The Journal *of* Immunology

Activation of Human NK Cells by the Bacterial Pathogen-Associated Molecular Pattern Muramyl Dipeptide

Verónica Athié-Morales, Geraldine M. O'Connor and Clair M. Gardiner

---

References This article **cites 66 articles**, 36 of which you can access for free at:
http://www.jimmunol.org/content/180/6/4082.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Activation of Human NK Cells by the Bacterial Pathogen-Associated Molecular Pattern Muramyl Dipeptide

Verónica Athié-Morales,* Geraldine M. O'Connor,[†] and Clair M. Gardiner[1][†]

Muramyl dipeptide (MDP) is a bacterial pathogen associated molecular pattern derived from both Gram-positive and -negative bacteria. It is a specific ligand for nuclear oligomerization domain 2, a pattern recognition receptor best characterized for its role in immunosurveillance in the gut. In this study, we demonstrate that human peripheral blood NK cells express nuclear oligomerization domain 2 and respond to MDP. NK cells naturally internalize MDP leading to direct cell activation, including signaling through NFκB: characterized by p50/p65 heterodimers at early stimulations times and sustained activation of p50 homodimers. Moreover, MDP synergizes with IFN-α and IL-12 to activate NK cells and stimulate IFN-γ secretion, suggesting a role for accessory cells in induction of an optimal NK cell response. Although IL-12 costimulation leads to a greater IFN-γ response by NK cells, higher levels of CD69 in response to MDP are induced in the presence of IFN-α, suggesting that different pathogen-induced cytokine profiles will affect downstream NK cell responses. In contrast, MDP alone or in combination with either IFN-α or IL-12 only poorly increases NK cell cytotoxicity. In summary, this report identifies MDP as a bacterial pathogen associated molecular pattern that activates human NK cells.   *The Journal of Immunology,* 2008, 180: 4082–4089.

Natural killer (NK) cells are lymphocytes widely recognized for their essential role in the innate immune response against virally infected and tumor cells (1–3). However, participation of NK cells is not restricted to the immune response to viral infections as highlighted by the recurrent mycotic, parasitic, and bacterial infections that occur in NK -deficient patients (4). The important role of NK cells and NK cell-derived IFN-γ in eradication of bacterial infections has also been demonstrated in in vivo models of *Listeria monocytogenes*, *Shigella flexneri*, *Bordetella pertussis*, and *Legionella pneumophila* (5–11). In most cases, bacterial infection leads to accessory cell-derived cytokines, mainly IL-12, resulting in NK cell activation and IFN-γ secretion. There is also increasing evidence that NK cells become activated by direct recognition of bacteria and bacterial products in the human system (12, 13). In the case of the outer membrane protein A from *Klebsiella pneumoniae* and flagellin, direct recognition is mediated by the functional expression of the pattern recognition receptors TLR2 and TLR5 on NK cells (12).

It is now recognized that TLRs, along with RIG-I-like receptors and nuclear oligomerization domain (Nod)[2] proteins, are the three main families of mammalian pattern recognition receptors (14–16). Within the Nod family, Nod1 and Nod2 function as receptors for bacterial derived pathogen associated molecular patterns (PAMPs). Nod2 is a cytoplasmic receptor with expression reported on human monocytes and dendritic cells at the mRNA level and on intestinal epithelial cells and murine macrophages at the protein level (17–23). Nod2 plays an essential role in the detection of invasive bacteria in the gut, as demonstrated by increased bacterial dissemination during *L. monocytogenes* infection in Nod2 knock out mice (24). Nod2 mutations are associated with susceptibility to inflammatory conditions such as Crohn's disease and Blau syndrome (16, 25, 26).

Muramyl dipeptide (MDP), the specific ligand for Nod2 (24, 27–29), is a naturally occurring stem peptide of peptidoglycan from both Gram-positive and Gram-negative bacteria. Peptidoglycan is synthesized and remodeled during bacterial cell growth, making it available for cleavage by host lysozyme, thereby producing muropeptides that generate MDP through the action of bacterial and host amidases (30). These enzymes are present in human blood, suggesting bloodstream availability of MDP during bacterial infections. In monocytic and epithelial cells, stimulation by MDP triggers Nod2 oligomerization and consequent recruitment of the adaptor protein Rip2, which leads to activation of the NF-κB signaling pathway (16).

The contribution of accessory cell help in the NK cell response to infection is well documented. Depending on the type of infection, NK cells play effector and/or regulatory roles mediated through cytotoxicity and cytokine secretion. During viral infections, NK cells primarily exert a direct cytotoxic effector role that can be further enhanced by accessory cell-derived cytokines, such as IL-12 (1–3). The cytotoxic role of NK cells is less involved in bacterial infections, whereas production of cytokine seems to be more important, in particular against intracellular bacteria where secretion of IFN-γ and TNF-α facilitate macrophage activation and consequent pathogen eradication (31, 32). Nod2 is mainly known for its role in bacterial sensing in the gut. However, peripheral immune cells also express functional Nod2 and can be activated in response to MDP (17, 20, 24, 33). This together with the likely availability of MDP in the bloodstream during bacterial infections suggests that in addition to immunosurveillance in the gut, Nod2 functions may also extend to the peripheral innate immune response. To test this hypothesis, we investigated the potential role of Nod2 in human peripheral blood NK cells.

---

*Xoma Ireland Limited and †School of Biochemistry and Immunology, Trinity College, Dublin, Ireland

[1] Address correspondence and reprint requests to Dr. Clair Gardiner, School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland. E-mail address: clair.gardiner@tcd.ie

[2] Abbreviations used in this paper: Nod, Nuclear oligomerization domain; MDP, muramyl dipeptide; PAMP, pathogen associated molecular pattern; gDNA, genomic DNA.

Materials and Methods

Chemicals and cell culture

Unless specified, chemicals were obtained from Sigma-Aldrich. Conditions for cell culture and purification of primary NK cells, sorted NK cells, and primary monocytes were as previously described (34). NK cells and monocytes were purified from peripheral blood using magnetic beads (Miltenyi Biotec) and are referred to as "purified primary NK cells" (routinely 85–97% pure $CD56^+CD3^-$ containing 0.8–3% $CD14^+$ cells) and "primary monocytes" (routinely $\geq 92\%$ $CD14^+$ cells with $\leq 1.8\%$ $CD56^+CD3^-$ cells). "Sorted primary NK cells" were routinely greater than 99% pure, with no contaminating $CD14^+$ cells. Purity was assessed by flow cytometry.

RT-PCR

Total RNA was extracted with Tri reagent (Molecular Research Center). cDNA was generated with random hexamers using ImProm-II reverse transcription system (Promega). PCR was conducted on cDNA samples using *Taq* DNA polymerase (Promega) and specific primers for Nod2 (F: 5′-AGCCATTGTCAGGAGGCTC-3′; R: 5′-CGTCTCTGCTCCATCATAGG-3′) (18) and β-actin (F: 5′-CGCGAGAAGATGACCCAGATC-3′; R: 5′-TTGCTGATCCACATCTGCTGG-3′). As a control for genomic DNA (gDNA) contamination, we used specific primers for KIR3DL1 (F: 5′-CCATCGGTCCCATGATGCT-3′; R: 5′-AGAGAGAAGGTTTCTCATATG-3′), which amplify a 1600-bp fragment if gDNA is present. gDNA from PBMC was used as a positive control for KIR3DL1 amplification.

