Engagement of TLR3, TLR7, and NKG2D Regulate IFN-γ Secretion but Not NKG2D-Mediated Cytotoxicity by Human NK Cells Stimulated with Suboptimal Doses of IL-12[1]

María V. Girart, Mercedes B. Fuertes, Carolina I. Domaica, Lucas E. Rossi, and Norberto W. Zwirner[2]

NK cells express different TLRs, such as TLR3, TLR7, and TLR9, but little is known about their role in NK cell stimulation. In this study, we used specific agonists (poly(I:C), loxoribine, and synthetic oligonucleotides containing unmethylated CpG sequences to stimulate human NK cells without or with suboptimal doses of IL-12, IL-15, or IFN-α, and investigated the secretion of IFN-γ, cytotoxicity, and expression of the activating receptor NKG2D. Poly(I:C) and loxoribine, in conjunction with IL-12, but not IL-15, triggered secretion of IFN-γ. Inhibition of IFN-γ secretion by chloroquine suggested that internalization of the TLR agonists was necessary. Also, secretion of IFN-γ was dependent on MEK1/ERK, p38 MAPK, $p70^{S6}$ kinase, and NF-κB, but not on calcineurin. IFN-α induced a similar effect, but promoted lesser IFN-γ secretion. However, cytotoxicity (^{51}Cr release assays) against MHC class I-chain related A (MICA)$^-$ and MICA$^+$ tumor targets remained unchanged, as well as the expression of the NKG2D receptor. Excitingly, IFN-γ secretion was significantly increased when NK cells were stimulated with poly(I:C) or loxoribine and IL-12, and NKG2D engagement was induced by coculture with MICA$^+$ tumor cells in a PI3K-dependent manner. We conclude that resting NK cells secrete high levels of IFN-γ in response to agonists of TLR3 or TLR7 and IL-12, and this effect can be further enhanced by costimulation through NKG2D. Hence, integration of the signaling cascades that involve TLR3, TLR7, IL-12, and NKG2D emerges as a critical step to promote IFN-γ-dependent NK cell-mediated effector functions, which could be a strategy to promote Th1-biased immune responses in pathological situations such as cancer. *The Journal of Immunology,* **2007, 179: 3472–3479.**

Natural killer cells are the third largest lymphoid population, and they provide protection against viral infections and tumors. These functions are exerted by their ability to mediate cytotoxicity against susceptible target cells and by secretion of cytokines such as IFN-γ. These effector functions are regulated by receptors such as NKG2D, a molecule expressed by NK cells, $\gamma\delta$ T lymphocytes, and TCR$\alpha\beta$ CD8$^+$ T lymphocytes (1–5).

Different NKG2D ligands (NKG2DLs)[3] have been described (1–6). One of them is MHC class I-chain related A (MICA), a stress-regulated polymorphic, non-Ag-presenting molecule not associated with β_2-microglobulin (2, 7) that is almost not expressed by normal cells, but is up-regulated by neotransformation (8–10), cell activation (11, 12), or during the DNA damage response (13). Such expression has been shown to provide protection against tumor initiation and progression (14–18), highlighting the importance of stimulating NK cells to elicit an appropriate antitumor response.

However, besides expressing different NKG2DLs, most tumors grow in healthy individuals due to complex tumor immune escape mechanisms that promote immunosuppression (2, 18–20). Therefore, novel strategies to trigger NK cell effector functions may result in the promotion of a better immunosurveillance.

TLRs are germline-encoded molecules that recognize pathogen-associated molecular patterns (21). Specific ligands for most TLRs have been identified. TLR3, TLR7, TLR8, and TLR9 have been identified on NK cells (22, 23). They recognize dsRNA produced during viral replication (24), ssRNA and imidazoquinolines (25), and unmethylated CpG motifs (26), respectively. However, their role in NK cell immunobiology has been poorly investigated. Most studies were performed using NK cells cocultured with dendritic cells (DCs) stimulated in vitro or after in vivo administration of different pathogen-associated molecular patterns (22, 27, 28). Some TLR agonists have the capacity to trigger NK cell-mediated effector functions through indirect mechanisms that involve maturation of DCs or monocytes (22, 29, 30). In other cases, a direct effect also was demonstrated (22, 30, 31). However, the underlying mechanisms involved in TLR-mediated NK cell stimulation and whether NKG2D has the ability to cooperate in the activation of NK cells in response to these agonists are not known. Information in this field may lead to the development of novel strategies to improve the immunosurveillance and promote a sustained tumoricidal capacity of NK cells. Therefore, the aim of the present work was to investigate the role of agonists of TLRs expressed by NK

Laboratorio de Inmunogenética, Hospital de Clínicas "José de San Martín," and Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires (UBA), Buenos Aires, Argentina

Received for publication December 7, 2006. Accepted for publication July 2, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was funded with grants from National Agency for Promotion of Science and Technology, Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, and Universidad de Buenos Aires (all to N.W.Z.). We also thank Fundación Sales for additional financial support. M.V.G., M.B.F., and C.I.D. are fellows of Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina. L.E.R. holds a fellowship of ANPCYT. N.W.Z. is a member of the Researcher Career of Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina.

[2] Address correspondence and reprint requests to Dr. Norberto W. Zwirner, Laboratorio de Inmunogenética, Hospital de Clínicas "José de San Martín," Av. Córdoba 2351, 3er piso., C1120AAF Buenos Aires, Argentina. E-mail address: nwz@sinectis.com.ar

[3] Abbreviations used in this paper: NKG2DL, NKG2D ligand; CsA, cyclosporine; DC, dendritic cell; IC, isotype-matched negative control; MICA, MHC class I-chain related A; ODN, synthetic oligonucleotide containing unmethylated CpG sequences; Sz, sulfasalazine.

