Human NK cells directly recognize Mycobacterium bovis via TLR2 and acquire the ability to kill monocyte-derived DC

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Abstract

NK cells are important players of the early innate defense against various pathogens. In this study, we investigated the interaction between human NK cells and Mycobacterium bovis [bacille Calmette–Guérin (BCG)] and we determined whether and how such an interaction might impact on NK cell activation, cytokine production and cytotoxicity. We show that highly purified NK cells, upon short-term co-culture with BCG, expressed activation markers including CD69 and CD25. Moreover, these NK cells released IFN-gamma and tumor necrosis factor-alpha and killed more efficiently different targets including monocyte-derived immature dendritic cell. All these functions were strongly up-regulated in the presence of exogenous IL-12. Although more efficient responses were detected in NK cell populations displaying an NCRbright phenotype, no direct evidence of an involvement of triggering NK receptors in BCG recognition could be obtained. On the other hand, anti-toll-like receptor (TLR)2 mAb inhibited NK cell responses to BCG, suggesting that NK cells may express a functional TLR2, which plays a role in their mechanism of direct BCG recognition. Taken together, these data suggest that BCG, by inducing simultaneous activation of NK and antigen-presenting cells via their ‘shared’ TLR2, can promote efficient bidirectional NK–dendritic cell interactions necessary for subsequent priming of T₈₁ responses.

Introduction

There is increasing evidence that components of innate or natural immunity, in particular NK cells, are important players in the early, non-specific defenses against a number of microorganisms, including mycobacteria (1–6). NK cells contribute to the innate resistance to infections through various mechanisms, including secretion of pro-inflammatory and immunoregulatory cytokines and chemokines lysis of host cells infected with intracellular pathogens and, in particular cases, direct killing of the microorganism (7). Recent data highlighted the role of the interaction between NK cells and other cells of the innate immunity that occur during the early phases of acute inflammatory responses secondary to infection (8). In particular, various studies have been focused on the ‘cross-talk’ between NK cells and monocyte-derived dendritic cells (DCs) or macrophages upon their recruitment into tissues invaded by pathogens. DCs are the major antigen-presenting cells (APCs) (9) involved in the induction of cellular responses to intracellular pathogens, such as mycobacteria. Several studies have also shown that they play a central role in the modulation of adaptive immune responses. Along this line, the interaction of DC with microbial antigens represents a major step in the induction of T₈₁-oriented protective responses (10–13). Upon contact with mycobacteria, DCs acquire an enhanced capability of releasing pro-inflammatory cytokines and chemokines and become potent inducers of IFN-gamma-producing cells in vivo (14). Recently, interest has been focused on how cytokines released from NK cells may contribute to host resistance and influence the subsequent development of antigen-specific T cell responses (15, 16). Immature dendritic cells (DCs), upon exposure to either extracellular or intracellular bacteria, undergo maturation and release high amounts of tumor necrosis factor (TNF)-alpha and IL-12 (5, 17, 18) that also promote IFN-gamma and TNF-alpha production by...
NK cells (15, 16, 19). In turn, this early NK cell-mediated cytokine production would promote optimal DC maturation resulting in priming of T<sub>H</sub>1 adaptive responses. These polarized responses have been shown to correlate with resistance to disease in various experimental models (20–22). The interaction between NK and DCs is also characterized by the ability of activated NK cells to kill iDCs while sparing mature dendritic cells (mDCs) (23, 24). Thanks to this mechanism, referred to as NK cell-mediated DC editing, NK cells can select, among DCs undergoing maturation, those displaying optimal surface expression of HLA (and of co-stimulatory molecules) (8, 25, 26). Several lines of evidence indicate that NK cells may play a role in immunity to mycobacterium. For example, it has been shown that the NK cell activity increases in peripheral blood and in the pleural effusions of patients with pulmonary tuberculosis, thus, suggesting that a state of NK cell activation exists during active disease (27–29). Moreover, purified NK cells or lymphokine-activated killer cells are capable of lysing human monocytes infected with live Mycobacterium bovis bacille Calmette–Guérin (BCG), with Mycobacterium avium complex or with Mycobacterium tuberculosis (30–32). Interestingly, it has been shown that NK cells can even promote killing of intracellular mycobacteria (33–35). More recent studies (36, 37) revealed that NKp46- and NKG2D-activating receptors mediate lysis of M. tuberculosis-infected mononuclear phagocytes by NK cells. An important role for toll-like receptor (TLR)2 expressed on macrophages was revealed by the observation that this receptor contributed to the up-regulation of ULBP1, a major ligand of NKG2D (37). In a murine model, the in vivo depletion of NK cell function by specific antibodies resulted in enhanced division of M. avium complex (38).

