinase activity. MDP pretreatment reduced IRAK-1 kinase activity in macrophages restimulated with MDP or lipid A from 17 of 24 (70%) or 10 of 21 (48%) individuals, respectively. In cells from individuals that demonstrated down-regulation of IRAK-1 kinase activity after MDP pretreatment, myelin basic protein (MBP) phosphorylation was reduced to 29% of the original levels upon MDP restimulation and 21% of the original levels upon lipid A restimulation (Fig. 3). The reduced IRAK-1 activity is not simply due to delayed kinetics in tolerant cells because MDP pretreatment reduced IRAK-1 activity even 60 min after MDP or lipid A retreatment (SI Fig. 9B and C). Therefore, acute stimulation of Nod2 results in an induction of IRAK-1 kinase activity, whereas chronic Nod2 stimulation results in a persistent reduction of this kinase activity in macrophages restimulated with bacterial products.

IRAK-M Is Up-Regulated in Control Individuals After Prolonged MDP Treatment, and Down-Regulation of IRAK-M Expression Partially Reverses Nod2-Mediated Tolerance. To address the mechanisms contributing to Nod2-mediated tolerance, we examined whether the tolerance is a result of down-regulation of receptors to bacterial products. Surface levels of TLR2 ($n = 8$) and TLR4 ($n = 10$),

Fig. 4. Monocyte-derived macrophages up-regulate IRAK-M upon prolonged treatment with MDP, and silencing of IRAK-M expression reduces Nod2-mediated self-tolerance. (A) Human primary monocyte-derived macrophages were left unstimulated or treated with 100 $\mu\text{g/ml}$ MDP for 48 h, and cell lysates were analyzed by Western blotting for the expression of IRAK-M. GAPDH served as the loading control. Cells from three representative individuals are shown. (B) Human primary macrophages from control individuals were stimulated with 100 $\mu\text{g/ml}$ MDP for 24 h or left untreated, then transfected with siRNA for IRAK-M or control siRNA. Cells were lysed 24 h later and analyzed by Western blotting for IRAK-M expression. GAPDH served as the loading control. Data are representative of eight individuals. (C) Human primary macrophages from control individuals were stimulated with 100 $\mu\text{g/ml}$ MDP for 24 h or left untreated, transfected with scrambled siRNA or siRNA for IRAK-M, and restimulated 24 h later for an additional 24 h with 100 $\mu\text{g/ml}$ MDP or 0.1 $\mu\text{g/ml}$ lipid A. The supernatants were assayed for IL-8. The dotted line represents the normalized level of cytokine secretion by scrambled siRNA-transfected, non-MDP-pretreated cells acutely stimulated for 24 h with MDP or lipid A (= 100%). The data are representative of individuals that demonstrated a $>15\%$ reversal in Nod2-mediated self-tolerance or cross-tolerance to lipid A. tx, treatment; M, MDP; L, lipid A. IL-8 secretion of MDP-pretreated scrambled and siRAK-M-transfected cells was compared, and significance was calculated by using the *t* test. ***, $P < 0.001$.

and expression of Nod2 ($n = 13$) after 48 h of MDP treatment did not exhibit a significant decrease (SI Fig. 10).

Because chronic Nod2 stimulation down-regulates IRAK-1 kinase activity, we questioned whether one of the mechanisms mediating MDP-induced tolerance could be up-regulation of proteins that inhibit IRAK-1 activation, such as IL-1 receptor-associated kinase M (IRAK-M) (25). We observed up-regulation of IRAK-M protein expression upon a 48-h treatment with MDP (Fig. 4A) in monocyte-derived macrophages from 13 of 28 (46%) control individuals up to 4.4-fold.

To further examine the role of IRAK-M in Nod2-mediated tolerance, we assessed whether down-regulation of IRAK-M reversed tolerance. IRAK-M small interfering RNA (siRNA) transfection reduced IRAK-M protein levels to a mean of 40% of control levels (Fig. 4B). We find that macrophages transfected with scrambled siRNA demonstrated Nod2-mediated tolerance (Fig. 4C). However, cells transfected with IRAK-M siRNA demonstrated a reduction of MDP self-tolerance in 14 of 26 (54%) individuals, such that IL-8 increased from 14.8% to 64.2% of the cytokine levels observed upon acute MDP stimulation (Fig. 4C). Similarly, IRAK-M down-regulation resulted in a reduction of MDP to lipid A cross-tolerance in 15 of 26 (58%) individuals, such that IL-8 levels in transfected and restimulated cells increased from 13.5% to 46.2% compared with IL-8 levels of cells stimulated acutely with lipid A (Fig. 4C). Taken together, in a significant percentage of individuals tested, IRAK-M contributes to Nod2-mediated tolerance upon restimulation by both MDP and lipid A.

