NLRP1 and NLRP3 inflammasomes are essential for distinct outcomes of decreased cytokines but enhanced bacterial killing upon chronic Nod2 stimulation

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Hedl M, Abraham C. NLRP1 and NLRP3 inflammasomes are essential for distinct outcomes of decreased cytokines but enhanced bacterial killing upon chronic Nod2 stimulation. Am J Physiol Gastrointest Liver Physiol 304: G583–G596, 2013. First published January 3, 2013; doi:10.1152/ajpgi.00297.2012.—Upon chronic microbial exposure and pattern-recognition receptor (PRR) stimulation, myeloid-derived cells undergo a distinct transcriptional program relative to acute PRR stimulation, with proinflammatory pathways being downregulated. However, other host-response pathways might be differentially regulated, and this concept has been relatively unexplored. Understanding mechanisms regulating chronic microbial exposure outcomes is important for conditions of ongoing infection or at mucosal surfaces, such as the intestine. The intracellular PRR nucleotide oligomerization domain 2 (Nod2) confers the highest genetic risk toward developing Crohn’s disease (CD). We previously identified mechanisms mediating downregulation of proinflammatory pathways upon chronic Nod2 stimulation; here we sought to define how chronic Nod2 stimulation regulates bacterial killing. We find that, despite downregulating cytokine secretion upon restimulation through PRR and live bacteria, chronic Nod2 stimulation of human monocyte-derived macrophages enhances bacterial killing; this dual regulation is absent in CD Nod2-risk carriers. We show that chronic Nod2-mediated reprogramming of human monocyte-derived macrophages to a state of enhanced bacterial killing requires upregulated reactive oxygen/nitrogen species pathway function through increased p67phox/p47phox/nitric oxide synthase-2 expression; selectively knocking down each of these genes reverses the enhanced bacterial killing. Importantly, we find that, during chronic Nod2 stimulation, NLRP3/NLPR1 inflammasome-mediated caspase-1 activation with subsequent IL-1 secretion is essential for the subsequent bifurcation to downregulated proinflammatory cytokines and upregulated bacterial killing. Therefore, we identify mechanisms mediating the distinct inflammatory and microbicidal outcomes upon chronic stimulation of the CD-associated protein Nod2.

human; monocytes/macrophages; pattern-recognition receptors

THE HIGHEST GENETIC RISK for developing Crohn’s disease (CD), characterized by intestinal inflammation and dysregulated microbial responses (1, 2), is conferred by polymorphisms in NOD2 (1). How nucleotide oligomerization domain 2 (Nod2) contributes to the balance between downregulation of inflammation and upregulation of microbial defenses under the chronic bacterial exposure inherent to environments with heavy microbial population, such as the intestine, is incompletely understood. Acute Nod2 stimulation with muramyl dipeptide (MDP), a component of bacterial cell wall peptidoglycan, induces proinflammatory cytokines (1) and contributes to bacterial killing (3, 6, 24, 39, 48). Consistent with a role in bacterial killing, Nod2-deficient mice demonstrate increased susceptibility to pathogens (1, 30). The mechanisms mediating this susceptibility in the intestinal environment have been primarily attributed to the Nod2-mediated regulation of intestinal epithelial cell-associated antimicrobial proteins (1, 30). However, how Nod2 regulates bactericidal activity in macrophages, cells that play a critical and direct role in bacterial killing, specifically under chronic Nod2 stimulation conditions, is not known. As peripheral monocytes migrate into the intestinal lamina propria, they are chronically exposed to microbial products, including MDP. We and others have found that chronic Nod2 stimulation of human myeloid cells results in downregulation of cytokine secretion upon restimulation with specific PRR ligands, and several mechanisms have been proposed to contribute to this cytokine downregulation (21, 22, 54). Prolinflammatory cytokines enhance bacterial killing (7); it is unknown if cytokine downregulation after chronic Nod2 stimulation adversely affects bacterial killing. Reports indicate that chronic Toll-like receptor (TLR) 4 stimulation of human and mouse myeloid-derived cells results in distinct transcriptional outcomes, wherein cytokine genes are downregulated, while select transcripts in antimicrobial pathways are upregulated (12, 43). The mechanisms and consequences of cytokine downregulation after chronic PRR stimulation have been well investigated. However, whether chronic PRR-mediated enhanced expression of select antimicrobial genes results in significant modulation of important immunologic functions and the specific antimicrobial pathways that might contribute to the modulated functional outcomes under these chronic stimulatory conditions have not been examined. Moreover, transcriptional and functional regulation of bacterial killing upon chronic stimulation of Nod2 has not been examined. While multiple PRR ligands are present in the intestine, Nod2 has an important and nonredundant role in intestinal immune homeostasis and in contributing to CD. We hypothesized that, despite downregulated cytokines, chronic Nod2 stimulation would, nevertheless, enhance bactericidal activity of primary human monocyte-derived macrophages (MDM).

To dissect the consequences of chronic Nod2 stimulation on bacterial killing, we utilized primary human peripheral monocytes that were differentiated to macrophages. We performed our studies in primary human cells, because Nod2 is important in human disease and because mouse (28, 29, 54) and human (22, 33, 54) macrophages can respond differently to Nod2 stimulation in vitro. We first asked if stimulation with live bacteria reverses Nod2-mediated tolerance and found that chronic Nod2 stimulation of MDM downregulates cytokine induction, even upon subsequent exposure to live bacteria.
Importantly, in contrast to reducing proinflammatory cytokines, chronic Nod2 stimulation enhances bacterial killing; this mechanism is absent in Nod2 Leu1007insC homozygote/compound heterozygote carrier cells. We determine that mechanisms contributing to increased antimicrobial activity after chronic Nod2 stimulation include a transcriptional reprogramming of human MDM that results in upregulated expression of the reactive oxygen species (ROS) pathway members p47phox and p67phox and the reactive nitrogen species (RNS)-generating enzyme nitric oxide synthase (NOS)-2; knockdown of each of these genes reversed chronic Nod2-mediated enhanced bacterial killing. While we previously showed that autocrine IL-1 contributes to cytokine downregulation in Nod2-tolerant MDM (21) and that MDP-induced autocrine IL-1 is caspase-1-dependent (18), in the present study we found that caspase-1 activation upon chronic Nod2 stimulation in primary human MDM is required for cytokine downregulation and the ROS/RNS pathway upregulation mediating enhanced bacterial killing. Significantly, we found that NLRP3 and NLRP1 are specific inflammasome components required for Nod2-mediated caspase-1 activation and IL-1 secretion and the subsequent induction of the ROS/RNS pathway and enhancement of bactericidal activity in primary human MDM. As such, chronic Nod2 stimulation provides for improved bacterial killing in situations of ongoing bacterial exposure while simultaneously minimizing tissue damage through downregulation of inflammatory cytokines.