Cell stimulation

Unless otherwise stated, cells were stimulated at a density of 1×10^6 cells/ml with 1 ng/ml -10 μg/ml MDP, 0.5–50 U/ml rhIFN-α (Strathmann Biotec AG), and 10 pg/ml -1 ng/ml rhIL-12 (Strathmann Biotec AG) either alone or in combination as indicated in the corresponding figure legends. PBS and IL-2 (200 U/ml) were used as negative and positive controls, respectively. For inhibition of lysosomal acidification, cells were incubated with chloroquine (20 μg/ml) for 1 h before stimulation. For transfection of MDP, cells were stimulated with PBS or MDP (10 μg/ml) mixed with Gene juice (1/100; Novagen) in serum-free RPMI 1640 for indicated times.

Total cell lysates

Cells were lysed in total cell lysis buffer (1% (v/v) Nonidet P-40, 50 mM HEPES, (pH 7.4) 10 mM NaF, 10 mM iodoacetamide, 75 mM NaCl, 1 mM PMSF, 1 mM Na_2VO_3, and 1 μg/ml each pepstatin, chymostatin, leupeptin, and antipain) and mix rotated at 4°C for 20 min. Cell nuclei and debris were eliminated by centrifugation at 10,000 g for 5 min. Reducing sample buffer (30 mM Tris, (pH 6.8) 1% (w/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and 2.5% (v/v) 2-ME) was then added to a final concentration of 20%. Where indicated, total protein content in the lysate was standardized using a protein assay (Bio-Rad), according to the manufacturer's instructions.

Affinity precipitation with biotinylated oligonucleotides

Affinity precipitation of DNA binding proteins was performed as previously described (35) using the consensus binding sequence for NF-κB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (36).

SDS-PAGE and Western blotting

Proteins were separated by SDS-PAGE using 10% acrylamide/0.03% bis for IκBα, and the various NF-κB subunits, or 7.5% acrylamide/0.03% bis for Nod2. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) and blocked in 4% dried milk/TBST (for Nod2); 5% dried milk, 0.05% Tween 20/PBS (for IκBα), or 3% BSA/TBST (for p50, p65, and c-Rel). The following Abs were used: anti-Nod2 rabbit polyclonal Ab (HM2563; a gift from Dr. Daniel K. Podolsky, Massachussetts General Hospital and Harvard Medical School, Boston, MA) (19); anti-IκBα mouse mAb (a gift from Prof. Ron Hay, St. Andrews University, Scotland); anti-p50 (SC-114X), anti-p65 (SC-109), and anti-cRel (SC-71X) (Santa Cruz Biotechnology); and anti-β-actin (A2066; Sigma-Aldrich). HRP-conjugated anti-mouse or anti-rabbit Abs were used. Proteins were visualized using the West Pico ECL development system (Pierce). For sequential detection, membranes were stripped in 100 mM 2-ME, 2% SDS, and 62.5 mM Tris (pH 6.8) for 1 h at 50°C.

^{51}Cr-release cytotoxicity assay

Standard ^{51}Cr-release assay was performed as previously described (34) using 1×10^4 Daudi target cells at an E:T ratio of 10:1.

CD107a staining

A total of 1×10^5 purified primary NK cells were stimulated, as described, under "cell stimulation" for 18 h in 96-round-bottom well plates. Freshly re-suspended K562 cells (10:1 E:T ratio) and anti-CD107a-FITC (4 μl/well; BD Pharmingen) or IgG1-FITC (4 μl/well; BD Pharmingen), as a control, were then added. Plates were centrifuged at 150g for 5 min and incubated for 1 h, before the addition of 10 μM monensin. Plates were incubated for further 5 h before extracellular staining and flow cytometric analysis. All incubations were at 37°C.

Staining of cell surface molecules for flow cytometric analysis

Cells were incubated with optimal concentrations of the following anti-human Abs: CD14-FITC, CD56-PE, CD3-PerCP, CD69-FITC, CD69-PerCP, or the corresponding isotype control Abs (all from BD Pharmingen) in 50 μl of 2% FCS/PBS at 4°C for 20 min. Cells were washed twice with PBS and acquired on a FACSCalibur flow cytometer. Events were collected and stored ungated in list-mode and analyzed using Cellquest sotfware (BD Biosciences).

IFN-γ intracellular staining for flow cytometric analysis

Cells were stimulated, as described, under "cell stimulation" for 18 h, the last 12 h in the presence of Golgi-Plug (BD Pharmingen). Cells were washed once in PBS and FcRs were blocked by incubating with 10% human AB serum/40 μg/ml rabbit IgG/40 μg/ml mouse IgG/2% FCS/PBS. Cell surface staining was performed as above, followed by intracellular staining with IFN-γ-FITC or IgG2a/b-FITC (both from BD Pharmingen) as control using the Cytofix/Cytoperm Plus kit (BD Pharmingen) according to the manufacturer's instructions.

Human IFN-γ ELISA

Production of IFN-γ by NK cells was assessed by ELISA using the human IFN-γ Ready-Set-Go kit (eBioscience) on cell culture supernatants according to the manufacturer's instructions.

Statistical analyses

One-way ANOVA followed by Newman-Keuls multiple comparison post test were used to test for statistical significance of differences between experimental groups; ∗, $p < 0.01$; ∗∗, $p < 0.001$.

Results

Nod2 is constitutively expressed in primary NK cells, NK cell lines, and primary monocytes

The present study aimed to investigate whether functional expression of Nod2 is a mechanism used by NK cells to sense bacterial Ags. Clear expression of Nod2 mRNA was found in all three NK cell lines (Fig. 1*A*) and in all donors in both purified primary NK cells (Fig. 1*A*) and monocytes (Fig. 1*A*). Nod2 protein was detected in all three NK cell lines, with NK92 cells consistently showing weaker expression compared with NKL and YT cells (Fig. 1*B*). Nod2 protein was also found in all donors tested in both purified primary NK cells (Fig. 1*C*) and monocytes (Fig. 1*D*). Lower total cell protein was required for detection in primary monocytes (54 μg) as compared with primary NK cells (130 μg), indicating that Nod2 is more abundant in the monocyte population. Nod2 protein was detected as a band running at ~140–145 kDa in all cell types analyzed. This apparent molecular mass is higher than the calculated molecular mass for Nod2 (115.3 kDa) suggesting the presence of post-translational modification(s). A lower band running at ~130–135 kDa was also detected in the case of purified primary NK cells and monocytes. Detection of two Nod2 bands in the primary cells most likely reflects translation from the two initiation sites previously reported for Nod2 (21). The present data show that Nod2 is constitutively expressed at both mRNA and