IL-1 β Autocrine Pathways Contribute to MDP Proinflammatory Responses. Nod2 stimulation produces IL-1 β (SI Fig. 6), and IL-1 β , in turn, signals through IRAK-1 to induce proinflammatory

Fig. 5. Intestinal monocyte-derived cells are tolerant to MDP stimulation but phagocytose bacteria. (A) Human peripheral ($n = 17$) or intestinal ($n = 6$) monocyte-derived cells from control individuals were stimulated for 16 h with 100 $\mu\text{g/ml}$ MDP or 0.1 $\mu\text{g/ml}$ lipid A. Supernatants were collected and assayed for TNF- α by ELISA. M, MDP; L, lipid A. Significance was calculated by using the *t* test comparing untreated cells to those treated with MDP or lipid A. $\dagger$, $P < 1 \times 10^{-5}$. (B) Freshly isolated intestinal cells were cultured for 16 h and incubated with heat-killed FITC-labeled *E. coli*, and monocyte-derived cells were analyzed by flow cytometry. The shaded area represents unlabeled bacteria, and the solid line represents FITC-labeled *E. coli*. Data are representative of six individuals.

cytokines. Given down-regulated IRAK-1 activation in macrophages chronically stimulated through Nod2, we considered that the decreased production of proinflammatory cytokines in these cells may be due in part to down-regulation of possible IL-1 β -mediated autocrine responses. We find that IL-1 β autocrine responses significantly contribute to the proinflammatory cytokines produced upon Nod2 stimulation. Combined treatment with IL-1 receptor antagonist and anti-IL-1 β antibody blocked MDP-induced TNF- α , IL-8, and IL-6 to 22.6%, 24.7%, and 20.4% of the original levels, respectively (SI Fig. 11). We assessed IL-6 responses to confirm the role of IL-1 β in an additional proinflammatory cytokine induced upon Nod2 stimulation. These results indicate that down-regulated IRAK-1 kinase activity may contribute to Nod2-mediated tolerance by decreasing IL-1 β autocrine signaling.

Intestinal Macrophages Exhibit Tolerance to MDP and Lipid A but Phagocytose Bacteria. Given the importance of Nod2 in mediating intestinal immune homeostasis, we questioned whether resident intestinal macrophages are tolerant to Nod2 stimulation. Because the intestinal environment *in vivo* is constantly exposed to bacterial stimulation, we hypothesized that the cytokine response of freshly isolated enteric macrophages would resemble that of peripheral monocyte-derived macrophages chronically stimulated with MDP. In contrast to peripheral-derived macrophages, we observe that acute stimulation with MDP, similar to lipid A, does not induce TNF- α secretion in intestinal macrophages (Fig. 5A). The intestinal macrophages were, however, able to avidly phagocytose bacteria (Fig. 5B), indicating that they maintain specific functional host-defense mechanisms consistent with previous studies (14).

Discussion

A predominant feature of the intestinal immune system is its ongoing adjustment in response to chronic bacterial exposure. As such, the requirements of the innate immune system in maintaining intestinal homeostasis are particularly rigorous be-

cause perturbations in bacterial recognition could disrupt the fine balance between tolerance and inflammation. Support for this concept is the strong association of Nod2 mutations and Crohn's disease, a chronic relapsing inflammation of the intestine. We have defined that chronic stimulation of the Nod2 pathway results in a significant decrease in the production of proinflammatory mediators in response to subsequent Nod2, TLR2, or TLR4 restimulation in primary monocyte-derived macrophages from a large number of individuals. Importantly, TNF- α -induced signaling pathways remain intact, demonstrating selectivity in Nod2-mediated tolerance pathways. The Nod2-initiated tolerance to TLR2 and TLR4 was abolished in cells homozygous for the Crohn's disease-associated Leu1007insC Nod2 mutation. As a result, we have shown that an intact wild-type Nod2 LRR region is required for signals in response not only to the acute activation of proinflammatory cytokines but also to tolerance mechanisms induced upon prolonged Nod2 stimulation. Therefore, in addition to mechanisms such as stromal cell-derived TGF- β (14), our data suggest that Nod2-mediated tolerance may be an alternative or additional mechanism contributing to intestinal macrophage tolerance.

Synergy in cytokine production has been previously described after a concomitant stimulation of Nod2 and TLR4 or TLR2 (8, 23). We observe a synergistic or additive effect with lipid A after pretreatment with MDP for short time periods (i.e., 3 h). Therefore, in the early time period after MDP pretreatment, induced signaling pathways enhance the effects of subsequent TLR stimuli. This may be due to the initial up-regulation of a number of signaling molecules in the pathway including MyD88 (5, 26). In addition, Nod2 interacts with a number of proteins such as procaspase-1, RIP2, and GRIM-19 (27–29), which could participate in proinflammatory pathways separate from TLR signaling. In contrast, upon longer MDP pretreatment, we observed increasing self- and cross-tolerance. This is likely due to the up-regulation of inhibitory signaling molecules such as IRAK-M that begin to dominate at later time points, consistent with what would be expected from the induction of a negative feedback mechanism leading to down-regulation of inflammatory pathways.