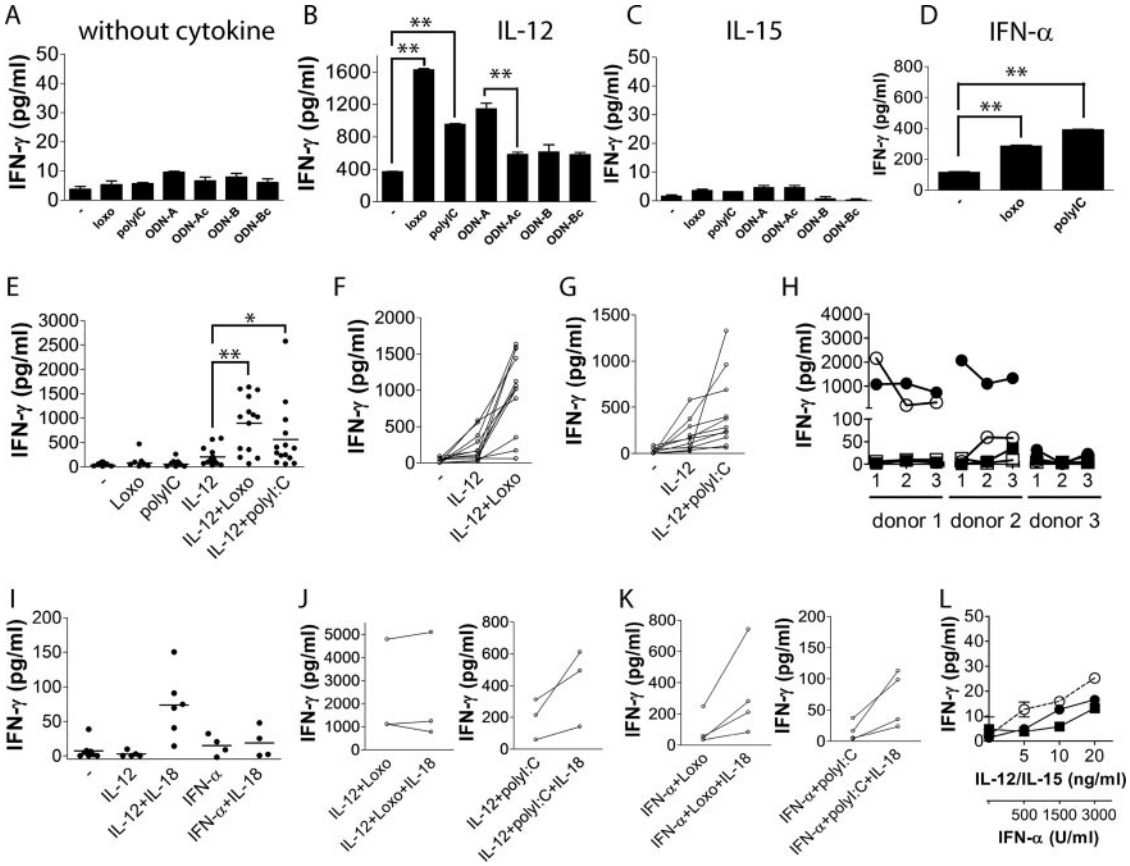

FIGURE 1. IL-12 and IFN-α, in combination with agonists of TLR3 or TLR7, promote a strengthened IFN-γ secretion by isolated human NK cells. IFN-γ production was assessed by ELISA in culture supernatants of NK cells stimulated without (*A*) or with IL-12 (*B*), IL-15 (*C*), or IFN-α (*D*) and agonists of TLR3 (poly(I:C)), TLR7 (loxoribine), TLR9 (ODN-A or ODN-B), or their respective control ODNs (ODN-Ac and ODN-Bc) for 24 h. Results are representative of three independent experiments. Also, IFN-γ secretion by NK cells from 14 different donors stimulated with loxoribine, poly(I:C), IL-12, IL-12 and loxoribine, or IL-12 and poly(I:C) was assessed, as described (*E*). -, Unstimulated NK cells. *A–E*, *, $p < 0.05$; **, $p < 0.01$. When not indicated, differences were NS. Also, individual behavior of NK cells of 12 different donors in response to IL-12, IL-12 and loxoribine (*F*), or IL-12 and poly(I:C) (*G*), and reproducibility of the response (IFN-γ secretion) to IL-12 and loxoribine (●), IL-12, and poly(I:C) (○) or IL-12 alone (□) assessed in NK cells of three different donors (*H*) is shown. ■, NK cells without stimulation. Also, cooperation of IL-18 with IL-12 or IFN-α (*I*), with IL-12 and loxoribine (*J*, *left panel*) or poly(I:C) (*J*, *right panel*), or with IFN-α and loxoribine (*K*, *left panel*) or poly(I:C) (*K*, *right panel*) for IFN-γ secretion by NK cells from different donors was assessed, as well as the effect of increasing doses of IL-12 (●), IL-15 (■), or IFN-α (○) on IFN-γ secretion by NK cells (*L*). In *I–K*, each dot or line represents the result obtained with NK cells from a different donor.

cells as direct stimulators of NK cell-mediated IFN-γ secretion and cytotoxicity, and the existence of cooperative effects between TLRs and NKG2D in human NK cells to enhance their effector functions. We observed that NK cells can be directly stimulated to secrete IFN-γ by a combination of IL-12 and agonists of TLR3 or TLR7, and this effect can be further strengthened by engagement of NKG2D by MICA expressed on tumor cells.

Materials and Methods

Reagents

The following Ab and reagents were used: anti-NKG2D (1D11), PE-labeled anti-TLR3, and anti-TLR9 from eBioscience; anti-IL-12Rβ1 and anti-IL-12Rβ2 from BD Biosciences; anti-IFN-αR (64G12, mouse IgG1; provided by P. Eid, Villejuif, France); isotype-matched negative control (IC) mAb from Southern Biotech; anti-TLR7 from Imgenex; agonist of TLR3 (poly(I:C)) from Amersham Biosciences; agonists of TLR7 (loxoribine) and TLR9 (synthetic oligonucleotides containing unmethylated CpG sequences (ODNs), type A or B) from InvivoGen; human rIL-12, rIL-15, and rIL-18 from PeproTech; human rIFN-α from Biosidus; U0126, SB202190, and rapamycin from Calbiochem; cyclosporine (CsA) provided by Novartis Argentina; and sulfasalazine (Sz), chloroquine, and Ly294002 from Sigma-Aldrich. ODN type A (ODN2216 or ODN-A) and ODN type B (ODN2006 or ODN-B), as well as the negative control ODNs (ODN-Ac and ODN-Bc) were used.

NK cell isolation and stimulation

Human peripheral blood NK cells were isolated from healthy volunteers using the RosetteSep NK cell enrichment mixture (StemCell Technologies) and Ficoll-Paque Plus (Amersham) centrifugation. Studies have been approved by the institutional review committee. NK cells were washed with RPMI 1640 (Sigma-Aldrich) and resuspended in RPMI 1640 supplemented with 10% FCS, sodium pyruvate, glutamine, and penicillin-streptomycin. $CD3^+$ cells represented 0.19 ± 1.70%, and $CD3^-$ cells represented 96.52 ± 2.26% of the isolated cells. In some experiments, monocytes were further depleted by passage through nylon wool columns. In these cases, contaminating $CD14^+$ cells represented 1.72 ± 0.12% of the cells. NK cells (10^6 cells/ml) were used unstimulated or were stimulated with 1 ng/ml IL-12, IL-15, or IL-18; 1000 U/ml IFN-α; and TLRs agonists (50 μg/ml poly(I:C), 1 mM loxoribine, or 10 μg/ml ODN-A, ODN-B, ODN-Ac, or ODN-Bc) for 24 h. Thereafter, expression of NKG2D, TLRs, IL-12Rβ1, IL-12Rβ2, and IFN-αR; IFN-γ secretion; and cytotoxicity against $MICA^+$ and $MICA^-$ melanoma target cells were assessed. In other experiments, NK cells were stimulated with IL-12 alone or with IL-12 and loxoribine or poly(I:C) in the absence or in the presence of chloroquine (20 μg/ml), or inhibitors of MEK1/ERK (U0126), p38MAPK (SB202190), NF-κB (Sz), calcineurin (CsA), or the $p70^{S6}$ kinase (rapamycin). To assess the contribution of the simultaneous engagement of NKG2D, NK cells (5×10^5 cells/ml) were stimulated as described in the presence of a clone of the IIB-MEL-LES human melanoma (32) transfected with MICA (clone 1) or a clone of the same melanoma transfected with empty plasmid (clone C),