MATERIALS AND METHODS

Patient recruitment. Informed consent was obtained per protocol approved by the Yale University Institutional Review Board. We performed genotyping by TaqMan single-nucleotide polymorphism genotyping (Applied Biosystems, Foster City, CA) or Sequenom platform (Sequenom, San Diego, CA). Unless otherwise stated, we utilized cells from healthy individuals not homozygous for R702W, G908R, or Leu1007insC Nod2 mutations.

Primary MDM isolation. Monocytes were purified from human peripheral blood mononuclear cells by positive CD14 selection (Miltenyi Biotec, Auburn, CA), tested for purity, and cultured as described elsewhere (21).

Cytokine secretion. For tolerance studies, 4 × 10^5 MDM were pretreated with MDP (Bachem, King of Prussia, PA), lipid A (Peptide International, Louisville, KY), polyinosinic-polycytidylic acid, pretreated with MDP (Bachem, King of Prussia, PA), lipid A (Peptide International, Louisville, KY), polyinosinic-polycytidylic acid, or combined anti-IL-10 and anti-TGF-β antibodies or appropriate isotypes (R & D Systems, Minneapolis, MN). Cells were then infected in triplicate with S. typhimurium and AIEC at 10:1 MOI or S. aureus at 1:1 MOI for 1 h, washed three times with PBS, and incubated in HBSS containing 50 µg/ml gentamicin for 1 h. Cells were washed three times, lysed with 1% Triton X-100 (Sigma), and plated on MacConkey agar.

Protein expression analysis. Western blot analysis was performed as described elsewhere (22) using anti-p67phox, anti-p47phox, anti-NOS2, anti-caspase-1 p20, anti-IL-1β (Cell Signaling Technology, Beverly, MA), anti-p40phox, or anti-GAPDH (Abcam, Cambridge, MA) antibody.

mRNA expression. Total RNA was isolated, and quantitative PCR was performed as described elsewhere (21), with levels normalized to GAPDH.

Intracellular ROS measurement. ROS was measured by flow cytometry using 10 µM 2’,7’-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR).

Bacterial killing. MDM were plated at 3 × 10^5 cells per well in 24-well tissue culture plates. Prior to infection, cells were cultured with MDP, E. coli LPS (Sigma Aldrich, St. Louis, MO), N-acetyl-cysteine (NAC), ascorbic acid, N^2-nitro-l-arginine methyl ester hydrochloride (l-NAME; Sigma Aldrich), IL-1 receptor antagonist (Genscript, Piscataway, NJ), Ac-YVAD-CHO (American Peptide, Sunnyvale, CA), or combined anti-IL-10 and anti-TGF-β antibodies or appropriate isotypes (R & D Systems, Minneapolis, MN). Cells were then infected in triplicate with S. typhimurium and AIEC at 10:1 MOI or S. aureus at 1:1 MOI for 1 h, washed three times with PBS, and incubated in HBSS containing 50 µg/ml gentamicin for 1 h. Cells were washed three times, lysed with 1% Triton X-100 (Sigma), and plated on MacConkey agar.

Construction and transfection of small interfering RNAs. Pooled small interfering RNA (siRNA) containing four different siRNAs against p47phox, p67phox, p40phox, NOS2, NLRP3, NLRP1, ASC, NLRC4, or Nod2 each (SMARTpool, Dharmacon, Lafayette, CO) or scrambled siRNA (Dharmacon) was transfected into untreated macrophages or macrophages treated for 24 h with MDP using the Nucleofector kit (Amaxa, San Diego, CA).

RESULTS

Live bacteria cannot disrupt Nod2-mediated downregulation of proinflammatory cytokines. We previously established that Nod2 stimulation for 48 h (chronic) with 100 µg/ml MDP optimally downregulates cytokine secretion in human MDM upon Nod2, TLR2, TLR4 (22), and IL-1 receptor (21) restimulation (Nod2-mediated tolerance); this MDP concentration approximates levels of muramic acid in stool (50). However, macrophages generally encounter live bacteria, which results in stimulation of multiple PRR, and it is possible that live and/or invasive bacteria might break Nod2-mediated tolerance. We first established that chronic Nod2 stimulation cross-tolerizes to additional PRR, including TLR3, TLR5, TLR7, TLR9, and Nod1 (Fig. 1, A and B). We verified cell viability by annexin and propidium iodide staining after chronic Nod2 stimulation (data not shown). We then questioned if live invasive bacteria, such as S. typhimurium, abrogate Nod2-mediated tolerance. We also investigated stimulation with AIEC, as this invasive E. coli strain colonizes the ileum of CD patients with increased frequency (9), as well as stimulation with a noninvasive E. coli strain, DH5α. We found that chronic MDP treatment significantly downregulated TNF-α upon subsequent infection with S. typhimurium and both E. coli strains (Fig. 1, C and D); IL-8, IL-6, and IL-1β were similarly downregulated (Fig. 1D). As Nod2 recognizes peptidoglycan, which is abundant in gram-positive bacteria, we examined Nod2-mediated tolerance to S. agalactiae and S. aureus. We found that Nod2 downregulated cytokine secretion induced by these bacteria as well (Fig. 1, C and D). Mononuclear cells from CD-risk Leu1007insC Nod2 homozygote and compound heterozygote individuals show dramatically attenuated cytokine production (5, 21, 22, 26, 33, 40) following MDP stimulation and do not undergo Nod2-mediated tolerance upon PRR restimulation (21, 22). We found that chronic Nod2-stimulated MDM from these individuals also fail to downregulate cytokines following S. typhimurium infection (Fig. 1, E and F) but are able to undergo TLR4-mediated self-tolerance (Fig. 1, E and F), indicating that the defects in the Leu1007insC Nod2 cells are specifically in Nod2-mediated tolerance. Taken together, chronic Nod2 stimulation of MDM downregulates cytokine secretion following restimulation with not only ligands for multiple
PRR, but also with live bacteria; this downregulation is absent in
MDM from Leu1007insC Nod2 homozygote/compound heterozygote
individuals.