The present study was undertaken to investigate the effect of BCG on highly purified human NK cells. Previous studies suggested that BCG could induce NK cell activation indirectly, by triggering APC and inducing cytokine release (5, 14, 39, 40). Here we show that human NK cells can directly sense and respond to BCG in the absence of APC. This direct recognition results in NK cell activation, cytokine release and induction of cytolytic activity against tumor cells and iDCs. As suggested by the inhibitory effect of specific mAbs, TLR2 would play a key role in NK-mediated BCG recognition.

Methods

Antibodies and blocking reagents

The following mAbs, produced in our laboratory, were used in this study: JT3A (IgG2a, anti-CD3), BAB281 and KL247 (IgG1 and IgM, respectively, anti-NKp46), Z231 (IgG1, anti-NKp44), Z25 and F252 (IgG1 and IgM, respectively, anti-NKp30), ON72 (IgG1, anti-NKG2D), c127 (IgG1, anti-CD16), c218 and FS280 (IgG1 and IgG2a, respectively, anti-CD56), c227 and FS3 (IgG1 and IgG3, respectively, anti-CD69), MAR93 (IgG1, anti-CD25), PP35 (IgG1, anti-CD244), MA127 (IgG1, anti-NTBA), MAR206 (IgG1, anti-CD2), KRA236 (IgG1, anti-CD262), c284 (IgG1, anti-CD11a), Z270 and Z199 (IgG1 and IgG2b, respectively, anti-CD159a), GL183 (IgG1, anti-KIR2DL2/3), EB6 (IgG1, anti-KIR2DL1) and Z27 (IgG1, KIR3DL1).

Anti-CD83 (IgG2b) PE-conjugated, anti-CD86 (IgG2b), anti-IL-T3 (IgG1) PC5-conjugated, anti-CD14 (IgG2a) PE-conjugated mAbs and a mixture of PC5-conjugated anti-CD56 mAb and FITC-conjugated anti-CD3 mAb were purchased from Beckman Coulter Immunotech (Marseille, France).

Anti-CD107a (IgG1) PE-conjugated mAb and anti-human IFN-gamma mAb were purchased from BD Biosciences PharMingen (San Diego, CA, USA). Perforin and granzyme B expression analysis in NK cells was performed by purified anti-perforin mAb (Anell Corporation, Bayport, MN, USA) and purified anti-granzyme B mAb (Alexis Biochemicals, San Diego, CA, USA), respectively, after cells were fixed and permeabilized (BD Cytofix/Cytoperm Kit, San Diego, CA, USA).

The neutralizing anti-IL-12, anti-IL-15 and anti-IL-2 mAbs (goat polyclonal anti-human) were purchased from Pepro Tech (London, UK) and the antagonistic anti-TLR2 mAb (clone T2.5) from ebioscience (San Diego, CA, USA). The neutralizing anti-hIL-18 mAb (mouse IgG1 mAb) was purchased from MBL (Naka-ku Nagoya, Japan).

NK cell purification and culture conditions

Heparinized venous blood was obtained from healthy volunteers. These subjects had no history of previous BCG vaccination and exhibited a negative skin test reaction to purified protein derivative of M. tuberculosis.

Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the declaration of Helsinki.

NK cells were purified by NK Cell Separation Cocktails (Rosette Sep, Stem Cell Technologies Inc., Vancouver, BC) and/or by NK Cell Isolation Kit human (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of NK cells was >96% as assessed by flow cytometric analysis of cells stained with a mixture of anti-CD56-PC5 and anti-CD3-FITC, anti-CD14-PE and anti-ILT3-PC5 mAb. T cells, monocytes and DCs contamination in purified NK cells was <1%.

Freshly purified NK cells were re-suspended in RPMI 1640 medium, supplemented with 2 mM glutamine, 50 μg ml<sup>−1</sup> penicillin, 50 μg ml<sup>−1</sup> streptomycin and 10% heat-inactivated FCS (PA Laboratories GmbH, Linz, Austria) and stimulated with BCG (Pasteur Merieux, Lyon, France) (one NK per one bacterial body) alone or in combination with either neutralizing anti-IL-12 mAb or anti-IL-15 or anti-IL-2 (0.1 μg ml<sup>−1</sup>) and/or 0.5 ng ml<sup>−1</sup> rhl-12 (Pepro Tech). In some experiments, NK cells were also cultured in the presence of both IL-12 and rhl-18 at 0.1 μg ml<sup>−1</sup> (Biosource International Inc., CA, USA). These experiments were also performed either in the presence or in the absence of the antagonistic anti-TLR2 mAb (10 μg ml<sup>−1</sup>).

Cells were plated at 0.5 × 106 cells ml<sup>−1</sup> in flat-bottom 24-well tissue culture plates (Costar, Corning Incorporated, NY, USA). After 20- and/or 40-h culture, NK cells were directly assessed for cytolytic activity, cytokine production and surface phenotype.