FIGURE 1. Nod2 is constitutively expressed in primary NK cells, NK cell lines, and primary monocytes. *A*, RT-PCR was performed on RNA isolated from NKL, NK92, YT (*n* = 3), purified primary NK cells (*n* = 4), and primary monocytes (*n* = 3) using primers specific for Nod2. Numbers correspond to individual donors. RNA from PBMC was used as a positive control for Nod2. RT-PCR for β-actin is shown as a quality control. Specific primers for KIR3DL1 were used as a control for gDNA contamination. gDNA from PBMC is shown as a positive control. Western blot analysis of Nod2 in total cell lysates of NKL, NK92, and YT (*n* = 3, *B*), purified primary NK cells (*n* = 3, *C*), and primary monocytes (*n* = 3, *D*). *B*, HL-60 and THP-1 were used as controls for low and high expression of Nod2, respectively (18).
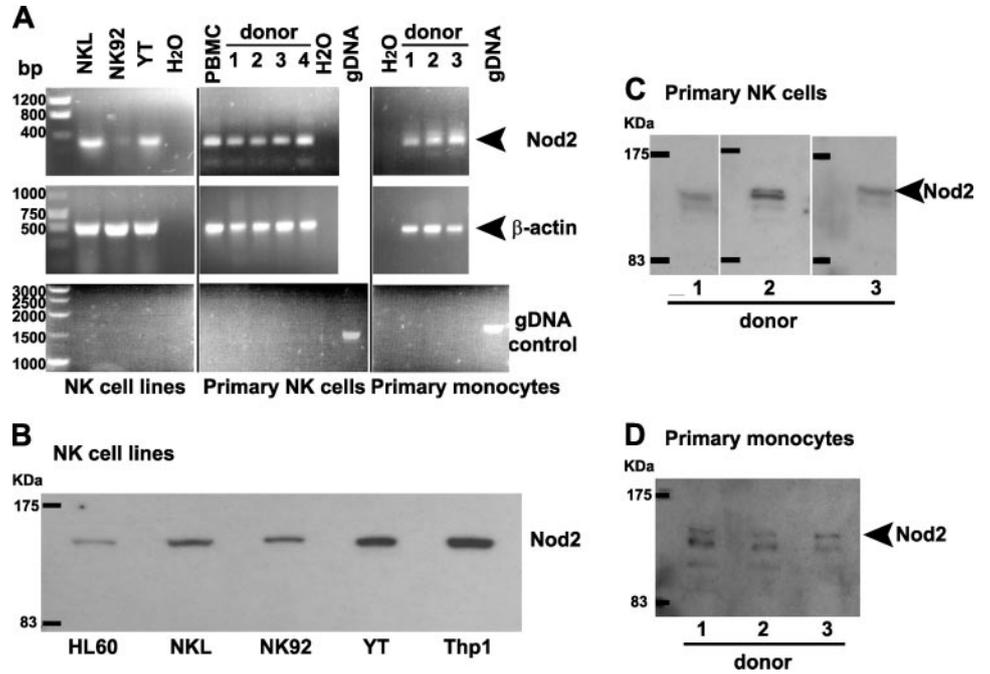
protein levels in NK cell lines, primary NK cells, and monocytes.

Human NKL cells, primary NK cells, and primary monocytes express functional Nod2

In intestinal epithelial cells and transient expression systems, MDP stimulates Nod2, leading to Rip-2-dependent IκB degradation and consequent activation of the NF-κB signaling pathway (16). IκB degradation as detected by Western blotting was used as a read out for NF-κB activation in response to MDP.

Degradation was clearly detected upon 60-min and 90-min stimulation of NKL cells with MDP (Fig. 2*A*, *left panel*) and at lower levels at 120-min stimulation (data not shown). IκB degradation was dose-dependent, being weak at 0.5, 1, and 100 μg/ml and maximal at 10 μg/ml MDP stimulation (Fig. 2*A*, *right panel*). To extend these findings to a more physiological system, we studied the direct activation of purified primary NK cells and monocytes by MDP. In the case of monocytic cells, this has been previously shown in mouse macrophages (24, 29) but not in the human system. IκB degradation in response to
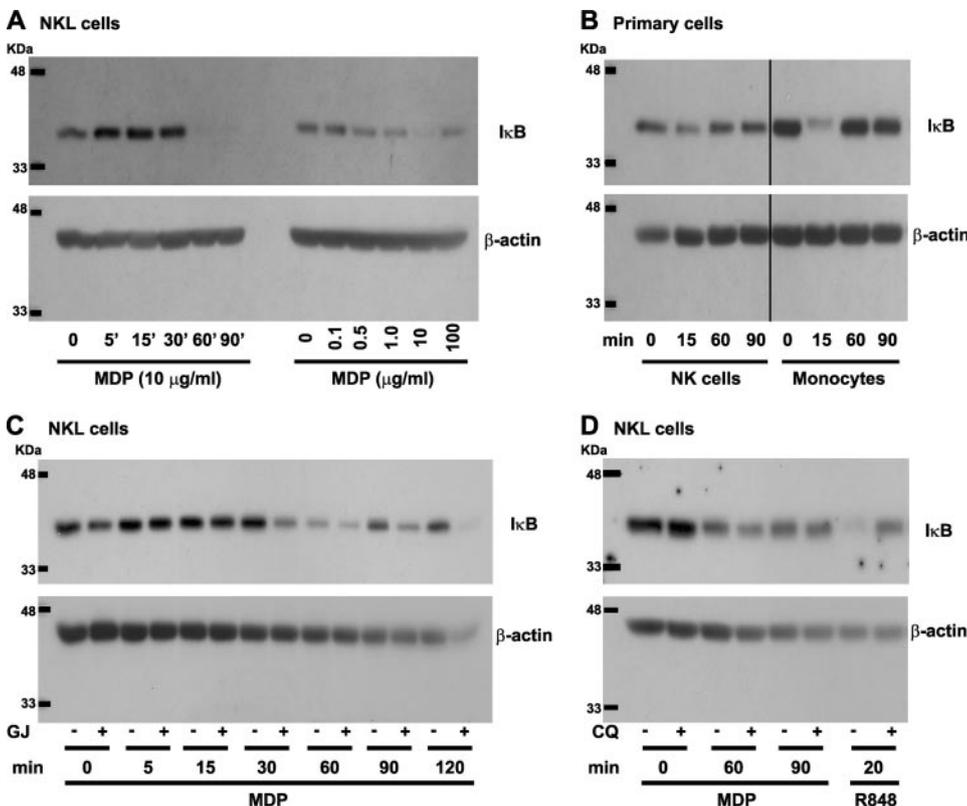

FIGURE 2. Human NKL cells, primary NK cells, and primary monocytes express functional Nod2. *A*, NKL cells were stimulated with PBS, 10 μg/ml MDP for indicated times or for 90 min with indicated concentrations of MDP. *B*, Purified primary NK cells (*left panel*) and primary monocytes (*right panel*) were stimulated with PBS or 10 μg/ml MDP for indicated times. *C*, For transfection of MDP, NKL cells were treated with a mixture of Gene juice (GJ):MDP (1:100; 10 μg/ml) or GJ: PBS as a negative control. *D*, NKL cells were pretreated with 20 μg/ml chloroquine (CQ) for 1 h before stimulation with 10 μg/ml MDP or 1 μg/ml R848 as a positive control. *A—D*, Following corresponding stimulation, Western blot analysis was performed on total cell lysates. Membranes were stained with an anti-IκB Ab (*upper panels*), followed by stripping and reprobing with anti-β-actin Ab (*lower panels*) as a loading control. Each figure is representative of three independent experiments.
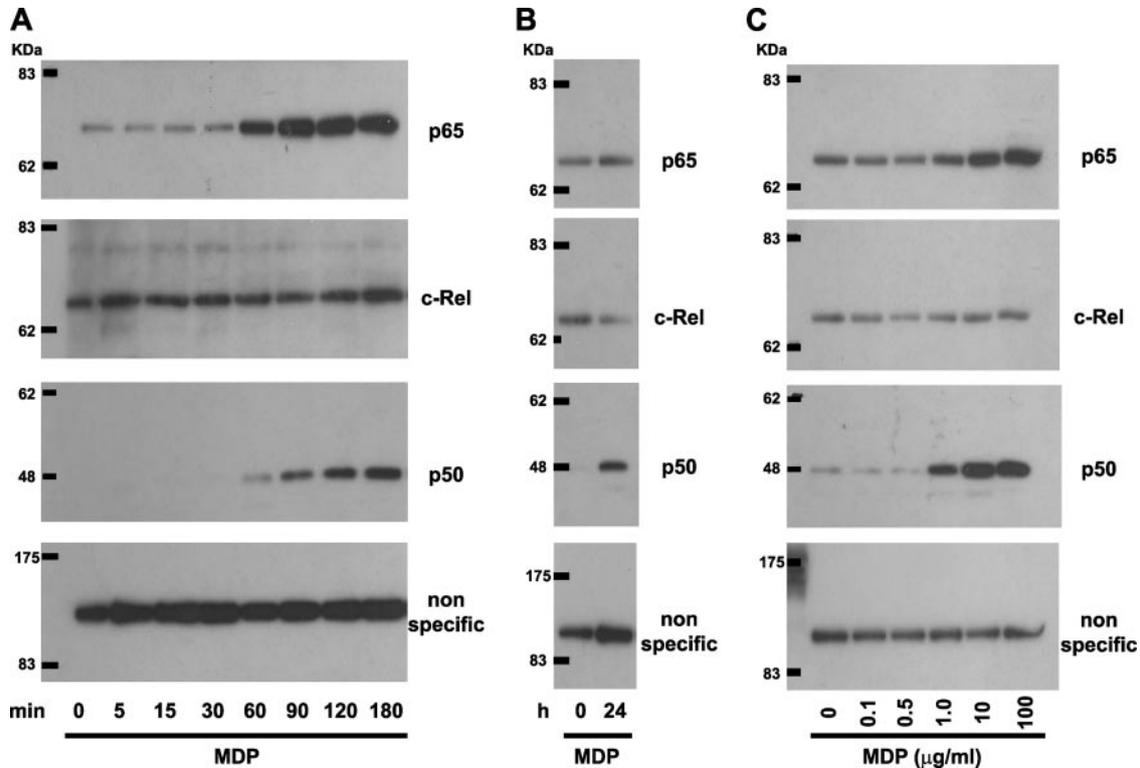
FIGURE 3. MDP induces activation of p50 and p65, but not c-Rel, in NK cells. NKL cells were stimulated with 10 μg/ml MDP for indicated times (*A* and *B*) or for 90 min with indicated concentrations of MDP (*C*). Whole cell lysates were prepared and proteins were DNA affinity purified using the consensus binding sequence for NF-κB, resolved on SDS-PAGE, and immunoblotted with anti-p65, followed by sequential stripping and reprobing with anti-c-Rel and anti-p50 Abs. A non-specific band detected with the anti-p65 Ab is shown as loading control (*bottom panels*). Each figure is representative of three independent experiments.