Chronic Nod2 stimulation enhances killing of S. typhimurium in
MDM from wild-type Nod2, but not Nod2Leu1007insC homozygote/compound heterozygote, individuals. Given that
proinflammatory cytokines contribute to bacterial killing (7), we
asked if chronic Nod2-stimulated MDM that show down-
regulated cytokines have a bactericidal defect. However, de-
spite the downregulated cytokines (Fig. 1), MDM chronically

treated with MDP and then cultured with S. typhimurium, AIEC, or S. aureus demonstrated enhanced killing of these
bacteria (Fig. 2A). MDP treatment enhanced killing as early as
6 h, which progressively increased over 48 h (data not shown).
Chronic MDP treatment did not affect cell viability or S.

Chronic MDP treatment enhanced killing of S. typhimurium,
Nod2 homozygote/compound heterozygote MDM show trends toward decreased S. typhimurium clearance, even
at baseline (Fig. 2B). To more clearly address the role of Nod2
in untreated MDM, MDM transfected with siRNA to Nod2 were
cocultured with S. typhimurium and similarly found to be
slightly defective in bacterial clearance (Fig. 2C). Importantly,
the enhanced killing after chronic Nod2 stimulation in wild-
type (WT) Nod2- or control siRNA-transfected cells was
abolished in Leu1007insC Nod2 homozygote/compound het-

The focus of this study was the role of ROS/RNS pathways. Mouse
studies (3, 16, 34) and overexpression studies in epithelial cell
lines (34) show that acute Nod2 signaling induces ROS/RNS
production. However, ROS/RNS may be differentially regu-
lated under chronic Nod2 stimulation conditions, in particular
given that 1) proinflammatory cytokines upregulate ROS/RNS
pathways (46), but proinflammatory cytokines are downregu-
lated after chronic Nod2 stimulation; 2) NOS2 transcripts are
downregulated in LPS-tolerant human (42) and mouse macro-
phages (12); and 3) human and mouse regulation of RNS
pathways can be very different (36), as can regulation of
pathways in primary MDM relative to cell lines or epithelial
cells. Therefore, it is critical to define ROS and RNS pathway
regulation upon Nod2 stimulation specifically in primary hu-
man MDM and under the chronic stimulation conditions in
the intestinal environment. As human macrophages produce sig-
ificantly lower RNS levels than mouse cells, making direct
RNS detection challenging (36), we first assessed ROS regu-
lation upon chronic MDP treatment. While we saw modest
ROS induction upon short-term MDP stimulation, this induc-
tion was significantly enhanced after 48 h of Nod2 stimulation
in WT, but not Leu1007insC, Nod2 homozygote/compound heterozygote MDM (Fig. 3). ROS induction to H2O2 treatment
in these mutant cells remained intact, indicating no overall
impairment in the ability of the cells to induce ROS (Fig. 3).
Taken together, ROS are not only maintained but, in fact,
significantly upregulated following chronic Nod2 stimulation
of MDM and might constitute a mechanism for the enhanced
bacterial killing observed in these MDM.

Chronic Nod2 stimulation increases dependency on ROS and
RNS pathways for bacterial killing. To assess whether the in-
creased ROS production contributes to enhanced killing fol-
lowing chronic Nod2 stimulation, we first utilized NAC, a ROS
scavenger. NAC decreased S. typhimurium killing in untreated
and chronic MDP-treated cells (Fig. 4A). Upon NAC treat-
ment, the enhanced bacterial killing observed with chronic
Nod2 stimulation was completely lost (Fig. 4A, left). Reanal-
ysis by normalization to the starting numbers of intracellular
bacteria in untreated or chronic MDP-treated cells, respec-
tively, revealed that ROS scavenging more strongly impaired
bacterial killing in chronic Nod2-stimulated MDM than un-
treated cells (Fig. 4A, right). Therefore, after chronic Nod2
stimulation, MDM are more dependent on ROS-generating
pathways to eliminate bacteria. Comparable results were ob-
tained using ascorbic acid, another ROS scavenger (Fig. 4B).
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Nod2 homozygote/compound heterozygote individuals, how-
ever, fail to upregulate ROS-dependent bacterial killing fol-
owing chronic MDP treatment, consistent with their inability
to demonstrate enhanced bacterial killing under these condi-
tions (Fig. 4C).