Cytokine production

IFN-gamma and TNF-alpha production by BCG-stimulated NK cells were measured in culture supernatants using ELISA (Biosource International Inc.).
For IFN-gamma and TNF-alpha secretion detection by cytfluorimetric analysis, NK cells, after exposure to BCG, were washed and labeled for 10 min at a concentration of $3 \times 10^6$ cells ml$^{-1}$ in ice-cold medium with 50 mg ml$^{-1}$ of anti-IFN-gamma or TNF-alpha/CD45 antibody-antibody conjugates (Miltenyi Biotec GmbH). Cells were suspended in 37°C warm medium to a final concentration of $5 \times 10^6$ cells ml$^{-1}$ and were allowed to secrete IFN-gamma or TNF-alpha for 40 min at 37°C. After capturing secreted cytokines at their surface, cells were centrifuged at $300 \times g$ for 5 min at 4°C and re-suspended at a concentration of 106 cells ml$^{-1}$ in ice-cold PBS containing 0.5% BSA and 5 mM EDTA (both from Sigma-Aldrich Corporation, MI, USA). The cells were then stained with 5 mg ml$^{-1}$ PE-conjugated anti-IFN-gamma or anti-TNF-alpha for 10 min at 4°C and analyzed by flow cytometry (FACS-Calibur; Becton-Dickinson). Viable IFN-gamma-secretting NK cells were also detected by using an intracellular staining after cells were fixed and permeabilized.

Cytolytic activity

NK cells that had been exposed to BCG alone or in combination with neutralizing anti-IL-12 mAb or with IL-12 cytokine were tested for cytolytic activity against various NK-susceptible tumor target cells including FO1, M14 and LCL 721.221 in a 4-h $^{51}$Cr-release assay as previously described (25) and in a de-granulation assay quantifying cell-surface CD107a expression. In this experiment, $2 \times 10^5$ NK cells, that had been cultured 40 h in the presence of BCG, were added to various targets, including FO1 and M14 cell lines, in round-bottom 96-well tissue culture microplates (200 microliter medium) at an effector-to-target (E/T) cell ratio of 1:1. Cells were mixed and spun down for 3 min at 300 r.p.m. and incubated for 3 h at 37°C in 5% CO$_2$. PE-anti-human CD107a mAb (LAMP-1) was included during the incubation. Thereafter, cells were spun down and stained with PC5-conjugated anti-CD56 mAb for 30 min on ice. The cells were washed and analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

In other experiments, their cytolytic activity was evaluated against allogeneic iDCs derived as previously described (19, 25). The concentration of anti-NKp30 and anti-NKp46 mAbs used for masking experiments was 10 mg ml$^{-1}$. The E/T ratios are indicated in the figure legend.

Generation of DCs

DCs were generated as previously described (19). Briefly, PBMC were derived from healthy donors, and plastic-adherent cells were cultured in RPMI-1640 containing 10% FCS, in the presence of IL-4 and granulocyte macrophage colony-stimulating factor (Pepro Tech) at final concentrations of 20 and 50 ng ml$^{-1}$, respectively. After 6 days of culture, cells were characterized by the CD14$^-$CD1a$^+$CD83$^-$ phenotype corresponding to iDCs (25).

TLR2 reverse transcription–PCR analysis

Total RNA was extracted using RNeasy mini Kit (Qiagen) from human purified NK cells, cultured for 40 h in the absence or in the presence of IL-12, BCG or IL-12 and BCG and the cDNA synthesis was performed on 1 mg of RNA using hexameric primers. In this study, to selectively amplify TLR2 transcript, we used the following primers: TLR2 up 5' ACT GGT AGT TGT GGG TTG AA/TLR2 down 5' AAG CAT CAA TCT CAA GTT CC. Amplification was performed for 35 cycles: 30' at 95°C, 30' at 60°C and 30' at 72°C. Notably, this set of primers does not amplify DNA, and therefore, also if the PCR protocol consisted in 35 cycles, no false positive can be detected. The amplification product (581 bp) was resolved in a 1.2% agarose gel, sub-cloned into pcDNA3.1/V5/His TOPO$^\text{TM}$ vector using the Eukaryotic TOPO TA Cloning Kit (Invitrogen Life Technologies, CA, USA) and sequenced to verify the specificity of the performed PCR reaction. DNA sequencing was performed using d-Rhodamine Terminator Cycle Sequencing Kit and a 3100 ABI automatic sequencer (PerkinElmer/Applied Biosystems, USA). The set of primers: b act-up 5' ACT CCA TCA TGA AGT GTG ACG/b act-down 5' CAT ACT CCT GGT TGC TGA TCC, that allowed to amplify a 250-bp fragment of human beta-actin transcript, has been used to verify the cDNA synthesis.