MDP was consistently detected in both primary populations and was weaker in primary NK cells (Fig. 2*B*). Both cell types show identical kinetics, with IκB being degraded following 15-min stimulation with MDP but not at later time points. Cryopyrin (also known as PYPAF1 and NALP3) has been proposed as an alternative receptor to Nod2 for MDP (37). However, cryopyrin mediates activation of caspase-1 and processing of pro-IL-1β but does not induce activation of the NF-κB signaling pathway (38–40). In contrast to monocytes, we found no evidence of IL-1β secretion from MDP-stimulated primary NK cells (data not shown). Although we were unable to knock-down Nod2 in NK cells using a range of commercial short interfering RNA and different transfection protocols (data not shown), previous demonstration that Nod2 is the receptor for MDP, activation of the NF-κB signaling pathway, lack of IL-1β secretion, and expression patterns of Nod2 in our experimental system all support that MDP is sensed through Nod2 in primary NK cells.

Nod2 expression at both mRNA and protein levels is induced in intestinal epithelial cells by LPS, IFN-γ, and TNF-α via an NF-κB-dependent mechanism (18, 19, 22). Activation of NF-κB in response to MDP in NK cells (Fig. 2*B*), along with a similar response in HL-60 cells (data not shown), suggested that MDP might mediate up-regulation of its own receptor. However, we did not find any changes on Nod2 protein expression levels following 24 h MDP stimulation in either NKL cells or primary NK cells (data not shown), indicating that activation of NF-κB is involved in, but not sufficient for, Nod2 up-regulation.

MDP is internalized by NK cells and does not require lysosomal acidification for signaling

Previous studies have shown that MDP is not naturally internalized by epithelial cells or the macrophage cell line RAW,

with intracellular delivery required for activation of the Nod2 signaling cascade (27, 28, 41). In our experiments, direct activation of Nod2 was achieved by extracellular addition of MDP to NKL cells, primary NK cells, and monocytes (Fig. 2, *A* and *B*), indicating that MDP is naturally internalized in these cells. However, there is a delay in the kinetics of IκB degradation in NKL cells compared with primary NK cells and monocytes. To test whether this delay was due to restricted MDP internalization, we compared IκB degradation in response to MDP, added extracellularly or delivered intracellularly by the transfection agent Gene juice (Fig. 2*C*). Intracellular delivery of MDP resulted in earlier IκB degradation, which was detected following 30 min of stimulation with MDP as compared with 60 min required for that added extracellularly. The response to transfected MDP was more pronounced at all time points tested, most likely reflecting availability of higher intracellular concentrations of MDP. Differences in the kinetics of IκB degradation between primary cells and NKL cells might reflect distinct mechanisms or different kinetics of MDP internalization in these cells.

Nod2 localizes in intracellular vesicles in intestinal epithelial cells (42, 43). As signaling by TLR7, TLR8, and TLR9, which are localized in phagolysosomal compartments, requires lysosomal acidification (34, 44), we investigated whether a similar mechanism was required for MDP/Nod2 signaling. Comparison of the MDP response in NKL cells pretreated with the lysosomal-acidification inhibitor chloroquine showed no difference in the levels of IκB degradation compared with control, indicating that MDP/Nod2 signaling, in contrast to TLR7/8, does not require lysosomal acidification (Fig. 2*D*). Taken together, these data demonstrate that NK cells internalize MDP and signaling is independent of the lysosomal pathway.

MDP induces activation of p50 and p65, but not of c-Rel, in NK cells

To further characterize the MDP/Nod2-induced NF-κB signaling pathway in NK cells, we studied activation of the NF-κB subunits p50, p65, and c-Rel, which can function as either homodimers or heterodimers. We used DNA-affinity purification assays with the consensus binding sequence for NF-κB. Only active forms of the NF-κB transcription factors are able to bind to this oligonucleotide and can therefore be affinity purified and detected by Western blot analysis with specific Abs. Comparative kinetics show parallel activation of p65 and p50 at early stimulation times in response to MDP (Fig. 3A). A clear increase in the active forms of both transcription factors is detected at 60 min and remains elevated for at least 3 h following MDP stimulation (Fig. 3A). At 24-h stimulation, p50 remains active, whereas p65 is only detected at background levels (Fig. 3B). Activation of these transcription factors was detected at 1, 10, and 100 μg/ml MDP and was maximal by 10 μg/ml (Fig. 3C). The kinetics of activation, as well as the optimal MDP dose, are in agreement with the IκB degradation results (Fig. 2A). Background levels of active c-Rel were consistently detected and these were not modified in response to MDP at any time or dose tested (Fig. 3, A–C).

MDP synergizes with IFN-α for activation of NK cells, and IL-12 for production of IFN-γ, but does not induce cytotoxicity in primary NK cells

To define whether direct activation of primary NK cells by MDP translates into a functional response, we studied CD69 up-regulation, induction of IFN-γ, and cytotoxicity of NK cells. Highly purified NK cell preparations were first used in functional experiments. Compared with PBS, MDP stimulation of purified primary NK cells induced some increase (1.5–2.7-fold increase) in the percentage of CD69 positive cells in only 5 of 15 donors (Fig. 4 and data not shown). Similarly, MDP alone induced minor to undetectable levels of IFN-γ, as studied by both ELISA and intracellular staining (Fig. 5). However, costimulation with suboptimal concentrations of IFN-α or IL-12 readily induced robust CD69 up-regulation and IFN-γ production, respectively (Figs. 4 and 5). A clear synergy between MDP and IFN-α was observed for CD69 up-regulation, whereas IL-12 had no appreciable effect on this response (Fig. 4, A and B). As MDP synergized with IFN-α to increase CD69 expression on purified NK cells, we tested whether contaminating accessory cells played a role in this response. NK cells were sorted to greater than 99% purity (with no detectable monocyte contamination). As expected, MDP on its own did not increase CD69 expression. IFN-α alone did increase CD69 expression, but the synergy observed between MDP and IFN-α was absent when cell sorted NK cells were used (Fig. 4C). This defines induction of CD69 on human NK cells by MDP as being accessory cell dependent.