To address the RNS contribution to the enhanced bacterial
crning through NOS2 inhibition by l-NAME. Under these
cditions, S. typhimurium killing was impaired in un-
treated and chronic Nod2-stimulated cells; however, the RNS
ctribution to bacterial killing was significantly greater after
chronic Nod2 stimulation (Fig. 4D). We next questioned if the
ROS and RNS pathways cooperate to enhance the bacterial
crning upon chronic Nod2 stimulation. MDM chronically
treated with MDP showed significantly decreased bacterial
crning upon inhibition of combined ROS and RNS pathways
compared with non-MDP-treated cells (Fig. 4E, left) and com-
pared with inhibition of either pathway alone (Fig. 4E, right).
NAC and l-NAME affected neither MAPK nor NF-κB acti-
nation under acute MDP treatment, nor did they affect cytokine
uction upon MDP treatment under the conditions of down-
regulated cytokines in Nod2-tolerant MDM (data not shown).
Fig. 2. MDM from WT Nod2 individuals, but not Leu1007insC homozygote/compound heterozygote carriers, show enhanced bacterial killing following prolonged Nod2 stimulation. A: MDM from control individuals were stimulated with 100 μg/ml MDP for 48 h and infected with S. typhimurium or AIEC at 10:1 MOI or S. aureus at 1:1 MOI. Left: S. typhimurium colony-forming units (CFU) for MDM from a representative 4 of 8 individuals. Right: percent live intracellular bacteria compared with untreated cells (represented by dashed line at 100%). Values are means ± SE (n = 8). Enhanced killing of S. typhimurium by MDP was confirmed in an additional 20 individuals. B: MDM from control individuals (n = 3) and Leu1007insC homozygote/compound heterozygote carriers (n = 3) were stimulated with 100 μg/ml MDP or 0.1 μg/ml LPS for 48 h and than infected with S. typhimurium at 10:1 MOI. C: MDM from WT Nod2 individuals (n = 6) were transfected with scrambled (scr) or Nod2 small interfering RNA (siRNA) for 48 h, stimulated with MDP for 48 h, and then infected with S. typhimurium. Unless otherwise indicated, significance comparisons were assessed relative to untreated cells: *P < 0.05; **P < 0.01; ***P < 0.001; †P < 1 × 10^{-5}; ††P < 1 × 10^{-6}.

Taken together, ROS and RNS pathways are critical in enhancing S. typhimurium killing by MDM after chronic Nod2 stimulation.

p67phox, p40phox, p47phox, and NOS2 are upregulated upon chronic Nod2 stimulation. To identify mechanisms through which ROS and RNS function is enhanced, we examined the induction of specific components of these pathways upon chronic Nod2 stimulation. The NADPH oxidase complex generates ROS and consists of p67phox, p40phox, p47phox, gp91phox, and p22phox subunits; NOS2 is crucial for producing RNS. TNF-α, IL-8, and IL-1β transcripts were decreased following chronic Nod2 stimulation (Fig. 5A), consistent with the decreased protein levels of these proinflammatory cytokines (Fig. 1). In contrast, p67phox, p40phox, p47phox, and NOS2, but not gp91phox or p22phox, mRNA expression was considerably upregulated following 48 h of Nod2 stimulation relative to 4 h of Nod2 stimulation (Fig. 5A). Furthermore, MDP retreatment of chronic Nod2-stimulated MDM further enhanced RNA expression of p47phox, p67phox, and p40phox (Fig. 5A). Consistent with the transcriptional upregulation of p67phox, p40phox, p47phox, and NOS2, we found that, relative to untreated cells, chronic Nod2 stimulation upregulated expression of these four proteins (Fig. 5B). Taken together, chronic Nod2 stimulation of MDM generates distinct transcriptional outcomes; proinflammatory cytokines are downregulated and ROS and RNS pathway members are upregulated. These bifurcated responses are consistent with the dual functional outcomes of decreased inflammation and increased antibacterial activity after chronic Nod2 stimulation.

p67phox, p47phox, and NOS2 are required for enhanced bacterial killing upon chronic Nod2 stimulation. To address the contribution of the upregulated NADPH oxidase components and NOS2 to the enhanced bacterial killing after chronic Nod2 stimulation, we silenced the expression of p67phox, p40phox, p47phox, and NOS2 by siRNA. We saw effective silencing at the RNA (Fig. 6A) and protein (Fig. 6B) levels. Silencing of p67phox, p47phox, and NOS2 individually significantly impaired bacterial killing in untreated and chronic MDP-treated MDM compared with cells transfected with scrambled siRNA (Fig. 6C). However, silencing of these genes more strongly impaired bacterial killing in chronic Nod2-stimulated MDM than in untreated cells, highlighting the increased function and more important role of these pathways in regulating bacterial killing after chronic Nod2 stimulation (Fig. 6C). In contrast, p40phox silencing did not significantly alter bacterial killing (Fig. 6C). ROS and RNS pathways cooperated to enhance bacterial killing after chronic Nod2 stimulation (Fig. 4E). We therefore questioned if combined silencing of p47phox, the NADPH oxidase subunit most dramatically affecting killing, and NOS2 further impairs Nod2-enhanced bacterial killing relative to silencing of each of these genes individually; we found that this was, indeed, the case (Fig. 6, D and E). Taken together, chronic Nod2 stimulation in MDM alters the transcriptional program, thereby upregulating p47phox, p67phox, and NOS2 expression, which, in turn, is necessary for the enhanced bacterial killing in these MDM.

Caspase-1 activation contributes to the bifurcated cytokine and bacterial killing responses following chronic Nod2 stimulation. We previously found that autocrine IL-1 secretion is one
mechanism mediating the chronic Nod2-induced downregulation of cytokines observed following PRR restimulation (21). In separate studies, we found that MDP-mediated autocrine IL-1 secretion is caspase-1-dependent in MDM (18). Whether caspase-1 contributes to chronic Nod2-enhanced bacterial killing and which specific inflammasome components might regulate this process in human MDM is not known. We hypothesized that caspase-1-dependent autocrine IL-1 secretion might contribute to the dual outcomes of cytokine downregulation and enhanced ROS/RNS-mediated bacterial killing following chronic Nod2 stimulation. We therefore first examined the regulation of pro-IL-1 by MDP in MDM. We found that pro-IL-1 was present in unstimulated MDM (Fig. 7A), consistent with the rapid secretion of active IL-1 in MDP-stimulated MDM (18, 20). Moreover, MDP stimulation further enhanced pro-IL-1 levels (Fig. 7A), consistent with our prior results showing MDP-mediated induction of IL-1 mRNA (18, 33). We then examined if Nod2 stimulation activates caspase-1 in primary human MDM; we found this to be the case, as indicated by the induction of the cleaved caspase-1 p20 subunit (Fig. 7B). To define if the activation of caspase-1 upon initiation of chronic Nod2 stimulation is required for the subsequent cytokine downregulation and enhanced bacterial killing, we wished to inhibit caspase-1 during chronic Nod2 stimulation but enable resumption of its function upon subsequent exposure to bacteria. We therefore utilized the reversible caspase-1 inhibitor Ac-YVAD-CHO to dissect the role of caspase-1 at the time of chronic Nod2 stimulation. We further verified the role of caspase-1 in the chronic Nod2-mediated outcomes through silencing of inflammasome components (see below). Ac-