Results

Analysis of the NK cell-surface phenotype after exposure to BCG

Highly purified peripheral blood NK cells were cultured for 40 h (a time interval compatible with in vivo innate immune responses) in the presence of BCG and/or cytokines. In particular, owing to the central role of IL-12 (41, 42), released by monocytes/macrophages and/or DCs (43), in the initiation and maintenance of immune responses to different pathogens, NK cells were cultured with BCG either in the presence or in the absence of exogenous hIL-12. At the end of the culture period, NK cells were harvested and analyzed for the expression of various informative markers including CD56, the natural cytotoxicity receptors (NCRs) NKp46 and NKp30 (44), killer Ig-like receptors (KIRs) and CD94/CD158a (NKG2A) (45–49). The levels of expression of the activating or of the HLA-class I-specific inhibitory receptors remained substantially unchanged after culture of NK cells under the different conditions analyzed. However, a significant up-regulation of CD56 surface expression was detected not only in NK cells cultured with BCG alone but also in those cultured with IL-12 alone (data not shown). On the other hand, no similar effects could be detected in the presence of other exogenous cytokines including IL-4 and IL-18. For this reason, in all subsequent experiments, neutralizing anti-IL-12 antibodies were added to NK cells cultured with BCG in order to exclude the possibility that even low levels of IL-12 released by residual (undetectable) APCs still present in the purified NK cell population could promote or up-regulate NK cell responses to BCG.

NK cells that had been cultured under different conditions were first analyzed for the expression of activation markers, including CD69, CD25 and NKp44 (50). The highest levels of expression of CD69 molecules were detected in NK cells cultured in the presence of both BCG and exogenous IL-12. However, a significant fraction of NK cells did express CD69 also after culture in the presence of BCG alone. Remarkably, the presence of neutralizing anti-IL-12 antibodies did not inhibit CD69 expression in these cultures (Fig. 1, panel A). On
the other hand, anti-IL-12 antibody did abrogate CD69 expression in NK cells cultured with IL-12 alone (data not shown). Significant levels of expression of CD25 were detected both in NK cells cultured with BCG + anti-IL-12 antibody and in those cultured with BCG and exogenous IL-12 (Fig. 1, panel B). Finally, expression of NKp44 was essentially confined to the CD56$^{bright}$ NK subset without major differences among the various culture conditions, although also this molecule was slightly more expressed in NK cells that had been cultured with both BCG and IL-12 (data not shown). Regarding the time required for induction of NK cell responses to BCG, no substantial differences as compared to 40 h were observed when NK cells were stimulated for 20 h (data not shown).

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**Fig. 1.** Expression of surface CD69 and CD25 activation markers on NK cells stimulated with BCG. Freshly isolated NK cells were purified and cultured for 40 h either in medium alone or in the presence of the indicated conditions and then analyzed by two-color immunofluorescence for the expression of CD69 (panel A) or CD25 (panel B) in combination with CD56. This experiment is representative of 10 independent experiments.
We further analyzed whether the ability of NK cells to respond to BCG was confined to cells expressing either KIRs or CD94-NKG2A. As shown in Fig. 2 (panels A and B), the acquisition of CD69 by BCG-stimulated NK cells did not appear to correlate with the expression of neither KIRs nor NKG2A. Thus, while only a fraction of NK cells appear to be directly activated by BCG, no relationship exists with the expression of a particular HLA-class I-specific receptor.

Cytokine release by NK cells exposed to BCG

We next analyzed whether purified NK cells, upon exposure to BCG, could also release cytokines. In these experiments, supernatants from NK cells that had been cultured under different conditions were tested (by ELISA) for the release of IFN-gamma and TNF-alpha. As shown in Fig. 3 (panels A and B), NK cells cultured in the presence of BCG and exogenous IL-12 released higher levels of IFN-gamma than NK cells cultured in the presence of BCG alone.

**Fig. 2.** KIR and CD94/NKG2A expression by NK cells stimulated with BCG. Freshly isolated NK cells were purified, cultured for 40 h as indicated and analyzed by two-color immunofluorescence for the expression of CD69 in combination with a mixture of anti-KIRs (panel A) or anti-CD94/NKG2A (panel B). The experiment shown is representative of four independent experiments using different donors.
cells stimulated with BCG alone or BCG + anti-IL-12. However, in some donors, high levels of IFN-gamma were also detected in NK cells cultured with BCG alone or BCG + anti-IL-12 (Fig. 3, panel A, left, and panel B). This was not true for other donors in which only the use of both BCG and IL-12 resulted in high levels of IFN-gamma production (Fig. 3, panel A, right, and panel B). This data further support the notion that IL-12 strongly enhances the IFN-gamma production by NK cells (42). However, it also provides evidence that IFN-gamma production by NK cells stimulated with BCG may not strictly require the presence of IL-12. Indeed, in donors characterized by high responsiveness, the IFN-gamma production in response to BCG alone could not be abrogated by neutralizing anti-IL-12 antibodies (Fig. 3, panel A, left). On the other hand, in donors displaying low responsiveness to BCG alone, the same antibodies abrogated the IFN-gamma production induced by the use of both BCG and exogenous IL-12 (Fig. 3, panel A, right). The release of IFN-gamma in the above culture conditions was evaluated after 20 and 40 h in most of the donors analyzed. Generally, after 40 h, there was a slight increase of cytokine production as compared to 20 h (Fig. 3, panel B, right). The production of TNF-alpha by NK cells stimulated with BCG displayed only minor variations in cells cultured in the presence or in the absence of exogenous IL-12. This finding supports the notion that production of TNF-alpha (51) by BCG-activated NK cells may be substantially independent or only partially dependent on the presence of IL-12 (Fig. 3, panel C). Remarkably, NK cells from donors characterized by high levels of IFN-gamma release in response to BCG alone also produced high amounts of TNF-alpha. In contrast, NK cells from donors displaying low IFN-gamma production in response to BCG alone were producing lower, although significant, levels of TNF-alpha.