In the case of IFN-γ production, the synergy with MDP was more pronounced in the presence of IL-12 compared with IFN-α with over 4 times higher percentages of IFN-γ positive cells and 10 times higher levels of secreted IFN-γ (Fig. 5). As expected, IFN-γ positive cells were detected primarily within the CD56^{bright} but not the CD56^{dim} population (Fig. 5 and data not shown). The percentages of CD69 and IFN-γ positive cells, as well as the concentrations of secreted IFN-γ, show high variability among individuals (Figs. 4 and 5 and data not shown) as has previously been reported for other experimental systems (45). However, the trend of the responses and the synergy were consistently observed over a wide range of concentrations on all donors tested. As low levels of accessory cells contribute to the
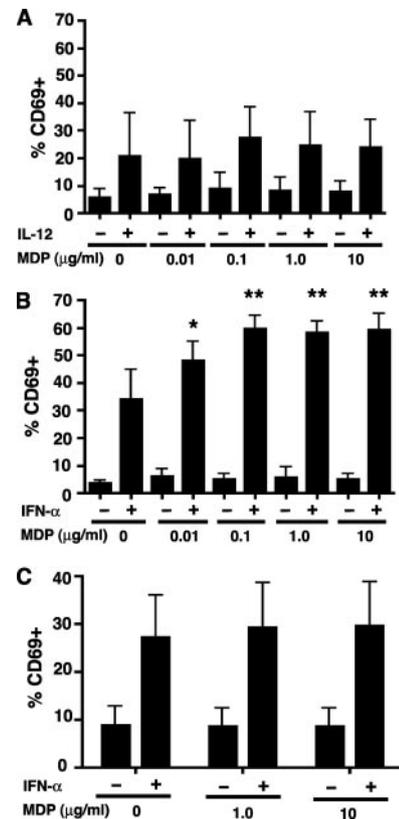

FIGURE 4. MDP synergizes with IFN-α for activation of NK cells. Purified primary NK cells were stimulated with indicated concentrations of MDP in the presence or absence of 1 ng/ml IL-12 (A) or 50 U/ml IFN-α (B) for 18 h. Sorted primary NK cells were stimulated with indicated concentrations of MDP in the presence or absence of 50 U/ml IFN-α for 18 h (C). Cell surface expression of CD69 was assessed by specific immunostaining and flow cytometric analysis. Data are expressed as percentage of CD3^{−}CD56^{+}CD69^{+} cells. Error bars show SEM. IFN-α synergy for CD69 expression is statistically significant as tested by one-way ANOVA followed by Newman-Keuls multiple comparison post test; *, $p < 0.01$; **, $p < 0.001$. The data represent the average values obtained for three (B and C) and six donors (A).

synergy between MDP and IFN-α in up-regulating CD69 expression on NK cells, we investigated the role of accessory cells in the synergy observed between MDP and IL-12 in inducing IFN-γ production by NK cells. As expected, MDP alone did not stimulate IFN-γ production in sorted NK cells. IL-12 alone induced some cytokine but, importantly, MDP synergized with IL-12 over a range of concentrations to stimulate production of IFN-γ by the sorted NK cells (Fig. 5E). This result clearly demonstrates a direct effect of MDP on NK cell activation as the synergy with IL-12 was observed in the definitive absence of any other cell type. Thus, in the presence of cytokine, NK cells can directly activate effector functions in response to the bacterial product MDP.

Various concentrations of MDP induced low levels of cytotoxicity on purified primary NK cells, as detected by both the ^{51}Cr-release assay and the percentage of CD107a positive NK cells compared with PBS stimulation (Fig. 6). However, MDP was unable to induce cytotoxicity on sorted primary NK cells (Fig. 6A) Contrary to the synergy observed for CD69 up-regulation and induction of IFN-γ, there was no change in the percentages of CD107a positive cells following costimulation with MDP and either IL-12 or IFN-α (Fig. 6B). Taken together, these data demonstrate that MDP directly acts on NK cells, where it synergizes with
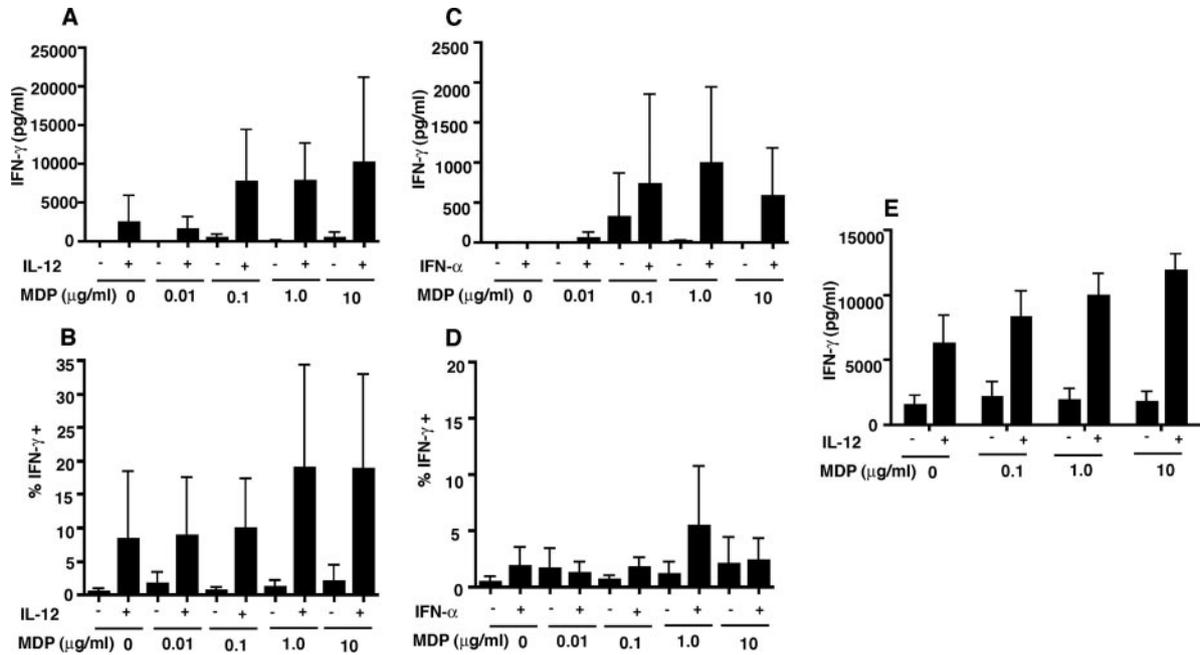
FIGURE 5. MDP synergizes with IL-12 and IFN-α for induction of IFN-γ in NK cells. Purified primary NK cells were stimulated with indicated concentrations of MDP in the presence or absence of 1 ng/ml IL-12 (A) or 50 U/ml IFN-α (B) for 18 h. Sorted primary NK cells were stimulated with indicated concentrations of MDP in the presence or absence of 1 ng/ml IL-12 for 18 h (E). The secretion and the percentage of IFN-γ producing cells were assessed by ELISA and expressed as pg/ml IFN-γ (A, C, and E) or intracellular staining followed by flow cytometric analysis and expressed as percentage CD3⁻CD56⁺IFN-γ⁺ cells (B and D). Error bars show SEM. The data represent the average values obtained for six (A), five (B and D), and three donors (C and E).