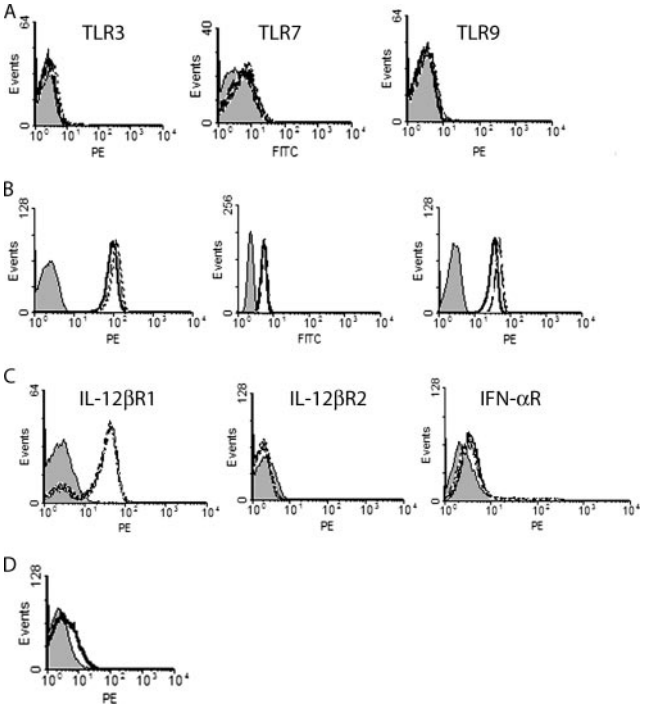

FIGURE 2. Expression of TLRs, IL-12Rβ1, IL-12Rβ2, and IFN-αR is not reciprocally regulated by stimulation with IL-12, IFN-α, or agonists of TLR3 or TLR7. Expression of TLR3, TLR7, and TLR9 in human NK cells (*A*, nonpermeabilized; *B*, permeabilized) assessed by flow cytometry after a culture of 24 h without (thick continuous line) or with IL-12 (dotted line) or IFN-α (dashed line). *C*, Expression of IL-12Rβ1, IL-12Rβ2, and IFN-αR on isolated human NK cells assessed by flow cytometry after a culture of 24 h without (thick continuous line) or with loxorinine (dotted line), poly(I:C) (dashed line), or ODN-A (dashed-dotted line). *D*, Expression of IL-12Rβ2 on activated $CD3^+$ T lymphocytes (thick line). Filled histogram: IC mAb. Results are representative of three independent experiments.

at a ratio of 10:1 (IIB-MEL-LES is a human melanoma cell line that expresses very low levels of NKG2DLs; Fuertes, M. B., M. V. Girart, and N. W. Zwirner, unpublished observations), either in the absence or in the presence of 50 μM PI3K inhibitor Ly294002.

Flow cytometry

Expression of NKG2D and IFN-αR was analyzed by flow cytometry with unlabeled mAbs and PE-labeled anti-mouse IgG (DakoCytomation). Expression of IL-12Rβ1, IL-12Rβ2, TLR3, and TLR9 was performed with the PE-labeled mAbs. IC mAbs were used as negative control. Expression of TLR7 was analyzed with rabbit polyclonal Ab and FITC-labeled goat anti-rabbit IgG (MP Biomedicals). Normal rabbit polyclonal IgG was used and negative control. For TLR3, TLR7, and TLR9, nonpermeabilized and permeabilized NK cells were used. For permeabilization, NK cells were treated with the Cytofix/Cytoperm reagent (BD Biosciences), following the instructions provided by the manufacturer. Cells were analyzed in a FACSCalibur (BD Biosciences) flow cytometer, and data were processed with Winmdi software.

ELISA

Secretion of IFN-γ by NK cells was analyzed by ELISA using a paired set of anti-IFN-γ mAbs and HRP-labeled streptavidin (all from Pierce). Standard curve was obtained using rIFN-γ. Results are presented as mean ± SD and analyzed by one-way ANOVA with Dunnett′s comparison test.

Cytotoxicity

Five-hour standard ^{51}Cr release assays were performed using 5000 target cells/well and human NK cells as effector cells, using different E:T ratios. The percentage of cytotoxicity was calculated as follows: 100 × ((experimental release − spontaneous release)/(maximum release − spontaneous release)). Maximum release was obtained from target cells lysed with 2% Triton X-100 (Sigma-Aldrich). Spontaneous release was always below 15% of maximum release. As target cells, clone 1 and clone C of IIB-MEL-LES were used.

Results

Agonists of TLR3, TLR7, and TLR9 promote IFN-γ secretion by NK cells stimulated with IL-12 and IFN-α

To address the biological function of TLRs expressed by NK cells, we first evaluated their capacity to modulate IFN-γ secretion in unstimulated NK cells and in NK cells stimulated for short periods (24 h) with suboptimal doses of IL-12, IL-15, or IFN-α (Fig. 1). We observed that the TLR3 agonist poly(I:C), the TLR7 agonist loxoribine, or the TLR9 agonist ODN-A, in the absence of cytokines, induced negligible IFN-γ secretion (<20 pg/ml; Fig. 1*A*). In contrast, stimulation of NK cells with suboptimal doses of IL-12 resulted in a moderate IFN-γ secretion (300–400 pg/ml on average for different donors); an effect that was strongly enhanced to values above 500 pg/ml by poly(I:C) and loxoribine (Fig. 1*B*). Although some IFN-γ secretion was observed with control ODNs, only ODN-A promoted TLR9-mediated IFN-γ secretion above the values produced by its control ODN. Conversely, stimulation of NK cells with suboptimal doses of IL-15 hardly induced IFN-γ secretion (Fig. 1*C*). Because poly(I:C) and loxoribine exhibited the strongest ability to promote IFN-γ secretion in the presence of IL-12, we investigated whether IFN-α could elicit a similar response (Fig. 1*D*). We observed that this cytokine promoted IFN-γ secretion (100–200 pg/ml), and that loxoribine and poly(I:C) further enhanced this effect. However, the amount of IFN-γ detected in these culture supernatants remained always below the amount detected in supernatants of NK cells stimulated with the same agonists and IL-12. Conversely, ODN-A and ODN-B did not enhance IFN-γ secretion by NK cells stimulated with IFN-α (data not shown). To discard the possibility of an indirect effect mediated by contaminating monocytes (we used NK cell preparations with 1.2 ± 0.95% of $CD14^+$ cells, with a maximum of 3% of $CD14^+$ cells), we performed additional experiments depleting monocytes by passage through nylon wool columns. After that, $CD14^+$ cells represented 1.40 ± 0.38% of the cells (starting values for these