In addition, by using a cytokine capture cytofluorimetric assay, we could directly assess that only a fraction of NK cells did release IFN-gamma after culture with BCG alone. Also in this experimental setting, no substantial decrements of IFN-gamma release could be detected when NK cells were cultured with BCG in the presence of anti-IL-12 mAb (Fig. 3, panel D). Finally, in agreement with the ELISA data, maximal IFN-gamma release was detected in NK cells cultured with BCG + IL-12; notably, virtually all NK cells cultured under these conditions produced IFN-gamma (data not shown).

Release of TNF-alpha was also confined to an NK cell subset cultured with BCG alone. Again, there were no

Fig. 3. Cytokine release by BCG-stimulated NK cells. Freshly isolated NK cells were purified and cultured in the absence or in the presence of the indicated stimuli. Next, supernatants were harvested and assessed for cytokine content by specific ELISA (n = 3, mean ± SD) (panels A, B and C). Panel (A) shows the results of IFN-gamma production after 40 h culture by two representative donors characterized by high (left) and low (right) responsiveness, respectively. In panel (B) (left), a dot plot representation of the IFN-gamma release by 15 different donors displaying either high or low responsiveness to BCG is shown. Above, the various donors were assessed in the absence of IL-12, while below the same donors were assessed in the presence of this cytokine. In the right panel, the IFN-gamma release by BCG-stimulated NK cells derived from two representative donors was evaluated after 20- and 40-h culture, in the presence (circles) or in the absence (triangles) of IL-12. One donor (black symbols) was a high responder while the other was a low responder (white symbols). In panel (C), the two donors depicted in panel (A) were assessed for TNF-alpha production. In panels (D and E), the secreted IFN-gamma and TNF-alpha, respectively, were captured and stained on the surface of the cells. The cells were counterstained with FITC-labeled FS280 (IgG2a, anti-CD56 mAb). This experiment is representative of five independent experiments performed on different healthy donors.
substantial differences in TNF-alpha production in NK cells cultured with BCG alone or BCG + anti-IL-12 (Fig. 3, panel E). These experiments directly assessing the cytokine release at the single-cell level would rule out the possibility that TNF-alpha detected by ELISA in supernatants of BCG-stimulated NK cells could be produced by contaminating cells. Some of these cytokine detection experiments were also paralleled by the analysis of intracellular staining, which substantially confirmed the results obtained with the cytokine capture analysis (data not shown).

The advantage of the cytokine capture assay is that it also provides direct evidence that upon stimulation with BCG, a subset of both CD56^dull and CD56^bright NK cells (52) release cytokines (Fig. 3, panels D and E). In agreement with these results and with the concept that KIR+ cells are essentially confined to the CD16^+ CD56^dull NK cell subset, no substantial differences were detected between KIR+ (or between NKG2A+ and NKG2A−) cell sub-populations in their ability to release cytokines in response to BCG stimulation (data not shown).

**Anti-tumor cytolytic activity of NK cells exposed to BCG**

We further evaluated whether NK cells that had been cultured in the presence of BCG also acquired a more potent cytolytic activity. To this end, we assessed their ability to kill a panel of tumor target cell lines in a [51Cr]-release assay. The cytotoxicity against representative target cells including F01 (HLA class I− melanoma), M14 (HLA class I+ melanoma) and LCL 721.221 (HLA class I− EBV cell line) was clearly increased. Notably, recognition of these target cells is mediated via distinct sets of triggering receptors (44). Thus, killing of F01 is primarily due to NK cell activation via NKp30 and NKG2D; M14 killing is primarily due to NKp46, while killing of LCL 721.221 involves NKp46 and several coreceptors including 2B4 (CD244), NTB-A and CD2. Interestingly, most of the donors displaying high responsiveness to BCG stimulation (in terms of cytokine release) were characterized by an NCR^bright phenotype (53). This could suggest a possible role of NCRs in NK cell responses to BCG. In this context, it is known that the ability of NK cells to kill given target cells may directly correlate with the surface density of NCRs (53). In this case, however, the level of surface expression of NCRs was not significantly different in NK cells cultured in the presence or in the absence of BCG. The ability to kill may also reflect higher content in perforin and granzyme B (54). However, we could not detect any significant difference in the expression of these cytolytic proteases in NK cells cultured either in the presence or in the absence of BCG (data not shown). Nevertheless, NK cells stimulated for 40 h with BCG alone (or BCG + anti-IL-12) increased their capability of killing both F01 and M14 melanomas (Fig. 4, panels A and B) as well as LCL 721.221 (data not shown). Cytotoxicity was further increased in NK cells cultured in the presence of BCG + IL-12, whereas IL-12 alone induced increments comparable to those detected in the presence of BCG alone (or BCG + anti-IL-12) (Fig. 4, panel A). In line with previous data on IL-2-activated NK cell populations and clones (44), the cytolytic activity of BCG-stimulated NK cells against tumor cell lines could be inhibited by antibodies directed against one or another triggering receptor (see e.g. the inhibition of M14 killing by anti-NKp46 antibodies, Fig. 4, panel B).