IL-12 to selectively drive cytokine production, rather than a cytotoxic response, which is consistent with a role for NK cells against bacterial infections.
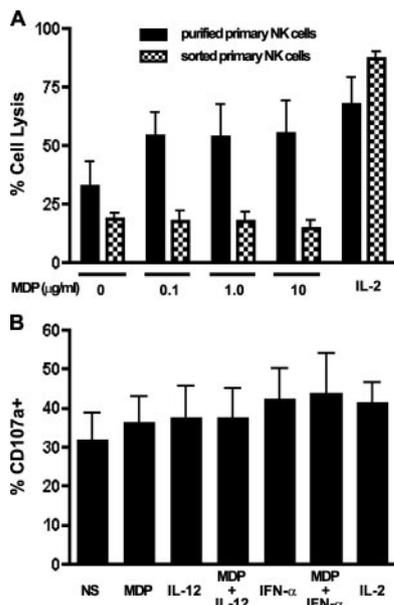

FIGURE 6. MDP does not synergize with IL-12 or IFN-α for NK cell cytotoxicity. Purified (A and B, filled histograms) or sorted (A, checkerboard histograms) primary NK cells were stimulated with indicated concentrations of MDP in the presence or absence of 1 ng/ml IL-12 or 50 U/ml IFN-α for 18 h (B). Cytotoxicity and cytotoxic capacity were studied using the ⁵¹Cr release assay (A) and CD107a staining (B). Data are expressed as percentage of cytotoxicity (A) and of CD3⁻CD56⁺CD107a⁺ cells (B). Stimulation with PBS and IL-2 (200 U/ml) were used as negative and positive controls, respectively. The data represent the average values obtained for three (A, checkerboard histograms) and four donors (A and B, filled histograms).

Discussion

Studies in NK cell-deficient patients and NK cell-depleted mouse models have established the importance of NK cells in eradication of bacterial infections (4–6, 9–11). In particular, IFN-γ production by NK cells has been shown to be critical for control of particular pathogens, e.g., *L. monocytogenes* (6), and in directing an appropriate Th1 type adaptive immune response required for full clearance of the infection (5). The mechanisms underlying the role played by NK cells are poorly understood but are thought to primarily involve indirect activation of NK cells by accessory cell-derived cytokines. However, data demonstrating direct activation of NK cells by bacterial products has started to emerge. The functional expression of TLR2 and TLR5 (12) provided the first clear evidence that NK cells are equipped with the mechanisms to directly respond to bacterial PAMPs. In the present study, we demonstrate functional expression of Nod2 in human NK cells and that stimulation with its specific ligand MDP leads to activation of NK cell responses. Although MDP can directly activate signaling in NK cells, optimal functional responses require costimulation by accessory cell-derived cytokines.

Studies on intestinal epithelial cells have shown that activation of Nod2 can only be achieved through intracellular delivery of MDP, either through invasive bacterial infection or transfection (27, 28, 41). A report using the colonic epithelial cell line Caco-2 suggested that the di-/tripeptidyl brush-border transporter hPepT1 mediates the intracellular transport of MDP (46). This transporter is not constitutively expressed in normal colonic epithelia, but its expression levels are increased in patients with chronic ulcerative colitis and Crohn's disease (47). In contrast, we show that MDP is naturally internalized by NKL cells, primary NK cells, and primary monocytes (Fig. 2, A and B). Our results indicate that both primary NK cells and monocytes are equipped to rapidly respond to bacterial-derived MDP and consequently have the potential to be directly activated by both Gram-positive and Gram-negative bacteria. This implies a clear difference in the response to MDP

between the immune system in the gut and the periphery. The constant exposure of gut epithelia to commensal bacterial Ags requires that responses to MDP are restricted to pathogenic conditions whereas immediate immune responses are required following bacterial sensing in the periphery.

CD69 is a commonly used activation marker and is up-regulated in response to MDP and cytokine in human NK cells (Fig. 4). Recent in vivo studies using CD69-deficient mice in an NK-sensitive tumor model have proposed a new non-redundant role for CD69 signaling in the induction of TGF-β (48, 49). Previous in vitro studies showing correlation of CD69 expression and increased NK cell cytotoxicity (50, 51) also support an activatory role for CD69. Therefore CD69 up-regulation seems to prepare NK cells for further responses, such as cytotoxicity or cytokine production. Bacterial products can weakly induce the expression of CD69 on NK cells and robust responses can only be achieved by costimulation with cytokines (12, 52–54). Maximal CD69 expression on NK cells in response to the outer membrane protein A from *K. pneumoniae*, flagellin, and unmethylated CpG motifs requires costimulation with IL-2 and IL-12 (12, 52–54). Consistent with a requirement for costimulation, MDP on its own poorly induces CD69 expression on NK cells, but this is increased in the presence of cytokine. Enhanced expression of CD69 in response to MDP and cytokine, and subsequent downstream production of TGF-β, may provide a mechanism to prepare NK cells for an optimal response to specific bacterial infections, e.g., TGF-β is an important mediator in the immune response to *L. monocytogenes* (55).

Induction of IFN-γ in T cells requires stimulation with multiple cytokines to drive activation of specific combinations of the transcription factors STAT4, NF-κB, NF-AT, AP-1, and CREB/activating transcription factor (56–63). Although the mechanisms regulating IFN-γ production in NK cells are still poorly understood, multiple cytokine stimulation is also required (61, 64, 65). In this study, we show that in NK cells, MDP can readily synergize with IL-12 and (to a lesser extent) with IFN-α for the induction of IFN-γ, while each stimulus alone induces undetectable to minor levels of this cytokine (Fig. 5). IL-12 and IFN-α both activate STAT4 in NK cells and here we show that MDP activates NF-κB (Figs. 2B and 3). Concomitant activation of STAT4 and NF-κB during combined stimulation with MDP and IL-12 or IFN-α may explain the observed synergy for IFN-γ production. This explanation is also supported by a previous report showing that in NK cells, IL-18 (which activates NF-κB) synergizes with IL-12 or IFN-α for IFN-γ production (66, 67). Similar to our findings with MDP, IL-18 also induces higher levels of IFN-γ production following costimulation with IL-12 compared with IFN-α (66). IL-12 induces a more sustained activation of STAT4 compared with IFN-α in both T and NK cells (35, 66). A sustained activation of STAT4 in response to IL-12 in NK cells could explain how, in response to MDP, this cytokine induced 4 times higher percentages of IFN-γ positive cells and 10 times higher levels of secreted IFN-γ compared with IFN-α (Fig. 5).

Interestingly, while IL-12 costimulation leads to a greater IFN-γ response by NK cells, higher levels of CD69 in response to MDP are induced in the presence of IFN-α. This suggests that immune responses to bacterial Ags that preferentially trigger early IFN-α may prime NK cells for TGF-β production (through up-regulation of CD69), whereas early IL-12 production may bias NK cells toward an IFN-γ response. Different effector NK cell responses may be required depending on the nature of the pathogen, e.g., IFN-γ production may be more important in the case of particular bacterial infections such as *Bordetella pertussis* (5). Varied combinations of bacterial PAMPs (including MDP) from different microorganisms will stimulate specific subsets of accessory cells to become activated and secrete distinct cytokine profiles. In parallel with trends seen with TLR activation of NK cells, it seems likely that a combination of cytokine and direct activation will optimally activate NK cells through Nod receptors. Indeed, this study adds MDP to the growing list of bacterial PAMPs that activate human NK cell functions to promote efficient clearance of pathogen.

