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

Verónica Athié-Morales,* Geraldine M. O’Connor,† and Clair M. Gardiner1†

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) 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.
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 performed on cDNA samples using TaqDNA polymerase (Promega) and specific primers for Nod2 (F: 5'-AACCAATTGTCAAGGAGGCTC-3'; R: 5'-GGTCCTGGTCCTACATA GG-3') (18) and β-actin (F: 5'-CGGAGAAGATGACCCGAAGATC-3'; R: 5'-TTGCTGTATCCAATCTGCTGG-3'). As a control for genomic DNA, (gDNA) contamination, we used specific primers for KIR3DL1 (F: 5'-CCATCGTCCATGATCGT-3'; R: 5'-AGAGAGAAGTTTCTCA TATG-3'), which amplify a 1600-bp fragment if gDNA is present. cDNA from PBMC was used as a positive control for KIR3DL1 amplification.

Cell stimulation
Unless otherwise stated, cells were stimulated at a density of 1 × 10⁶ cells/ml with 1 ng/ml IFN-γ, 50 μM 7HPE, (pH 7.4) 10 mM NaF, 10 mM iodacetamide, 75 mM NaCl, 1 mM PMSF, 1 mM Na₂VO₃, 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.

Total cell lysates
Cells were lysed in total lysis buffer (1% (v/v) Nonidet P-40, 50 mM HEPES, (pH 7.4) 10 mM NaF, 10 mM iodacetamide, 75 mM NaCl, 1 mM PMSF, 1 mM Na₂VO₃, 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'-AGTTGAGGGGACCTCCAGGC-3' (36). 

SDS-PAGE and Western blotting
Proteins were separated by SDS-PAGE using 10% acrylamide/0.03% bis for IgG, A, and the various NF-κB subunits, or 7.5% acrylamide/0.03% bis for Nod2. Proteins were transferred to polyvinylidine difluoride membranes (Immobilon-P; Millipore) and blocked in 4% dried milk/TBST (for Nod2); 5% dried milk, 0.05% Tween 20/ PBS (for IgG), 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-β2A mouse mAb (a gift from Prof. Ron Hay, St. Andrews University, Scotland); anti-p50 (SC-114X), anti-p65 (SC-109), and anti-c-Rel (SC-71X) (Santa Cruz Biotechnology); and anti-β-actin (A2066, Sigma-Aldrich). HRP-conjugated anti-rabbit 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.
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. 2A, 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. 2A, 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...
MDP was consistently detected in both primary populations and was weaker in primary NK cells (Fig. 2B). Both cell types show identical kinetics, with IκBα 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 NKL cells and primary NK cells (Fig. 2, A and B), indicating that MDP is naturally internalized in these cells. However, there is a delay in the kinetics of IκBα degradation in NKL cells compared with primary NK cells and monocytes. To test whether this delay was due to restricted MDP internalization, we compared IκBα degradation in response to MDP, added extracellularly or delivered intracellularly by the transfection agent Gene juice (Fig. 2C). Intracellular delivery of MDP resulted in earlier IκBα 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 IκBα 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 IκBα degradation compared with control, indicating that MDP/Nod2 signaling, in contrast to TLR7/8, does not require lysosomal acidification (Fig. 2D). 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 IkB 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 CD56bright but not the CD56dim 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 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 ⁵¹Cr-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...
IL-12 to selectively drive cytokine production, rather than a cytotoxic response, which is consistent with a role for NK cells against bacterial infections.

**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 can directly activate signaling in NK cells, optimal functional responses require costimulation by accessory cell-derived cytokines.

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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 cytokotyes (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 cytokotyes to drive activation of specific combinations of 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. 28 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 TLRs and 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.

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References