The up-regulation of BCG-induced NK cytolytic activity was further assessed in experiments of CD107a expression (54). A representative experiment is shown in Fig. 4 (panel C); it can be seen that the basal NK cell degranulation observed in response to F01 target cells (13%) was increased in NK cells stimulated with BCG alone (28%) or BCG + anti-IL-12 (30%), whereas the NK cell degranulation in the presence of M14 could be observed only after NK cell triggering with one or another of the above stimuli. Finally, as expected, double staining for CD107 and CD69 indicated...
that CD107+ cells were contained within the ‘activated’ CD69+ cell subset (data not shown).

NK cells exposed to BCG acquire the ability to kill myeloid iDCs

NK cells are thought to participate in cell-to-cell interactions leading to the generation of mDCs capable of inducing adaptive Tp1 immune responses (referred to as NK-mediated editing of DCs) (8). The NK-mediated editing of DCs undergoing maturation appears to be based on the ability of NK cells to selectively kill iDCs while sparing mature DCs (25, 55–57). In order to become capable of killing autologous iDCs, NK cells require activation. However, NK cells recruited from blood into inflamed peripheral tissues may not necessarily be activated and would thus require appropriate activating signals in order to kill iDCs. Along this line, it has been shown that an alternative mode of NK cell activation, independent on the recognition of NK-susceptible target cells (such as tumors or virus-infected cells), can be provided by the engagement of TLRs expressed by NK cells (58). Thus, direct pathogen recognition may enable NK cells to kill iDCs and to initiate their editing program by which they can select the most suitable DCs that mature after antigen uptake (4, 59). Since BCG can directly activate NK cells, we evaluated whether this could represent a novel pathway to induce NK cell-mediated cytotoxicity against iDCs. To this aim, NK cells cultured in the presence of BCG for 40 h under the various conditions described above were assessed for their cytotoxicity against iDC. The cytolytic test was performed either in the absence or in the presence of mAbs against triggering NK receptors in a [51Cr]-release assay.

As shown in Fig. 5 (panel A), BCG alone induced the NK cell cytotoxicity against iDCs and this activity was further increased when NK cells were cultured in the presence of both BCG and exogenous IL-12. In agreement with previous data on IL-2-activated NK cells (55, 60), killing of iDC by BCG-activated NK cells could be strongly inhibited by anti-NKp30 mAb and, in part, by anti-DNAM-1 mAb (Fig. 5, panel A), while neither anti-NKp46 nor anti-NKG2D mAb had any effect (data not shown).

Further analysis of CD107a expression provided data that are in line with the [51Cr]-release experiments. Indeed, as shown in Fig. 5 (panel B), the surface expression of this secretory lysosome marker could be detected only after exposure of NK cells to BCG or BCG + IL-12.

Involvement of TLR2 in the process of BCG recognition by NK cells

Controversial data were obtained in previous studies on the expression of mRNA for TLR2 by human NK cells (61–64). Since TLR2 plays a crucial role in BCG recognition by other innate immunity cells including APCs (17, 21, 65–68), we investigated whether it is expressed and functional also in NK cells.

First, the expression of TLR2 on freshly isolated or cultured NK cells was evaluated by cytofluorimetric analysis. The purity of the freshly isolated NK cell population was indicated by the expression of CD56 and NKp46 in the absence of detectable CD3 or CD14 staining (Fig. 6, panel A). Only low levels of TLR2 expression were usually detected in freshly isolated as well as in cultured NK cells. Only marginal increments were occasionally observed upon culture in the

![Fig. 5. Cytolytic activity of BCG-stimulated NK cells against iDC. NK cells cultured in the presence or in the absence of the indicated stimuli were assessed for cytolytic activity in a [51Cr]-release assay (panel A) or for CD107a expression (panel B) against iDC at an E/T ratio of 5:1 and 1:1, respectively. In panel (A), the cytolytic activity of NK cells was assessed either in the absence or in the presence of anti-NKp30 or anti-DNAM-1 antibodies as indicated. In panel (B), the values reported in the upper right panels indicate the percent of CD107a+ CD56+ NK cells. The SD did not exceed 4% in the [51Cr]-release assays (n = 3, mean ± SD). Data are representative of four and three independent experiments for [51Cr]-release and CD107a expression, respectively.](https://academic.oup.com/intimm/article-abstract/20/9/1155/666858)
presence of BCG and this increase was slightly more pronounced in the presence of IL-12 or BCG + IL-12 (Fig. 6, panel B).