References

1. Hamerman, J. A., K. Ogasawara, and L. L. Lanier. 2005. NK cells in innate immunity. *Curr. Opin. Immunol.* 17: 29–35.
2. Lanier, L. L. 2005. NK cell recognition. *Annu. Rev. Immunol.* 23: 225–274.
3. Moretta, L., C. Bottino, D. Pende, M. Vitale, M. C. Mingari, and A. Moretta. 2005. Human natural killer cells: molecular mechanisms controlling NK cell activation and tumor cell lysis. *Immunol. Lett.* 100: 7–13.
4. Orange, J. S. 2002. Human natural killer cell deficiencies and susceptibility to infection. *Microbes Infect.* 4: 1545–1558.
5. Byrne, P., P. McGuirk, S. Todryk, and K. H. Mills. 2004. Depletion of NK cells results in disseminating lethal infection with *Bordetella pertussis* associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur. J. Immunol.* 34: 2579–2588.
6. Dunn, P. L., and R. J. North. 1991. Early γ interferon production by natural killer cells is important in defense against murine listeriosis. *Infect. Immun.* 59: 2892–2900.
7. Harty, J. T., and M. J. Bevan. 1995. Specific immunity to *Listeria monocytogenes* in the absence of IFN γ. *Immunity* 3: 109–117.
8. Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the interferon-γ receptor. *Science* 259: 1742–1745.
9. Le-Barillec, K., J. G. Magalhaes, E. Corcuff, A. Thuizat, P. J. Sansonetti, A. Phalipon, and J. P. Di Santo. 2005. Roles for T and NK cells in the innate immune response to *Shigella flexneri*. *J. Immunol.* 175: 1735–1740.
10. Sporri, R., N. Joller, U. Albers, H. Hilbi, and A. Oxenius. 2006. MyD88-dependent IFN-γ production by NK cells is key for control of *Legionella pneumophila* infection. *J. Immunol.* 176: 6162–6171.
11. Way, S. S., A. C. Borczuk, R. Dominitz, and M. B. Goldberg. 1998. An essential role for γ interferon in innate resistance to *Shigella flexneri* infection. *Infect. Immun.* 66: 1342–1348.
12. Chalifour, A., P. Jeannin, J.-F. Gauchat, A. Blaecke, M. Malissard, T. N'Guyen, N. Thieblemont, and Y. Delneste. 2004. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers α-defensin production. *Blood* 104: 1778–1783.
13. Yun, C. H., A. Lundgren, J. Azem, A. Sjoling, J. Holmgren, A.-M. Svennerholm, and B. S. Lundin. 2005. Natural killer cells and *Helicobacter pylori* infection: bacterial antigens and interleukin-12 act synergistically to induce γ interferon production. *Infect. Immun.* 73: 1482–1490.
14. Creagh, E. M., and A. O'Neill, L. 2006. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol.* 27: 352–357.
15. O'Neill, L. A. J. 2006. How toll-like receptors signal: what we know and what we don't know. *Curr. Opin. Immunol.* 18: 3–9.
16. Strober, W., P. J. Murray, A. Kitani, and T. Watanabe. 2006. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat. Rev. Immunol.* 6: 9–20.
17. Fritz, J. H., S. E. Girardin, C. Fitting, C. Werts, D. Mengin-Lecreulx, M. Caroff, J. M. Cavaillon, D. J. Philpott, and M. Adib-Conquy. 2005. Synergistic stimulation of human monocytes and dendritic cells by toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur. J. Immunol.* 35: 2459–2470.
18. Gutierrez, O., C. Pipaon, N. Inohara, A. Fontalba, Y. Ogura, F. Prosper, G. Nunez, and J. L. Fernandez-Luna. 2002. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-κ B activation. *J. Biol. Chem.* 277: 41701–41705.
19. Hisamatsu, T., M. Suzuki, H. C. Reinecker, W. J. Nadeau, B. A. McCormick, and D. K. Podolsky. 2003. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology* 124: 993–1000.
20. Maeda, S., L.-C. Hsu, H. Liu, L. A. Bankston, M. Iimura, M. F. Kagnoff, L. Eckmann, and M. Karin. 2005. Nod2 mutation in Crohn's disease potentiates NF-κB activity and IL-1β processing. *Science* 307: 734–738.
21. Ogura, Y., N. Inohara, A. Benito, F. F. Chen, S. Yamaoka, and G. Nunez. 2001. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-κ B. *J. Biol. Chem.* 276: 4812–4818.
22. Rosenstiel, P., M. Fantini, K. Brautigam, T. Kuhbacher, G. H. Waetzig, D. Seegert, and S. Schreiber. 2003. TNF-α and IFN-γ regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology* 124: 1001–1009.

23. Voss, E., J. Wehkamp, K. Wehkamp, E. F. Stange, J. M. Schroder, and J. Harder. 2006. NOD2/CARD15 mediates induction of the antimicrobial peptide human β-defensin-2. *J. Biol. Chem.* 281: 2005–2011.

24. Kobayashi, K. S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R. A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307: 731–734.

25. Kambe, N., R. Nishikomori, and N. Kanazawa. 2005. The cytosolic pattern-recognition receptor Nod2 and inflammatory granulomatous disorders. *J. Dermatol. Sci.* 39: 71–80.

26. Watanabe, T., A. Kitani, and W. Strober. 2005. NOD2 regulation of toll-like receptor responses and the pathogenesis of Crohn's disease. *Gut* 54: 1515–1518.

27. Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti. 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* 278: 8869–8872.

28. Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, et al. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2: implications for Crohn's disease. *J. Biol. Chem.* 278: 5509–5512.

29. Watanabe, T., A. Kitani, P. J. Murray, and W. Strober. 2004. NOD2 is a negative regulator of toll-like receptor 2[ndash]mediated T helper type 1 responses. *Nat. Immunol.* 5: 800–808.

30. Inohara, Chamaillard, C. McDonald, and G. Nunez. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu. Rev. Biochem.* 74: 355–383.

31. Robertson, M. J., and J. Ritz. 1990. Biology and clinical relevance of human natural killer cells. *Blood* 76: 2421–2438.

32. Yokoyama, W. M. 1999. *Natural Killer Cells.* Lippincott-Raven Publishers, Philadelphia.

33. Tada, H., S. Aiba, K. Shibata, T. Ohteki, and H. Takada. 2005. Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect. Immun.* 73: 7967–7976.

34. Hart, O. M., V. Athie-Morales, G. M. O'Connor, and C. M. Gardiner. 2005. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFNγ production. *J. Immunol.* 175: 1636–1642.

35. Athie-Morales, V., H. H. Smits, D. A. Cantrell, and C. M. Hilkens. 2004. Sustained IL-12 signaling is required for Th1 development. *J. Immunol.* 172: 61–69.

36. Lenardo, M. J., and D. Baltimore. 1989. NF-κ B: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 58: 227–229.

37. Martinon, F., L. Agostini, E. Meylan, and J. Tschopp. 2004. Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. *Curr. Biol.* 14: 1929–1934.

38. Jeru, I., H. Hayrapetyan, P. Duquesnoy, T. Sarkisian, and S. Amselem. 2006. PYPAF1 nonsense mutation in a patient with an unusual autoinflammatory syndrome: role of PYPAF1 in inflammation. *Arthritis Rheum.* 54: 508–514.

39. Kanneganti, T.-D., N. Ozoren, M. Body-Malapel, A. Amer, J.-H. Park, L. Franchi, J. Whitfield, W. Barchet, M. Colonna, P. Vandenabeele, et al. 2006. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440: 233–236.