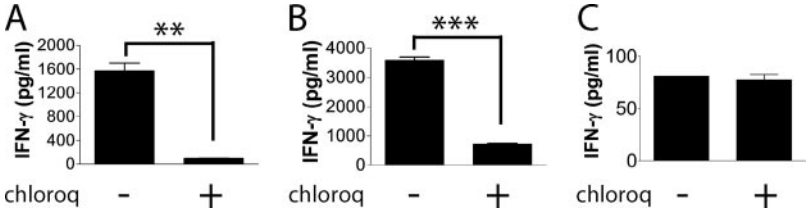

FIGURE 3. IFN-γ secretion by human NK cells stimulated with IL-12 and agonists of TLR3 or TLR7 depends on internalization and recognition of their ligands in a chloroquine-sensitive compartment. IFN-γ production by NK cells stimulated with IL-12 and loxoribine (*A*), with IL-12 and poly(I:C) (*B*), or with IL-12 alone (*C*) was assessed by ELISA in the absence (−) or in the presence (+) of 20 μg/ml chloroquine. Results are representative of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

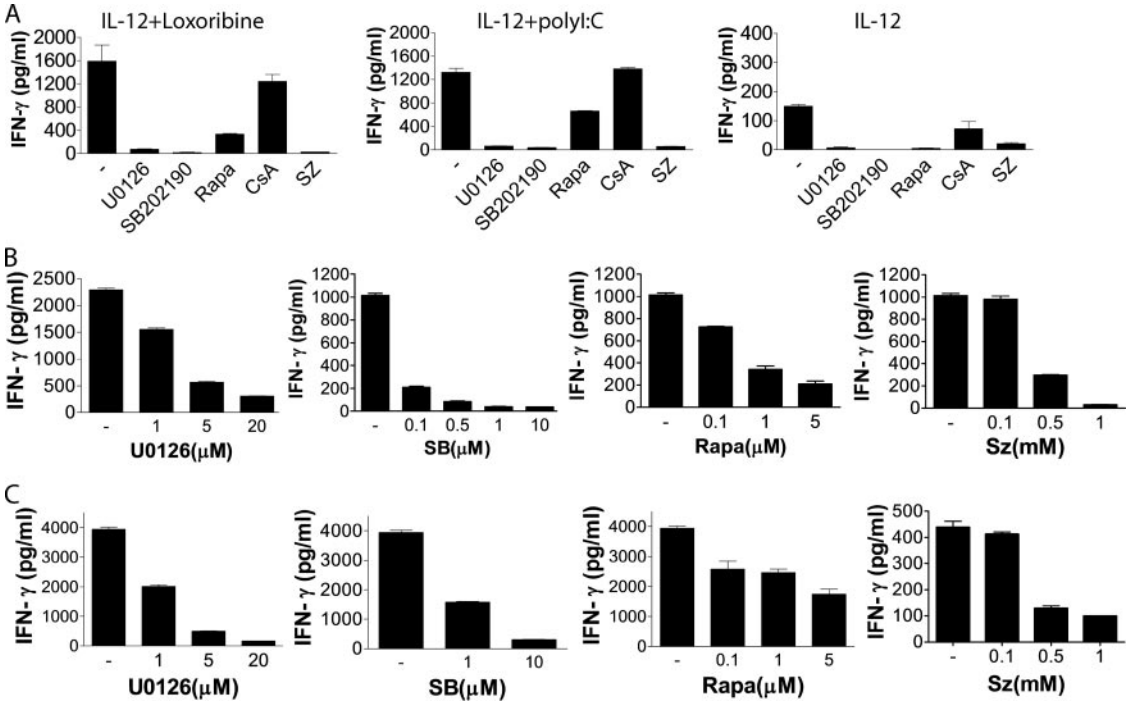

FIGURE 4. Signaling routes involved in IFN-γ production by human NK cells stimulated with IL-12 and loxoribine or poly(I:C). *A*, NK cells were stimulated with IL-12 and loxoribine (*left panel*), IL-12 and poly(I:C) (*middle panel*), or IL-12 alone (*right panel*) in the absence (−) or in the presence of 20 μM U0126, 10 μM SB202190, 5 μM rapamycin, 1 μM CsA, or 1 μM Sz. U0126, SB202190, rapamycin, and Sz vs -, $p < 0.01$; CsA vs -, NS. *B*, Dose-dependent inhibition of IFN-γ secretion by NK cells stimulated with IL-12 and loxoribine in the presence of different doses of U0126, SB202190 (SB), rapamycin (Rapa), or Sz. *C*, Dose-dependent inhibition of IFN-γ secretion by NK cells stimulated with IL-12 and poly(I:C) in the presence of different doses of U0126, SB202190 (SB), rapamycin (Rapa), or Sz. Results are representative of three independent experiments.

new experiments as follows: 1.93 ± 1.64% of $CD14^+$ cells). Of note, when the original cell preparation contained <1.5% of monocytes, the passage through the column did not lead to a further enrichment in NK cells. When these purified NK cells were used for the stimulations with IL-12 or IFN-α and loxoribine or poly(I:C), we observed that they produced similar amounts of IFN-γ than NK cells from the same donor not passed through the nylon wool columns (data not shown).

The IFN-γ secretion triggered by IL-12 and agonists of TLR3 or TLR7, and the heterogeneity in the response of human NK cells to different stimuli reported by different authors encouraged us to investigate the behavior of NK cells from more healthy donors. NK cells from 14 different donors systematically secreted significant IFN-γ upon stimulation with IL-12 and loxoribine or poly(I:C) (Fig. 1*E*). Individual analysis of NK cells from 12 of these donors confirmed these observations for IL-12 and loxoribine (Fig. 1*F*) and for IL-12 and poly(I:C) (Fig. 1*G*). Such response was reproducible along time (Fig. 1*H*), as follows: a donor that responded to IL-12 plus loxoribine and IL-12 plus poly(I:C) did so regardless of the time of blood drawing; a donor that responded only to IL-12 plus loxoribine, too; and a donor that did not respond to any of these stimuli remained unresponsive over time. Because similar cooperative effects were described for IL-12, IFN-α, and IL-18 (33, 34), we addressed the effect of IL-18 in our experimental setting. First, we observed that IL-18 alone or in combination with agonists of TLR3, TLR7, or TLR9 did not promote IFN-γ secretion by human NK cells (data not shown). However, IL-18 enhanced IFN-γ secretion by NK cells stimulated with IL-12 or IFN-α, although the effect of IFN-α was weaker that the effect of IL-12 (Fig. 1*I*). Also, IL-18 further enhanced the secretion of IFN-γ by NK cells stimulated with IL-12 and poly(I:C), but not with IL-12 and loxoribine (Fig. 1*J*), or with IFN-α and loxoribine or poly(I:C) (Fig. 1*K*). In addition, increasing concentrations of IL-12, IL-15, or IFN-α alone only elicited low amounts of IFN-γ secretion (Fig. 1*L*). These results support the idea that resting human NK cells become strong IFN-γ-secreting cells upon coactivation with some cytokines (IL-12 or IFN-α) and agonists of specific TLRs.