![Fig. 3. Chronic Nod2 stimulation increases reactive oxygen species (ROS) production. A and B: human MDM from healthy controls (n = 12) or Leu1007insC homozygote/compound heterozygote carriers (n = 3) were left untreated or treated with 100 µg/ml MDP for 1 and 48 h and analyzed by flow cytometry utilizing the ROS-detecting dye 2',7'-dichlorodihydrofluorescein diacetate. According to the manufacturer’s instructions, 1 mM H2O2 was included as a control for ROS induction. A: representative flow cytometry plots showing mean fluorescence intensity values. B: summarized data represented as fold ROS induction normalized to untreated cells. Values are means ± SE. *P < 0.05; **P < 0.01.

![Fig. 4. Chronic Nod2 stimulation results in increased dependency on ROS and reactive nitrogen species (RNS) pathways for bacterial killing. A–C: MDM from control individuals [n = 8 (A) and n = 4 (B)] or Leu1007insC homozygote/compound heterozyzogote carriers (n = 3; C) were left unstimulated or stimulated with 100 µg/ml MDP for 48 h, washed, treated with 20 mM N-acetylcysteine (NAC) or 1 mM ascorbic acid (AA), and infected with S. typhimurium at 10:1 MOI. Data are represented as percent intracellular S. typhimurium compared with untreated (A and B, left, and C) and MDP-pretreated (A and B, right) cells without NAC or ascorbic acid (represented by dashed line at 100%). Values are means ± SE. D and E: MDM from control individuals were left unstimulated or stimulated with 100 µg/ml MDP for 48 h, washed, treated with 10 mM N-nitro-L-arginine methyl ester (L-NAME, D, n = 8) or 10 mM L-NAME + 20 mM NAC (E, n = 8), and infected with S. typhimurium at 10:1 MOI. Data are represented as percent live intracellular S. typhimurium compared with untreated (D and E, left) or MDP-pretreated (E, right) cells without ROS and RNS inhibitors (represented by dashed line at 100%). Values are means ± SE. **P < 0.01; ††P < 1 × 10⁻⁵.
YVAD-CHO eliminated IL-1β secretion upon acute MDP stimulation (data not shown); to ensure reversibility, we confirmed that IL-1β secretion was intact in cells pretreated with the inhibitor, washed, and then treated with MDP (Fig. 7C, left). We found that, upon caspase-1 inhibition during chronic MDP stimulation, the cytokine downregulation upon restimulation with MDP was partially reversed, such that cytokine secretion increased from 5–9% to 34–42% of that observed in cells stimulated with MDP for 24 h (Fig. 7C, left). Despite the elevated cytokines in these cells and the known contribution of proinflammatory cytokines to bacterial killing, caspase-1 inhibition during chronic Nod2 stimulation impaired the enhanced bacterial killing (Fig. 7D, left), ROS induction (Fig. 7E, left), and p67phox, p47phox, and NOS2 expression (Fig. 7F, left) relative to that in the absence of caspase-1 blockade. As opposed to caspase-1 directly regulating the chronic Nod2-stimulated increased bacterial killing, it remained possible that cytokine tolerance and bacterial killing may depend on each other, such that reversal of cytokine tolerance might lead to reduced bacterial killing. To address this, we investigated a separate mechanism of reversing chronic Nod2-induced tolerance. We previously found that early induction of anti-inflammatory cytokines contributes to chronic Nod2-mediated tolerance (21). These anti-inflammatory mediators are induced during acute, but dramatically attenuated during chronic, Nod2 stimulation (21). We therefore neutralized early IL-10 and TGF-β secretion effects at the onset of chronic MDP treatment to reverse Nod2-mediated tolerance (Fig. 7C, right). However, unlike caspase-1 inhibition, reversal of Nod2-mediated tolerance by neutralizing these inhibitory mediators did not reduce but, in fact, enhanced bacterial killing, ROS induction, and p47phox, p67phox, and NOS2 expression (Fig. 7D–F, right). This likely reflects induced antimicrobial functions such as ROS/RNS pathways (Fig. 7, E and F, right), which remain intact after chronic Nod2 stimulation, combined with the enhanced proinflammatory cytokine secretion, which can also contribute to antimicrobial function. Therefore, reversal of cytokine downregulation does not, in and of itself, reverse chronic Nod2-mediated enhanced bacterial killing. Taken together, these findings indicate that caspase-1 activation is a critical mechanism during chronic Nod2 stimulation that allows for the subsequent bifurcation into decreased cytokine production and increased bacterial killing responses.

**IL-1 secretion contributes to bacterial killing following chronic Nod2 stimulation.** We next questioned if the IL-1 generated upon Nod2-initiated caspase-1 activation accounted for the enhanced bacterial killing observed upon chronic Nod2 stimulation. We first examined if chronic IL-1 treatment is sufficient to enhance bacterial killing. We determined that, similar to chronic MDP stimulation, the addition of exogenous IL-1 enhanced bacterial killing in MDM (Fig. 8A). We next asked if the autocrine IL-1 produced during chronic Nod2 stimulation contributes to the subsequent enhanced killing. We therefore blocked IL-1-mediated signaling by the physiological IL-1 receptor antagonist. As IL-1 is secreted following short-term Nod2 stimulation but suppressed after chronic Nod2 stimulation (Fig. 7C), we blocked IL-1 at the onset of Nod2 stimulation. IL-1 blockade resulted in a partial reversal of the enhanced bacterial killing observed following chronic Nod2 stimulation (S. typhimurium colonies increased from 62% during Acute Tx to 58% during Acute Tx + IL-1 block), indicating that caspase-1-mediated cleavage of pro-IL-1 and subsequent IL-1 secretion contribute to the enhanced bacterial killing observed following chronic Nod2 stimulation. The NLRP3-NLRP1-ASC inflammasome is required for optimal Nod2-mediated enhanced bacterial killing. Specific inflammasome components required for Nod2-mediated signaling in primary human MDM have not been well defined. Given the important role of caspase-1 activation and autocrine IL-1 in