40. O'Connor, W., Jr., J. A. Harton, X. Zhu, M. W. Linhoff, and J. P. Ting. 2003. Cutting edge: CIAS1/cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 is an inducible inflammatory mediator with NF-κ B suppressive properties. *J. Immunol.* 171: 6329–6333.

41. Oh, H. M., H. J. Lee, G. S. Seo, E. Y. Choi, S. H. Kweon, C. H. Chun, W. C. Han, K. M. Lee, M. S. Lee, S. C. Choi, and C. D. Jun. 2005. Induction and localization of NOD2 protein in human endothelial cells. *Cell. Immunol.* 237: 37–44.

42. Barnich, N., J. E. Aguirre, H. C. Reinecker, R. Xavier, and D. K. Podolsky. 2005. Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-κB activation in muramyl dipeptide recognition. *J. Cell Biol.* 170: 21–26.

43. Barnich, N., T. Hisamatsu, J. E. Aguirre, R. Xavier, H. C. Reinecker, and D. K. Podolsky. 2005. GRIM-19 interacts with nucleotide oligomerization domain 2 and serves as downstream effector of anti-bacterial function in intestinal epithelial cells. *J. Biol. Chem.* 280: 19021–19026.

44. Latz, E., A. Schoenemeyer, A. Visintin, K. A. Fitzgerald, B. G. Monks, C. F. Knetter, E. Lien, N. J. Nilsen, T. Espevik, and D. T. Golenbock. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* 5: 190–198.

45. Korbel, D. S., K. C. Newman, C. R. Almeida, D. M. Davis, and E. M. Riley. 2005. Heterogeneous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. *J. Immunol.* 175: 7466–7473.

46. Vavricka, S. R., M. W. Musch, J. E. Chang, Y. Nakagawa, K. Phanvijhitsiri, T. S. Waypa, D. Merlin, O. Schneewind, and E. B. Chang. 2004. hPepT1 transports muramyl dipeptide, activating NF-κB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. *Gastroenterology* 127: 1401–1409.

47. Merlin, D., M. Si-Tahar, S. V. Sitaraman, K. Eastburn, I. Williams, X. Liu, M. A. Hediger, and J. L. Madara. 2001. Colonic epithelial hPepT1 expression occurs in inflammatory bowel disease: transport of bacterial peptides influences expression of MHC class 1 molecules. *Gastroenterology* 120: 1666–1679.

48. Esplugues, E., D. Sancho, J. Vega-Ramos, C. Martinez, U. Syrbe, A. Hamann, P. Engel, F. Sanchez-Madrid, and P. Lauzurica. 2003. Enhanced antitumor immunity in mice deficient in CD69. *J. Exp. Med.* 197: 1093–1106.

49. Sancho, D., M. Gomez, and F. Sanchez-Madrid. 2005. CD69 is an immunoregulatory molecule induced following activation. *Trends Immunol.* 26: 136–140.

50. Clausen, J., B. Vergeiner, M. Enk, A. L. Petzer, G. Gastl, and E. Gunsilius. 2003. Functional significance of the activation-associated receptors CD25 and CD69 on human NK-cells and NK-like T-cells. *Immunobiology* 207: 85–93.

51. Lanier, L. L., D. W. Buck, L. Rhodes, A. Ding, E. Evans, C. Barney, and J. H. Phillips. 1988. Interleukin 2 activation of natural killer cells rapidly induces the expression and phosphorylation of the Leu-23 activation antigen. *J. Exp. Med.* 167: 1572–1585.

52. Chace, J. H., N. A. Hooker, K. L. Mildenstein, A. M. Krieg, and J. S. Cowdery. 1997. Bacterial DNA-induced NK cell IFN-γ production is dependent on macrophage secretion of IL-12. *Clin. Immunol. Immunopathol.* 84: 185–193.

53. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168: 4531–4537.

54. Sivori, S., M. Falco, M. D. Chiesa, S. Carlomagno, M. Vitale, L. Moretta, and A. Moretta. 2004. CpG and double-stranded RNA trigger human NK cells by toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *PNAS* 101: 10116–10121.

55. Nakane, A., M. Asano, S. Sasaki, S. Nishikawa, T. Miura, M. Kohanawa, and T. Minagawa. 1996. Transforming growth factor β is protective in host resistance against *Listeria monocytogenes* infection in mice. *Infect. Immun.* 64: 3901–3904.

56. Barbulescu, K., C. Becker, J. F. Schlaak, E. Schmitt, K. H. Meyer zum Buschenfelde, and M. F. Neurath. 1998. IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-γ promoter in primary CD4+ T lymphocytes. *J. Immunol.* 160: 3642–3647.

57. Chang, J. T., B. M. Segal, K. Nakanishi, H. Okamura, and E. M. Shevach. 2000. The costimulatory effect of IL-18 on the induction of antigen-specific IFN-γ production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor β2 subunit. *Eur. J. Immunol.* 30: 1113–1119.

58. Nakahira, M., H.-J. Ahn, W.-R. Park, P. Gao, M. Tomura, C.-S. Park, T. Hamaoka, T. Ohta, M. Kurimoto, and H. Fujiwara. 2002. Synergy of IL-12 and IL-18 for IFN-γ gene expression: IL-12-induced STAT4 contributes to IFN-γ promoter activation by up-regulating the binding activity of IL-18-induced activator protein 1. *J. Immunol.* 168: 1146–1153.

59. Sareneva, T., S. Matikainen, M. Kurimoto, and I. Julkunen. 1998. Influenza A virus-induced IFN-α/β and IL-18 synergistically enhance IFN-γ gene expression in human T cells. *J. Immunol.* 160: 6032–6038.

60. Sica, A., L. Dorman, V. Viggiano, M. Cippitelli, P. Ghosh, N. Rice, and H. A. Young. 1997. Interaction of NF-κ B and NFAT with the interferon-γ promoter. *J. Biol. Chem.* 272: 30412–30420.

61. Strengell, M., S. Matikainen, J. Siren, A. Lehtonen, D. Foster, I. Julkunen, and T. Sareneva. 2003. IL-21 in synergy with IL-15 or IL-18 enhances IFN-γ production in human NK and T cells. *J. Immunol.* 170: 5464–5469.

62. Sweetser, M. T., T. Hoey, Y.-L. Sun, W. M. Weaver, G. A. Price, and C. B. Wilson. 1998. The roles of nuclear factor of activated T cells and ying-yang 1 in activation-induced expression of the interferon-γ promoter in T cells. *J. Biol. Chem.* 273: 34775–34783.

63. Yang, J., T. L. Murphy, W. Ouyang, and K. M. Murphy. 1999. Induction of interferon-γ production in Th1 CD4+ T cells: evidence for two distinct pathways for promoter activation. *Eur. J. Immunol.* 29: 548–555.

64. Fehniger, T. A., W. E. Carson, and M. A. Caligiuri. 1999. Costimulation of human natural killer cells is required for interferon γ production. *Transplant. Proc.* 31: 1476–1478.

65. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* 162: 4511–4520.

66. Matikainen, S., A. Paananen, M. Miettinen, M. Kurimoto, T. Timonen, I. Julkunen, and T. Sareneva. 2001. IFN-α and IL-18 synergistically enhance IFN-γ production in human NK cells: differential regulation of Stat4 activation and IFN-γ gene expression by IFN-α and IL-12. *Eur. J. Immunol.* 31: 2236–2245.

67. Mavropoulos, A., G. Sully, A. P. Cope, and A. R. Clark. 2005. Stabilization of IFN-γ mRNA by MAPK p38 in IL-12- and IL-18-stimulated human NK cells. *Blood* 105: 282–288.