Next, we investigated whether stimulation of NK cells with IL-12 or IFN-α affects the expression of TLR3, TLR7, and TLR9, and vice versa, whether stimulation of NK cells with agonists of TLR3, TLR7, or TLR9 affects the expression of IL-12Rβ1, IL-12Rβ2, or IFN-αR. Although expression of TLRs was detected only using permeabilized NK cells (Fig. 2, *A* and *B*), we observed that unstimulated NK cells expressed similar amounts of these three TLRs as NK cells stimulated with IL-12 or IFN-α. In addition, unstimulated NK cells expressed similar amounts of IL-12Rβ1 as NK cells stimulated with agonists of TLR3, TLR7, or TLR9 (Fig. 2*C*, *left panel*). However, we were unable to detect expression of IL-12Rβ2 on resting and on stimulated NK cells (Fig. 2*C*, *middle panel*). As control, we assessed the expression of IL-12Rβ2 on activated T cells (Fig. 2*D*), in which we observed that this receptor was expressed at low levels, as has been reported (35). For IFN-αR, both unstimulated and stimulated NK cells expressed very little amounts of this receptor (Fig. 2*C*, *right panel*).

Therefore, TLR3, TLR7, and, in less extent, TLR9 engagement promoted IFN-γ secretion by NK cells in the presence of suboptimal doses of IL-12 or IFN-α, but not IL-15 or IL-18, in a process that did not involve a reciprocal up-regulation of IL-12Rβ1, IL-12Rβ2, or IFN-αR by agonists of TLRs, and of TLRs by IL-12 or IFN-α. Moreover, IL-18 further cooperated with IL-12 or IFN-α and the agonist of TLR3 or TLR7 (except in the case of IL-12 plus loxoribine) for IFN-γ secretion in our experimental setting.

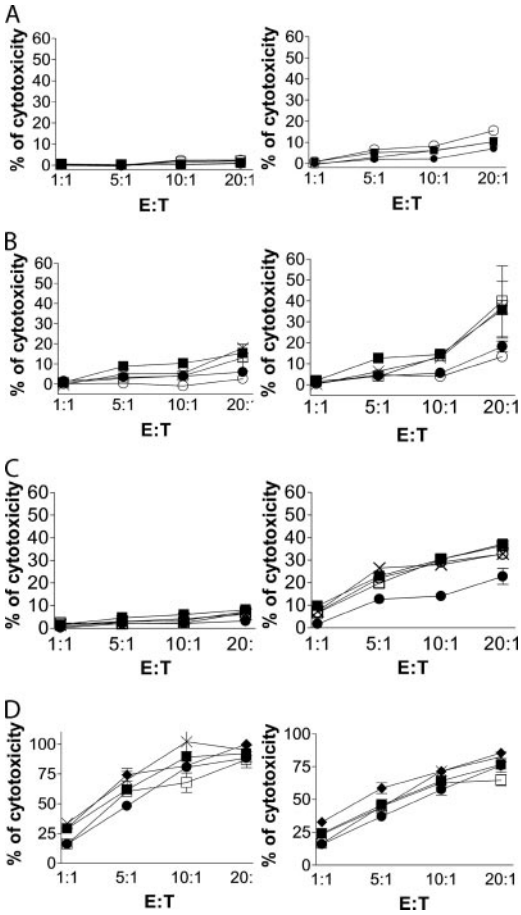

FIGURE 5. Cytokines alone, but not agonists of TLRs, regulate NKG2D-dependent NK cell-mediated cytotoxicity. NK cell-mediated cytotoxicity against MICA$^-$ clone C (*left graphs*) and against MICA$^+$ clone 1 (*right graphs*) at different E:T ratios (*x*-axis) was assessed. Human NK cells were used unstimulated (*A*) or were stimulated with IL-12 (*B*), IL-15 (*C*), or IFN-α (*D*) in the absence of TLR agonists and cytokine (○) or in the presence of loxoribine (●), poly(I:C) (■), or ODN-A (□). x, Represents the cytotoxicity obtained with cytokines alone (IL-12 for *B*; IL-15 for *C*; and IFN-α for *D*). ODN-Ac (data not shown) produced cytotoxicity values identical than those produced by unstimulated NK cells (*A*) or by NK cells stimulated with cytokine alone (*B–D*). Results are representative of three independent experiments.

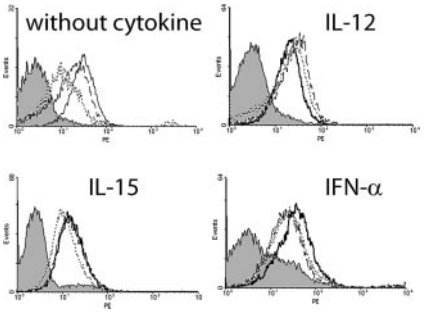

FIGURE 6. NKG2D expression on NK cells is not regulated by cytokines or agonists of TLRs. Expression of NKG2D on isolated human NK cells assessed by flow cytometry after a culture of 24 h without cytokines or with IL-12, IL-15, or IFN-α in the absence or presence of loxoribine (dotted line) or poly(I:C) (dashed line). The continuous line represents the basal staining for NKG2D (NK cells cultured in the absence of these agonists in each case). ODN-A and ODN-Ac produced similar histograms as NK cells cultured in the absence of these stimuli in each case (data not shown). Filled histogram: IC mAb. Results are representative of three independent experiments.

The effect of agonists of TLR3 and TLR7 depends on internalization and MEK1/ERK, p38 MAPK, p70^{S6} kinase, and NF-κB

Signaling through TLR3 and TLR7 in DCs involves TLR recognition in endosomes. To investigate whether this also occurs in NK cells, we stimulated human NK cells with IL-12 and loxoribine or poly(I:C) in the absence or in the presence of chloroquine (Fig. 3). In these experiments, we observed a strong inhibition of the IFN-γ secretion induced by the drug in cells stimulated with IL-12 and the agonists of TLRs (Fig. 3, *A* and *B*). Conversely, the low amount of IFN-γ secretion promoted by IL-12 alone was not prevented by chloroquine (Fig. 3*C*). These results indicate that signaling through TLR3 and TLR7 in NK cells depends on recognition of their putative ligands in a chloroquine-sensitive intracellular compartment.

To gain insight into the intracellular mediators involved in the IFN-γ secretion, we stimulated NK cells with IL-12 and loxoribine or poly(I:C) in the absence or in the presence of pharmacologic inhibitors of different signaling routes (Fig. 4). Inhibition of MEK1/ERK with U0126, p38 MAPK with SB202190, p70^{S6} kinase with rapamycin, and NF-κB with Sz strongly prevented IFN-γ secretion, but inhibition of calcineurin with CsA had no effect (Fig. 4*A*). The effects of U0126, SB202190, rapamycin, and Sz were dose dependent on NK cells stimulated with IL-12 and loxoribine (Fig. 4*B*) or with IL-12 and poly(I:C) (Fig. 4*C*).