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**Fig. 5. Chronic Nod2 stimulation upregulates p67phox, p40phox, p47phox, and nitric oxide synthase 2 (NOS2) expression.** A: MDM from control individuals (n = 8–12) were left unstimulated, stimulated with 100 μg/ml MDP for with 100 μg/ml MDP 4 h (acute), or pretreated with 100 μg/ml MDP for 48 h, washed, and left untreated or restimulated with MDP for an additional 4 h. TNF-α, IL-8, IL-1β, p67phox, p40phox, p47phox, p91phox, p22phox, and NOS2 mRNA expression was assessed by RT-PCR. Data are represented as fold mRNA induction compared with untreated cells. Values are means ± SE. B: MDM from control individuals were left unstimulated, stimulated for 24 h (acute), or pretreated with 100 μg/ml MDP for 48 h, washed, and left untreated or restimulated with MDP for an additional 24 h. Representative Western blots of p67phox, p40phox, p47phox, and NOS2 protein expression are shown. *P < 0.05; **P < 0.01; ***P < 0.001; ††P < 1 × 10⁻³.
Nod2-mediated NLRP1 activation has not been reported in NLRP1 in THP-1 (25) and HEK-293 (17) cells; however, constructs, Nod2 was implicated as a specific activator of and overexpression studies. In two reports utilizing transfected observed in some (27, 38), but not all (25), mouse macrophage The requirement for NLRP3 in Nod2-induced IL-1 has been be operational after chronic Nod2 stimulation is not known. Moreover, which of these inflammasome components are IL-1 secretion in primary human MDM have not been exam- be operational after chronic Nod2 stimulation is not known.

Fig. 6. NOS2, p67phox, and p47phox are necessary for enhanced bacterial killing following chronic Nod2 stimulation. A and B: MDM from control individuals were transfected with scrambled, p67phox, p40phox, p47phox, and NOS2 siRNA or p47phox + NOS2 siRNA. A: mRNA expression (n = 8) assessed by RT-PCR and represented as percent mRNA expression compared with cells transfected with scrambled siRNA (represented by dashed line at 100%). Values are means ± SE. B: Western blots showing protein expression in MDM stimulated with 100 μg/ml MDP for 48 h and transfected with scrambled, p67phox, p40phox, p47phox, and NOS2 siRNA. C–E: MDM from control individuals (n = 8) were left unstimulated or stimulated with 100 μg/ml MDP for 24 h, transfected with scrambled, p67phox, p40phox, p47phox, and NOS2 siRNA (C) or p47phox + NOS2 siRNA (D and E), left for an additional 24 h, and then infected with S. typhimurium. Data are represented as percent intracellular S. typhimurium compared with untreated (C and D) or 48-h MDP-treated (E) cells (represented by dashed line at 100%). Values are means ± SE. ***P < 0.001; †† †† †† P < 1 × 10⁻⁴; †† †† P < 1 × 10⁻³.

Nod2-enhanced bacterial killing (Figs. 7 and 8), we next sought to define the inflammasome components required for chronic Nod2-mediated enhanced bacterial killing. While interactions of Nod2 with select inflammasome components have been described in mouse cells and cell lines (17, 25, 27, 38, 53), specific outcomes of these interactions on Nod2-mediated IL-1 secretion in primary human MDM have not been examined. Moreover, which of these inflammasome components are required for the enhanced bacterial killing that we now find to be operational after chronic Nod2 stimulation is not known. The requirement for NLRP3 in Nod2-induced IL-1 has been observed in some (27, 38), but not all (25), mouse macrophage and overexpression studies. In two reports utilizing transfected constructs, Nod2 was implicated as a specific activator of NLRP1 in THP-1 (25) and HEK-293 (17) cells; however, Nod2-mediated NLRP1 activation has not been reported in primary cells, including primary human MDM, which can demonstrate regulation very different from that of cell lines. We first sought to determine the effects of these individual inflammasome components on Nod2-induced inflammasome activation as measured by IL-1β secretion. We therefore silenced their expression through siRNA and show effective knockdown of each component in Fig. 9A. We found that individual silencing of NLRP3, NLRP1, and ASC, a protein required for NLRP3 and NLRP1 inflammasome assembly (14), abolished MDP-induced IL-1β secretion (Fig. 9B). NLRC4 silencing, which interacts with and inhibits Nod2-mediated NF-κB activation in overexpression systems (8), did not affect Nod2-induced secretion of IL-1β in primary MDM but, as expected, decreased flagellin-induced IL-1β secretion (Fig. 9B). To ensure that the inflammasome components regulate Nod2-mediated IL-1β secretion by regulating caspase-1 acti-
Fig. 7. Activation of caspase-1 during chronic Nod2 stimulation is required for subsequent decreased cytokine induction and upregulated bacterial killing through ROS/RNS pathways. A and B: MDM from healthy controls were left unstimulated or treated with 100 μg/ml MDP for 2 or 4 h (A) or 100 μg/ml MDP for 6 h (B). Protein expression of pro-IL-1β (with GAPDH run separately to assess equal loading; A) or caspase-1 p20 (B) was assessed by Western blot analysis. Representative blots from 2 of 6 donors are shown. C–F: MDM from healthy controls (n = 8–12) were left unstimulated or pretreated with 100 μg/ml MDP in the presence or absence of 50 μM Ac-YVAD-CHO (YVAD, a reversible caspase-1 inhibitor; left) or anti-IL-10 (5 μg/ml, isotype goat IgG) + anti-TGF-β (25 μg/ml, isotype goat IgG) antibodies (right) for 48 h. After treatment, inhibitors/antibodies were thoroughly washed out. C: cells were restimulated for an additional 24 h with 100 μg/ml MDP (acute). Data are represented as percent cytokine secretion normalized to acutely stimulated cells (represented by dashed line at 100%). Values are means ± SE. D: cells were infected with S. typhimurium at 10:1 MOI. Data are represented as percent live intracellular S. typhimurium compared with untreated cells (represented by dashed line at 100%). Values are means ± SE. E: cells were analyzed by flow cytometry utilizing 2',7'-dichlorodihydrofluorescein diacetate. Summarized data are represented as fold ROS induction normalized to untreated cells (represented by dashed line at 1). Values are means ± SE. F: p67phox, p47phox, and NOS2 mRNA expression was assessed by RT-PCR. Data are represented as fold mRNA induction compared with untreated cells (represented by dashed line at 1). Values are means ± SE. D–F: controls include 48-h Ac-YVAD-CHO or antibody treatment followed by extensive wash and restimulation with DMSO or IgG isotypes after 3 h to ensure reversibility. Lines over adjacent bars indicate identical P value for these bars: *P < 0.05; **P < 0.01; ***P < 0.001; †P < 1 × 10^-5; ††P < 1 × 10^-5.
Fig. 8. Autocrine IL-1 is required for subsequent upregulated bacterial killing during chronic Nod2 stimulation. MDM from healthy controls (n = 8) were left unstimulated or treated with 100 μg/ml MDP or 10 ng/ml IL-1β for 48 h (A) or 100 μg/ml MDP for 48 h in the presence or absence of 50 μM Ac-YVAD-CHO or IL-1 receptor antagonist (IL-1Ra, 0.5 μg/ml; B). Cells were extensively washed and then infected with S. typhimurium at 10:1 MOI. Data are represented as percent live intracellular S. typhimurium compared with untreated cells (represented by dashed line at 100%). Values are means ± SE. Controls include 48 h of treatment with Ac-YVAD-CHO, IL-1Ra, or DMSO followed by extensive wash and coculture with bacteria. ††P < 1 × 10⁻⁵.