The expression of TLR2 in human NK cells was also analyzed by reverse transcription–PCR. In these experiments, total RNA extracted from NK cells cultured in the absence of stimulus or in the presence of IL-12, BCG or BCG + IL-12 were retrotranscribed and amplified using a set of primers specific for detection of TLR2 transcript. As shown in Fig. 6 (panels C and D), similar to freshly isolated NK cells (63, 64), cultured polyclonal NK cell populations, as well as NK clones, expressed TLR2 transcript. The results obtained using NK cell clones suggest that an heterogeneity in terms of TLR2 mRNA transcript may exist among NK cells clones derived from the same individual (Fig. 6, panel D) as well as among NK cell clones derived from different donors (data not shown). Notably, IL-12 increases TLR2 transcription. Therefore, this result may, at least in part, explain the additive effect observed in tests of cytokine production and cytotoxicity using both IL-12 + BCG stimuli.

Fig. 6. Expression of TLR2 in cultured NK cell populations and NK cell clones. Purified NK cells characterized by the CD14−, CD3−, CD56+ and NKp46+ phenotype (panel A) were cultured under different conditions, as indicated, and subsequently assessed for surface expression of TLR2 by cytofluorimetric analysis (panel B) and for TLR2 transcript expression (panels C and D). Reverse transcription–PCR analysis of TLR2 (upper panels C and D) and beta-actin (lower panels C and D) transcripts were performed on total RNA isolated from cultured human NK cells. (Panel C) NK bulk population cultured in the absence (lane 1) or in the presence of IL-12 (lane 2), BCG (lane 3) or BCG + IL-12 (lane 4). The neuroblastoma SHSY5Y (lane 5) has been used as positive control. (Panel D) Lanes 1 and 2 negative controls, no template control and DNA, respectively, lanes 3–10, NK cell clones derived from the same individual. Molecular markers are indicated on the left.
In order to directly verify the involvement of TLR2 in BCG-induced NK cell activation, purified NK cells were exposed to BCG (using the various culture conditions described above) either in the presence or in the absence of neutralizing anti-TLR2 mAb. Cells were subsequently analyzed for acquisition of activation markers and for the up-regulation of functional activities. In most instances, the expression of CD69 by BCG-stimulated NK cells was substantially inhibited by anti-TLR2 mAb (Fig. 7, panel A).

More importantly, these experiments indicated that anti-TLR2 mAb could inhibit the induction of NK cell functions induced by BCG. Thus, anti-TLR2 mAb inhibited at least in part the cytolytic activity against M14 tumor target cells (Fig. 7, panel B) and the release of IFN-gamma by NK cells cultured in the presence of BCG alone (Fig. 7, panel C) or BCG + IL-12 (Fig. 7, panel D). The level of this inhibition was variable among different donors (Fig. 7, panel E), suggesting the possibility that additional receptors might exist.

![Fig. 7. Anti-TLR2 mAb inhibits BCG-induced NK cell responses.](https://academic.oup.com/intimm/article-abstract/20/9/1155/666858)
that cooperate with TLR2 in BCG recognition. In these experiments, the specificity of the inhibition mediated by anti-TLR2 was indicated by its ability to inhibit NK cell responses to BCG and BCG + IL-12, while leaving unaffected the IFN-gamma production induced by IL-12 + IL-18 (Fig. 7, panel F). Finally, antibodies directed to other TLRs expressed by NK cells did not modify NK cell responses to BCG (data not shown).

Discussion
In this study, we show that human NK cells can directly recognize BCG via TLR2. As a result, they acquire functional properties that allow their entry into the complex series of events involved in the interplay between innate and adaptive immune responses. In particular, recognition of BCG by NK cells promotes an effective NK–DC cross-talk capable of modulating the maturation/editing of DC before they migrate into secondary lymphoid compartments. Previous studies indicated that NK cells strictly require receiving activation stimuli in order to initiate their cross-talk with DCs. For example, these stimuli may be provided by tumor cells expressing surface ligands recognized by the activating NK receptors (69). Here we show that NK cells may also be activated directly by BCG and that this results in the expression of typical activation markers such as CD69 and CD25 and in the acquisition/induction of important effector functions. Our results are in agreement with previous data reporting that extracellular mycobacteria may induce CD69 expression on NK cells and promote IFN-gamma production and proliferation (2, 70, 71).