Agonists of TLR3, TLR7, and TLR9 in combination with IL-12, IL-15, or IFN-α do not enhance NKG2D-dependent NK cell-mediated cytotoxicity

Next, we investigated whether these agonists also modulate NKG2D-dependent NK cell-mediated cytotoxicity using a clone of the IIB-MEL-LES human melanoma cell line transfected with full-length MICA (clone 1) or a clone transfected with empty plasmid (clone C) as target cells. Unstimulated NK cells were not cytotoxic against MICA$^-$ clone C (Fig. 5*A*, *left panel*), but slightly killed MICA$^+$ clone 1 (Fig. 5*A*, *right panel*, clone C vs clone 1: $p < 0.001$). However, we were unable to detect higher cytotoxicity against these target cells by NK cells stimulated with poly(I:C), loxoribine, or ODN-A. When NK cells were stimulated with cytokines, a slightly higher cytotoxic response was detected against clone C if NK cells were stimulated with IL-12 (Fig. 5*B*, *left panel*), but not if NK cells were stimulated with IL-15 (Fig. 5*C*, *left panel*). However, both cytokines promoted a good cytotoxicity against clone 1 (Fig. 5, *B* and *C*, *right panels*, clone C vs clone 1: $p < 0.01$ for IL-12 and $p < 0.0001$ for IL-15), suggesting that IL-12 and IL-15 enhance NKG2D-dependent NK cell-mediated cytotoxicity. When NK cells were stimulated with IFN-α, both target cells were strongly lysed (Fig. 5*D*, clone C vs clone 1: NS), but, as in the absence of cytokines, we did not observe higher cytotoxicity of NK cells stimulated with IL-12, IL-15, or IFN-α and loxoribine, poly(I:C), or ODN-A (Fig. 5, *B–D*). Therefore, we conclude that agonists of TLR3, TLR7, or TLR9 do not regulate NKG2D-dependent cytotoxic response of NK cells against target cells that express low or high amounts of MICA.

NKG2D engagement on NK cells stimulated with agonists of TLR3 or TLR7 and IL-12 enhances IFN-γ secretion

The dissociation between the IFN-γ secretion and cytotoxicity observed with NK cells stimulated with IL-12 and TLR agonists

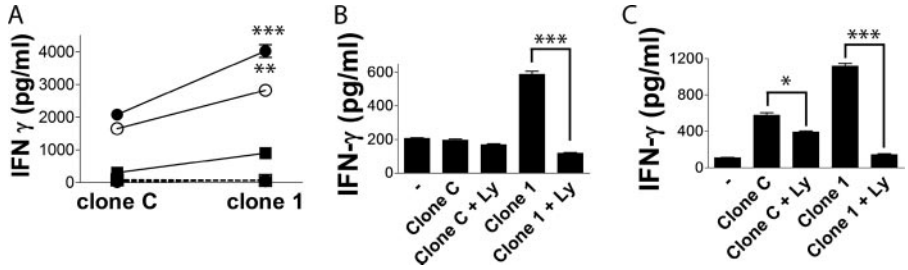

FIGURE 7. Enhancement of IFN-γ secretion by human NK cells stimulated with IL-12 and loxoribine or poly(I:C) upon engagement of NKG2D, and involvement of PI3K. *A*, NK cells were stimulated for 24 h with IL-12 (continuous line) or cultured without cytokine (dashed line) in the absence (■) or in the presence of loxoribine (●) or poly(I:C) (○) and in the presence of MICA$^-$ clone C or in the presence of MICA$^+$ clone 1. ***, $p < 0.001$ (clone 1 vs clone C for IL-12 and loxoribine); **, $p < 0.01$ (clone 1 vs clone C for IL-12 and poly(I:C)). *B* and *C*, IFN-γ secretion by NK cells stimulated with IL-12 and loxoribine (*B*) or poly(I:C) (*C*) and cocultured with clone C or clone 1 in the absence or in the presence of the PI3K inhibitor Ly294002 (Ly). -, Corresponds to the IFN-γ secreted by NK cells stimulated with IL-12 and loxoribine (*B*) or poly(I:C) (*C*) cultured in the absence of any clone. Results are representative of three independent experiments. ***, $p < 0.001$.

prompted us to investigate whether such stimuli regulate the expression of NKG2D. By flow cytometry, we observed that loxoribine and poly(I:C) induced a slight down-regulation of this receptor that was not detected on NK cells stimulated with the same agonists in the presence of IL-12, IL-15, or IFN-α (Fig. 6).

To investigate whether NKG2D engagement contributes to IFN-γ secretion triggered by agonists of TLR7 or TLR3 and IL-12, NK cells were stimulated with loxoribine or poly(I:C) and suboptimal doses of IL-12 in the presence of clone C or clone 1 of IIB-MEL-LES. We observed a significantly enhanced IFN-γ secretion if NKG2D engagement was induced by coculture with clone 1 vs clone C (Fig. 7*A*). This response involved the activation of the PI3K because it was abrogated by the PI3K inhibitor Ly294002 (Fig. 7*B* for IL-12 and loxoribine, and Fig. 7*C* for IL-12 and poly(I:C)).

Hence, our results indicate that selected agonists of TLRs expressed by NK cells (in particular, TLR7 and TLR3), in the presence of IL-12 or IFN-α can directly promote IFN-γ secretion in a process that involves internalization of the agonists and activation of ERK, p38 MAPK, p70^{S6} kinase, and NF-κB. Moreover, although the NKG2D-dependent cytotoxicity of these NK cells and the expression of NKG2D remained unaffected, IFN-γ secretion was additionally enhanced by engagement of NKG2D in a PI3K-dependent manner.

Discussion

Expression of TLRs on NK cells has been known for some time, but their functional relevance is still not completely elucidated (22, 23, 29–31). Also, there is no information about possible cooperative effects between these receptors and NK cell receptors such as NKG2D, a molecule that plays a pivotal role in their tumoricidal effects. In this work, we demonstrated that NK cells rapidly secrete high amounts of IFN-γ when stimulated simultaneously with suboptimal doses of IL-12 and agonists of TLR3, TLR7, or TLR9. In addition, IFN-α and loxoribine or poly(I:C) also promoted some IFN-γ secretion, but such response was barely induced by the agonists in the absence of these cytokines or in the presence of IL-15 or IL-18. IL-12 and loxoribine or poly(I:C) were stronger inducers of IFN-γ secretion than IL-12 and IL-18. Hence, it is possible that NK cells stimulated with IL-12 and agonists of TLR3 or TLR7 may acquire an immunoregulatory potential to skew the immune response toward a Th1 phenotype, as has been described for IL-12 and IL-18 (33, 34). The cooperative effects observed with IL-12 or IFN-α and agonists of TLR3, TLR7, or TLR9 did not involve a reciprocal up-regulation of TLRs, IL-12Rβ1, IL-12Rβ2, or IFN-αR. Even though we observed expression of IL-12Rβ1 (a component shared by receptors for other cytokines of the IL-12 family such as IL-23) (36) and the fact that low levels of IL-12Rβ2 were detected previously by others on NK cells stimulated with IL-2 (35), we were unable to detect expression of IL-12Rβ2 on resting and on NK cells stimulated with agonists of TLR3, TLR7, or TLR9. Thus, it remains intriguing why NK cells from a minority of the donors secreted IFN-γ in response to suboptimal doses of IL-12 alone, although stimulation through IL-12Rβ1 in the absence of IL-12Rβ2 has been associated with partial activation in T cells (37, 38). According to our results, the cooperative effects triggered by IL-12 or IFN-α, and the agonists of TLR3, TLR7, or TLR9 might occur due to a better coupling or integration of the intracellular routes triggered by both stimuli. It is not clear why ODN-A, but not ODN-B, triggered IFN-γ secretion, but similar results were obtained elsewhere (22). Mostly, NK cells from different donors responded better to loxoribine than to poly(I:C), although heterogeneity was observed in the amount of IFN-γ detected. Similar heterogeneity in cytokine secretion by NK cells was reported by others (22). The secretion of IFN-γ induced by control ODNs can be explained considering a nonspecific effect exerted through other (non-TLR9) receptors. It is unlikely that the effects observed in our study could be due to contaminating monocytes because we worked with NK cell preparations that contained 1.2 $\pm$ 0.95% of monocytes, and even when monocytes were further depleted, NK cells secreted high amounts of IFN-γ upon stimulation with IL-12 or IFN-α and loxoribine or poly(I:C). Conversely, if such monocytes or minute numbers of DCs would mediate the phenomena described in this work, we should have detected IFN-γ secretion induced by agonists of TLRs alone (without the addition of exogenous cytokines) because such cytokines would have been provided by the contaminating monocytes or DCs. Conversely, we observed a strict requirement of IL-12 (or, in less degree, IFN-α) for IFN-γ secretion, supporting our suggestion that the stimulatory effect was due to a direct stimulation of the NK cells. Two major populations of NK cells have been described (39, 40). Although the major population (>90%) of CD56^{dim} NK cells has been mostly associated with natural cytotoxicity, in our NK cell preparations we detected that CD56^{dim} cells constituted more that 95% of the cells recovered after the isolation procedure (data not shown). Therefore, this population of NK cells is likely to be responsible for the IFN-γ secretion detected in our experimental setting.