**DISCUSSION**

In this study, utilizing primary human MDM, we found that chronic Nod2 stimulation induces distinct transcriptional programs that lead to downregulation of inflammatory cytokines upon restimulation with multiple PRR ligands or live bacteria, on the one hand, and enhanced bacterial killing, on the other hand. Importantly, both outcomes are defective in MDM from homozygote/compound heterozygote CD-associated Leu1007insC Nod2 carriers. To compensate for the loss of proinflammatory cytokines, which promote bacterial killing, ROS and RNS pathway functions are upregulated following chronic Nod2 stimulation. Moreover, in contrast to downregulated cytokine transcripts following chronic Nod2 stimulation, mRNA and protein expression of the ROS and RNS pathway members p47phox, p67phox, and NOS2 is upregulated. Thus silencing of these genes, we demonstrate that p47phox, p67phox, and NOS2 are critical for the enhanced S. typhimurium elimination by MDM chronically stimulated through Nod2. Therefore, we discovered a link between chronic Nod2 stimulation in macrophages, induction of ROS and RNS pathways, and the specific ROS and RNS members regulated by chronic Nod2 stimulation. Furthermore, we determined that caspase-1 activation and subsequent IL-1β secretion contribute to the bifurcation in macrophage responses after chronic Nod2 stimulation. Moreover, we demonstrated that Nod2-induced inflammasome activation is specifically dependent on NLRP3, NLRP1, and ASC in primary human MDM. Finally, we determined that these inflammasome components are required for the enhanced bacterial killing observed after chronic Nod2 stimulation. These findings indicate that chronic Nod2 stimulation of human MDM leads to distinct regulation of two pathways critical to homeostasis in conditions of chronic microbial exposure, downregulation of PRR- and bacterial-induced proinflammatory cytokines and upregulation of bacterial killing, and we identified mechanisms leading to this dual regulation (Fig. 10).

We found that the dual effects of Nod2 are mediated at a transcriptional level, with cytokine transcripts being downregulated (“tolerized”) and transcripts contributing to antimicrobial function via ROS and RNS pathways being upregulated (“nontolerized”) (Fig. 5). Chronic LPS stimulation of human and mouse myeloid-derived cells leads to a similar bifurcation into tolerized and nontolerized genes at the level of mRNA (12, 43). While it has been hypothesized that such transcriptional programming may increase antimicrobial activity in chronic LPS-treated cells, the contributions of nontolerized genes to bacterial killing under these conditions have not been examined. We have clearly established the role of specific nontolerized genes that mediate enhanced bacterial killing in human MDM following chronic Nod2 stimulation. Nod2 remains active following chronic Nod2 stimulation, as evidenced by further upregulation of NADPH oxidase subunits and NOS2 following MDP restimulation (Fig. 5).

Importantly, chronic Nod2 stimulation in vivo improves experimental colitis (54). The mechanisms leading to this
outcome are not fully understood, but two hypotheses include downregulated cytokines and increased bacterial killing. These regulatory outcomes parallel the unique phenotype of intestinal macrophages, characterized by downregulated cytokine secretion but upregulated bacterial killing (47). While chronic Nod2 enhances bacterial killing in vitro by 40–50%, it is not known how chronic Nod2 stimulation contributes to intestinal macrophage bacterial clearance in...
vivo. However, small differences in bacterial killing in vitro can lead to a significant impact in vivo (49, 52, 55, 56); such in vivo differences might be particularly important in the high-density and chronic bacterial exposure encountered in the intestine.