In a significant portion of individuals, chronic Nod2 stimulation resulted in decreased IRAK-1 activation after retreatment with bacterial products. Down-regulated IRAK-1 activation, in turn, likely leads to down-regulation of both Nod2 and IL-1 β autocrine-induced production of proinflammatory cytokines. In fact, our data now demonstrates a significant role for IL-1 β autocrine pathways in Nod2-mediated proinflammatory cytokine secretion. Because down-regulation of IRAK-1 activity after chronic Nod2 stimulation is not uniform, it is likely that cells from the individuals who do not exhibit this down-regulation demonstrate inhibition of alternative pathways or pathways downstream of IRAK-1.

Several inhibitory proteins such as SOCS-1, Tollip, and IRAK-M (25, 30) can prevent proper activation of IRAK-1. Consistent with this, IRAK-M up-regulation is observed in 13 of 28 (46%) individuals upon 48 h of treatment with MDP. The loss of Nod2-mediated cytokine down-regulation upon silencing of IRAK-M expression further supports the role of this protein in Nod2-mediated tolerance. Consistent with the genetic variability between humans and heterogeneity of mechanisms mediating human responses, other mechanisms would be expected to mediate tolerance in those individuals who do not up-regulate IRAK-M. A number of inhibitory mechanisms contribute to TLR signaling down-regulation. Therefore, multiple mechanisms would be expected to function in macrophages from a given individual. In addition, various combinations and relative contributions of these mechanisms would lead to tolerance across different individuals.

In addition to Nod2-mediated cross-tolerance to TLR2 and TLR4, we observe a decrease in MDP-induced cytokines after pretreating macrophages with Pam₃Cys and lipid A (SI Fig. 12). Although multiple interacting mechanisms contribute to intestinal immune homeostasis, Nod2 clearly plays a nonredundant and important role in this process because Nod2 mutations are associated with Crohn's disease and dysregulation of intestinal immune homeostasis. The reduced TLR expression on the surface of intestinal compared with peripheral cells, altered ligand densities for Nod2 and TLRs in the small intestine, and/or unique signaling pathways initiated by Nod2 may lead to distinct Nod2-mediated contributions to intestinal tolerance. Critical to understanding the role of Nod2 in the intestinal environment is studying how it functions under chronic stimulatory conditions. These studies demonstrate that chronic, as opposed to acute, stimulation of Nod2 results in down-regulation of proinflammatory cytokine responses and provide insight into Nod2 function in intestinal immune homeostasis.

Materials and Methods

Patient Recruitment and Genotyping. Informed consent was obtained per protocol approved by the institutional review board at the University of Chicago. The Leu1007insC variant was confirmed by sequencing (7).

Primary Macrophage Cell Culture. Monocytes were purified from human peripheral blood mononuclear cells by negative selection (Miltenyi Biotec), and purity was confirmed by CD11c surface expression (>95%) and the absence of B cell and T cell markers CD19 and CD3 (BD Biosciences). Monocytes were cultured and differentiated into macrophages as described in ref. 7.

Primary Monocyte-Derived Macrophage Stimulation. Cultured macrophages were treated with MDP (Bachem) at the doses and times indicated. Cells were then washed with PBS twice and left unstimulated or retreated for 24 h with MDP, lipid A (Peptides International), or Pam₃Cys (Calbiochem). Supernatants were assayed for TNF- α or IL-8 secretion by ELISA (Pierce Biotechnology).

Protein Expression Analysis. Lysis and Western blot analysis were performed as described in ref. 24, using anti-IRAK-1 (Upstate Biotechnology), anti-IRAK-M, or anti-GAPDH antibodies (Abcam).

Immunoprecipitation and *in Vitro* Kinase Assay. IRAK-1 was immunoprecipitated from macrophage lysates by using anti-IRAK-1 antibody-bound protein A Sepharose (Upstate Biotechnology), and *in vitro* kinase assay was performed as described in ref. 31.

Constructions and Transfection of siRNAs. FAM-labeled siRNA against IRAK-M #858 or scrambled siRNA (Ambion) were transfected (1 nmol) into untreated or 24 h MDP-treated macrophages by using Amaxa nucleofactor technology. Cells were cultured for an additional 24 h and then restimulated with MDP or lipid A.

Intestinal Macrophage Phagocytosis Assay. Mononuclear cells were isolated from intestinal resection specimens by collagenase digestion as described in ref. 14 and further purified by HLA-DR positive selection (Miltenyi Biotec) with CD11c of >90% purity. Phagocytosis of FITC-labeled *E. coli* BioParticles (Molecular Probes) was performed by using flow cytometry as described in ref. 32 for 45 min at a multiplicity of infection of 25. Surface fluorescence was quenched with Trypan blue (EMD Chemicals).

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