Signaling of the agonists of TLR7 and TLR3 most likely required internalization because secretion of IFN-γ was inhibited by chloroquine. Conversely, the effect of IL-12 was not sensitive to this drug, as expected for a cell surface receptor that recognizes an

extracellular ligand. Because TLR3, TLR7, and TLR9 were detected only using permeabilized NK cells, we conclude that in NK cells, as in DCs, the agonists of TLR3, TLR7, and TLR9 exert their effect upon binding to its specific receptor in an intracellular, chloroquine-sensitive compartment that most likely would be the endosome. Hart et al. (23) using agonists of TLR7/8 reported that signaling of poly(I:C) in NK cells was independent of endocytosis. The discrepancy with our results could be due to the fact that they used the NKL cell line and that they evaluated the effect of chloroquine assessing the degradation of IκBα. Also, Pisegna et al. (41) observed that poly(I:C), together with IL-2, stimulated human NK cells to secrete IFN-γ in a p38 MAPK- and IFN regulatory factor-3-dependent way, whereas Schmidt et al. (31) observed that NK cells were stimulated by poly(I:C) in an APC-independent manner that required NF-κB (assessed as degradation of IκBα). However, there is no additional information about the signaling routes triggered by agonists of TLRs in NK cells and their functional outcome. In this study, we observed that IFN-γ secretion triggered by TLR3 or TLR7 in the presence of suboptimal IL-12 strictly depended on activation of MEK1/ERK, p38 MAPK, $p70^{S6}$ kinase, and NF-κB, but not on calcineurin. These signaling intermediates also appeared to participate in IFN-γ secretion promoted by IL-12 alone. Because the magnitude of IFN-γ secreted was much higher in the presence of loxoribine or poly(I:C), and the effect of the pharmacologic inhibitors led to an almost complete abrogation of IFN-γ secretion, we can speculate that activation of these intracellular mediators also might be triggered by the agonists of TLR3 and TLR7, and then integrate with the signals triggered by IL-12 and lead to the cooperative effects observed in this study.

To assess the contribution of NKG2D, we investigated the effect of the cytokines and agonists on the cytotoxicity of NK cells against target cells that express high or very low amounts of MICA. Although NK cells were not cytotoxic against the $MICA^-$ melanoma clone, but killed the $MICA^+$ melanoma clone, this effect was enhanced by IL-12, IL-15, and IFN-α. However, agonists of TLR3, TLR7, or TLR9 did not additionally modulate cytotoxicity. Accordingly, expression of NKG2D remained unchanged on NK cells upon stimulation with IL-12, IL-15, or IFN-α, and poly(I:C), loxoribine, or ODN-A. Therefore, our results indicate that IL-12, IL-15, and IFN-α, but not the agonists of TLR3, TLR7, or TLR9, enhance NKG2D-dependent NK cell-mediated cytotoxicity by inducing a more efficient activation of the signaling cascades triggered upon NKG2D engagement, perhaps inducing a cellular relocation or neosynthesis of adaptor proteins or signaling intermediates in NK cells. Other authors observed that agonists of different TLRs promote higher cytotoxicity of NK cells against target cells such as P815 with anti-CD16 mAb, K562, Daudi, or YAC-1 (22, 23, 29, 31, 41), but the contribution of NKG2D to this response was not addressed previously. In this study, we demonstrated for the first time that the cytotoxicity triggered by this pivotal receptor was enhanced by IL-12, IL-15, or IFN-α, but not further increased by agonists of the TLRs.

Contradicting results have been published about IFN-γ secretion by human NK cells upon engagement of NKG2D (3, 4, 42–45). Our results suggest that engagement of NKG2D by MICA expressed on tumor cells enhanced TLR7 or TLR3 plus IL-12-mediated IFN-γ secretion in a PI3K-dependent way. Although NKG2D-dependent PI3K pathway has been mainly associated with cytotoxicity (5), specific signaling routes that couple NKG2D engagement with IFN-γ secretion without inducing cytotoxicity may also exist (45) and be involved in our experimental observations.

The dichotomy between the NKG2D-mediated IFN-γ secretion promoted by the $MICA^+$ melanoma without stimulation of NKG2D-dependent cytotoxicity in human NK cells stimulated with agonists of TLR3 or TLR7 and IL-12 could be due to a rapid up-regulation of receptor mainly involved in IFN-γ secretion, but not in cytotoxicity such as KIR2DL4 (46), whose engagement by its putative ligand expressed on the melanoma cells would promote the observed effect. Alternatively, poly(I:C) and loxoribine in the presence of IL-12 may induce a better coupling of the signaling cascade triggered upon engagement of NKG2D by the $MICA^+$ melanoma and lead to augmented IFN-γ secretion. Intracellular relocation or neosynthesis of scaffolding or signaling proteins induced by these stimuli may contribute to this response. In any case, our results suggest that selected agonists of TLRs expressed by NK cells (in particular, TLR7 and TLR3), in the presence of IL-12 or, in less extent, IFN-α directly trigger a strong IFN-γ secretion without promoting NKG2D-dependent cytotoxicity, a process that involves internalization of the agonists and activation of MEK1/ERK, p38 MAPK, $p70^{S6}$ kinase, and NF-κB. Such IFN-γ secretion can be additionally enhanced by engagement of NKG2D, which in turn could be crucial to promote Th1-biased immune responses (33, 34, 47, 48). In light of the increasing experimental evidence that indicates that the immune response against tumors involving NKG2D is critical to achieve an efficient immunosurveillance (49), stimulation provided by agonists of TLR7 or TLR3 (safely administered exogenously), in conjunction with cytokines such as IL-12 in a tumor microenvironment, may be therapeutically useful as adjuvants to elicit an improved antitumor immune response mediated not only by NK cells, but also by T lymphocytes. Such strategy would be particularly suitable against tumors that express NKG2DLs, such as MICA, to promote a vigorous IFN-γ secretion and to provide an enhanced tumoricidal function.