In the present study, we provide clear experimental evidence that BCG can directly trigger a fraction of highly purified NK cells independent of the surface expression of different HLA class I-specific receptors. BCG-stimulated NK cells acquire functional features that render them suitable to initiate a bidirectional interaction with DCs (23, 24). They include the ability to release cytokines that can promote maturation of bystander DCs and potentiation of cytolytic activity against both tumor cells and iDCs. In particular, the acquisition of the capability of killing iDCs may represent an important property of BCG-activated NK cells. Notably, BCG alone is sufficient to induce this function. On the other hand, we also show that this BCG-mediated effect is strongly potentiated by exogenous IL-12. The source of this cytokine is likely to be represented by APCs, including recruited DCs that have been exposed to BCG. As shown in previous studies, IL-12 alone can induce a substantial increase of cytotoxicity in NK cells cultured in the absence of additional stimuli. Although this effect was documented for the ability of NK cells to mediate tumor cell lysis, it applies also to iDCs killing (19). Here we show that although BCG alone can induce NK cells to kill iDCs, the addition of IL-12 results in a synergistic effect that promotes optimal iDC killing. This is reminiscent of other bidirectional interactions between NK and DCs such as that promoted by the simultaneous engagement of TLR3 on both cell types. In this context, freshly isolated peripheral blood NK cells, in the presence of polyinosinic-polycytidylic acid and DC-derived IL-12, were shown to acquire potent cytolytic activity against iDCs (58). Remarkably, NK responses to BCG could be observed also in the presence of blocking antibody against IL-12, indicating that NK cells can directly respond even in the absence of this cytokine. Although additional cytokines, including IL-18, IL-2 and IL-15, may be involved in innate NK responses to BCG, blocking antibodies against these cytokines did not affect BCG-induced NK responses. A possible candidate acting as a BCG receptor on NK cells was represented by TLR2 since M. tuberculosis bacilli contain distinct ligands that can activate APCs either via TLR2 or TLR4 (17, 66–68). Along this line, it has been proposed that TLR2 expressed by APC could contribute significantly to the initiation and maintenance of innate and adaptive responses to mycobacteria infection.

We show that mRNA for TLR2 is expressed not only in freshly isolated NK cells but also in NK cells cultured in the presence of BCG or IL-12; under these culture conditions, its expression was up-regulated (Fig. 6, panel B). The surface density of this receptor, in both resting and cultured NK cells, is very low (Fig. 6, panel B) and in some donors even under the limit of detectability by cytofluorimetric analysis. Importantly, however, anti-TLR2 mAb inhibited, at least in part, NK cell responses to BCG strongly suggesting a role for TLR2 in BCG-induced NK cell activation. In this context, it is worth noting that antibody-mediated blocking of TLR2 not only down-regulated IFN-gamma production by NK cells stimulated by BCG alone but also it also sharply inhibited its production by NK cells co-stimulated by BCG and exogenous IL-12. Remarkably, anti-TLR2 mAb also inhibited the BCG + IL-12-mediated induction of NK-mediated cytotoxicity against both tumor and DC target cells as well as the expression of activation markers including CD69. Although, anti-TLR2 mAb down-regulated NK cell responses to BCG, in most individuals, a certain variability in the level of inhibition existed among different donors (Fig. 7, panel E). This may suggest the existence of additional, still undefined, receptors involved in BCG recognition by NK cells. In this context, it is worth noting that NK cells expressing an NCR$^\text{high}$ phenotype are usually more responsive to BCG stimulation. On the other hand, these receptors are unlikely to be directly involved in BCG recognition since mAb-mediated masking of one or more NCR did not prevent BCG-induced NK cell activation (data not shown).

In conclusion, our data demonstrate the existence of a direct recognition of BCG by NK cells and suggest that TLR2 plays a relevant role in the process of BCG-induced up-regulation of NK cell functions. In the presence of IL-12 released by BCG-activated APCs, these functions are potentiated as witnessed by the further increases of IFN-gamma secretion and cytolytic activity.

These results may have important implications in our understanding of the early phases of innate immune responses to mycobacterium and of the mechanisms initiating the cross-talk between NK cells and DC that eventually leads to the generation of protective T$_\text{h}1$ immunity. Moreover, since the immunotherapy with BCG represents one of the most effective treatments for preventing superficial bladder cancer recurrences after transurethral tumor resection, our present data may also serve to elucidate the NK activating mechanisms that are operative during BCG immunotherapy for bladder cancer (72–74).
NK cells recognize BCG via TLR2

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Abbreviations

- APC: antigen-presenting cells
- BCG: bacille Calmette–Guerin
- DC: dendritic cell
- miDC: mature dendritic cell
- iDC: immature dendritic cell
- NCR: natural cytotoxicity receptor
- KIR: killer Ig-like receptor
- TLR: toll-like receptor
- TNF: tumor necrosis factor
- E/T: effector-to-target

References