Recent findings demonstrated that select pathogenic bacteria can disrupt tolerance in mouse intestinal macrophages (15) and human intestinal myeloid cells (20) through IL-1 induction, which, in turn, is critical for mediating bacterial defenses (15). Consistently, we found that autocrine IL-1 is critical for the enhanced bacterial killing following chronic Nod2 stimulation (Fig. 8). We determined that, in addition to activating caspase-1, which cleaves preexisting pro-IL-1β (Fig. 7B), MDP also induces pro-IL-1β (Fig. 7A), which further contributes to IL-1 secretion. We showed that NLRP3, NLRP1, and ASC inflammasome components are required for Nod2-mediated inflammasome activation and enhanced bacterial killing in primary MDM (Fig. 9). Nod2-mediated NLRP1 activation has been reported in HEK-293 overexpression systems (11, 17) and in THP-1 cells (25). However, while THP-1 is a human monocytic leukemia cell line, its behavior and transcriptional profile are often different from the behavior and transcriptional profile of primary human macrophages (31), particularly regarding Nod2 outcomes. For example, in contrast to primary MDM (Fig. 1), MDP-stimulated THP-1 cells do not secrete TNF-α (35). Furthermore, although ASC activation is not required but does enhance MDP-induced NLRP1 inflammasome activation in a HEK-293 transfected system (11), we have found that ASC is absolutely required for Nod2-mediated inflammasome activation in primary MDM (Fig. 9). Moreover, NLRC4 has been shown to inhibit Nod2-induced NF-κB activity in HEK-293 overexpression systems (8) but did not affect Nod2-mediated IL-1β secretion or bacterial killing in primary MDM (Fig. 9). Interestingly, some (13, 45), but not all (4, 51), studies show that intracellular delivery of flagellin is needed to activate NLRC4 and stimulate IL-1 production in mouse macrophages. Interventions to deliver flagellin to the cytosol in human MDM were not necessary for NLRC4-dependent IL-1 secretion (Fig. 9). Also, unlike human MDM (22, 33, 40, 54), mouse macrophages (25, 28, 29, 37, 34) show minimal cytokine secretion following Nod1 and Nod2 stimulation, and chemically induced internalization of these ligands significantly improves cytokine secretion (37). Thus it is possible that microbial products are more efficiently internalized by human MDM. Finally, human, but not mouse, macrophages produce autocrine ATP following PRR stimulation (19, 44), which is necessary for subsequent inflammasome activation and IL-1β secretion. These differences highlight the need to study Nod2 regulation in primary human cells to clearly define Nod2 signaling and its role in disease. This is particularly important, given that mouse and human differences contribute to poor applicability of certain mouse models to human clinical studies (10). Our findings provide novel insight into the specific inflammasome components required for Nod2-mediated inflammasome activation in primary human MDM, cells directly relevant for studying human immune regulation.

RNS production has not been necessarily associated with bacterial killing responses in human cells (36), and its role in chronic LPS-mediated bacterial killing is controversial (12, 42, 58). We have determined that RNS clearly contribute to the enhanced bacterial killing after chronic Nod2 stimulation in human MDM (Figs. 4 and 6). Acute Nod2 stimulation was reported to upregulate ROS production in transfected intestinal epithelial cells (34), but this upregulation has not been examined in cells with direct bacterial killing ability, such as macrophages, or under chronic Nod2 stimulation conditions, which can lead to different outcomes relative to acute stimulation. We have shown that chronic Nod2 stimulation enhances bacterial killing through p47phox, p67phox, and NOX2 in primary human MDM (Fig. 6). Silencing p40phox did not impair the enhanced bacterial killing; this is consistent with the observation that p40phox is not required for NADPH oxidase activity in in vitro systems (57). Individuals with defective p47phox and p67phox are at risk for chronic granulomatous

Fig. 10. Model of dual functional outcomes of chronic Nod2 stimulation. Acute stimulation of MDM by MDP activates Nod2, which results in cytokine induction and a level of bacterial killing. During acute Nod2 stimulation, NLRP3 and NLRP1 inflammasomes are activated, resulting in caspase-1 activation, which leads to cleavage of preexisting and upregulated pro-IL-1β stores and subsequent IL-1β secretion. This provides one of the mechanisms contributing to the distinct outcomes observed after chronic Nod2 stimulation wherein 1) cytokine secretion is now downregulated and 2) bacterial killing is further enhanced through increased expression of the NADPH oxidase subunits p47phox and p67phox and of NOX2, which leads to increased ROS and RNS pathway function. Taken together, dual outcomes of decreased inflammation and enhanced bacterial killing following chronic Nod2 stimulation are modulated by the NLRP3 and NLRP1 inflammasomes.
disease, characterized by decreased neutrophil- and macrophage-mediated bacterial killing, and for colitis (23), further highlighting the importance of ROS in immune homeostasis. Moreover, while mice deficient in gp91phox or NOS2 show increased infectious susceptibility, gp91phox × NOS2 double-knockout mice demonstrate a more dramatic phenotype with large abscesses containing predominantly enteric organisms, resulting in increased mortality (46). Interestingly, some studies show that ROS and RNS downregulate inflammation (41). Therefore, the ROS pathway in environments exposed to ongoing bacterial colonization may contribute to bacterial killing without adversely affecting the suppressed inflammatory conditions. While multiple factors may mediate ROS- and RNS-induced bacterial killing, we found that chronic Nod2 stimulation is at least one mechanism that can upregulate these pathways, thereby providing defenses against microbial overgrowth/pathogen invasion.

Among the factors influencing intestinal immune function, Nod2 confers the highest genetic risk for developing CD (1). Given the differences in Nod2 signaling and tolerance across mouse and human primary cells and cell lines (22, 30, 54), it is important to study Nod2 in primary MDM. A major requirement of intestinal homeostasis is a proper balance between downregulation of inflammation and antimicrobial defenses. Nod2 participates in both processes, as evidenced by downregulation of intestinal inflammation with chronic MDP administration in vivo (54) and by increased susceptibility of Nod2-deficient mice to intestinal pathogens (30). We have shown that chronic Nod2 stimulation generates bifurcated pathways: downregulated proinflammatory cytokine production and enhanced bacterial activity. This dual outcomes mediated by Nod2 may contribute to improved bacterial clearance while minimizing tissue injury in the intestine.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.H. and C.A. are responsible for conception and design of the research; M.H. and C.A. performed the experiments; M.H. and C.A. analyzed the data; M.H. and C.A. interpreted the results of the experiments; M.H. and C.A. drafted the manuscript; M.H. and C.A. edited and revised the manuscript; M.H. and C.A. approved the final version of the manuscript.

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