Acknowledgments

We gratefully thank Dr. José Mordoh for the IIB-MEL-LES cell line; Dr. César Carbonetto (from Biosidus, Argentina) for IFN-α; Dr. Pierre Eid (Villejuif Cedex, France) for the anti-IFN-αR mAb; and Dr. Gabriel Rabinovich for his constant support.

Disclosures

The authors have no financial conflict of interest.

References

1. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727–729.
2. Zwirner, N. W., M. B. Fuertes, M. V. Girart, C. I. Domaica, and L. E. Rossi. 2007. Cytokine-driven regulation of NK cell functions in tumor immunity: role of the MICA-NKG2D system. *Cytokine Growth Factor Rev.* 18: 159–170.
3. Andre, P., R. Castriconi, M. Espeli, N. Anfossi, T. Juarez, S. Hue, H. Conway, F. Romagne, A. Dondero, M. Nanni, et al. 2004. Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors. *Eur. J. Immunol.* 34: 961–971.
4. Sutherland, C. L., N. J. Chalupny, K. Schooley, T. VandenBos, M. Kubin, and D. Cosman. 2002. UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. *J. Immunol.* 168: 671–679.
5. Hayakawa, Y., and M. J. Smyth. 2006. NKG2D and cytotoxic effector function in tumor immune surveillance. *Semin. Immunol.* 18: 176–185.
6. Cosman, D., J. Mullberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N. J. Chalupny. 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14: 123–133.
7. Bahram, S. 2000. MIC genes: from genetics to biology. *Adv. Immunol.* 76: 1–60.
8. Groh, V., R. Rhinehart, H. Secrist, S. Bauer, K. H. Grabstein, and T. Spies. 1999. Broad tumor-associated expression and recognition by tumor-derived γδ T cells of MICA and MICB. *Proc. Natl. Acad. Sci. USA* 96: 6879–6884.
9. Salih, H. R., H. Antropius, F. Gieseke, S. Z. Lutz, L. Kanz, H. G. Rammensee, and A. Steinle. 2003. Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. *Blood* 102: 1389–1396.
10. Pende, D., P. Rivera, S. Marcenaro, C. C. Chang, R. Biassoni, R. Conte, M. Kubin, D. Cosman, S. Ferrone, L. Moretta, and A. Moretta. 2002. Major

histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Res.* 62: 6178–6186.
11. Molinero, L. L., M. B. Fuertes, G. A. Rabinovich, L. Fainboim, and N. W. Zwirner. 2002. Activation-induced expression of MICA on T lymphocytes involves engagement of CD3 and CD28. *J. Leukocyte Biol.* 71: 791–797.
12. Maasho, K., J. Opoku-Anane, A. I. Marusina, J. E. Coligan, and F. Borrego. 2005. NKG2D is a costimulatory receptor for human naive $CD8^+$ T cells. *J. Immunol.* 174: 4480–4484.
13. Gasser, S., S. Orsulic, E. J. Brown, and D. H. Raulet. 2005. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436: 1186–1190.
14. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci. USA* 98: 11521–11526.
15. Busche, A., T. Goldmann, U. Naumann, A. Steinle, and S. Brandau. 2006. Natural killer cell-mediated rejection of experimental human lung cancer by genetic overexpression of major histocompatibility complex class I chain-related gene A. *Hum. Gene Ther.* 17: 135–146.
16. Diefenbach, A., E. R. Jensen, A. M. Jamieson, and D. H. Raulet. 2001. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413: 165–171.
17. Smyth, M. J., J. Swann, E. Cretney, N. Zerafa, W. M. Yokoyama, and Y. Hayakawa. 2005. NKG2D function protects the host from tumor initiation. *J. Exp. Med.* 202: 583–588.
18. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The three Es of cancer immunoediting. *Annu. Rev. Immunol.* 22: 329–360.
19. Khong, H. T., and N. P. Restifo. 2002. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat. Immunol.* 3: 999–1005.
20. Rabinovich, G. A., E. Sotomayor, and D. Gabrilovich. 2007. Immunosuppressive strategies that are mediated by tumor cells. *Annu. Rev. Immunol.* 25: 267–296.
21. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5: 987–995.
22. Sivori, S., M. Falco, M. Della 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. *Proc. Natl. Acad. Sci. USA* 101: 10116–10121.
23. 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 IFN-γ production. *J. Immunol.* 175: 1636–1642.
24. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* 413: 732–738.
25. Heil, F., P. Ahmad-Nejad, H. Hemmi, H. Hochrein, F. Ampenberger, T. Gellert, H. Dietrich, G. Lipford, K. Takeda, S. Akira, et al. 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur. J. Immunol.* 33: 2987–2997.
26. Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* 98: 9237–9242.
27. Zanoni, I., M. Foti, P. Ricciardi-Castagnoli, and F. Granucci. 2005. TLR-dependent activation stimuli associated with Th1 responses confer NK cell stimulatory capacity to mouse dendritic cells. *J. Immunol.* 175: 286–292.
28. Andoniou, C. E., S. L. van Dommelen, V. Voigt, D. M. Andrews, G. Brizard, C. Asselin-Paturel, T. Delale, K. J. Stacey, G. Trinchieri, and M. A. Degli-Esposti. 2005. Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. *Nat. Immunol.* 6: 1011–1019.
29. Gorski, K. S., E. L. Waller, J. Bjornton-Severson, J. A. Hanten, C. L. Riter, W. C. Kieper, K. B. Gorden, J. S. Miller, J. P. Vasilakos, M. A. Tomai, and S. S. Alkan. 2006. Distinct indirect pathways govern human NK-cell activation by TLR-7 and TLR-8 agonists. *Int. Immunol.* 18: 1115–1126.
30. 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.
31. Schmidt, K. N., B. Leung, M. Kwong, K. A. Zarember, S. Satyal, T. A. Navas, F. Wang, and P. J. Godowski. 2004. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J. Immunol.* 172: 138–143.
32. Kairiyama, C., I. Slavutsky, I. Larripa, V. Morvillo, A. I. Bravo, L. Bover, O. L. Podhajcer, and J. Mordoh. 1995. Biologic, immunocytochemical, and cytogenetic characterization of two new human melanoma cell lines: IIB-MEL-LES and IIB-MEL-IAN. *Pigm. Cell. Res.* 8: 121–131.
33. Mailliard, R. B., Y.-I. Son, R. Redlinger, P. T. Coates, A. Giermasz, P. A. Morel, W. J. Storkus, and P. Kalinski. 2003. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. *J. Immunol.* 171: 2366–2373.
34. Mailliard, R. B., S. M. Alber, H. Shen, S. C. Watkins, J. M. Kirkwood, R. B. Herberman, and P. Kalinski. 2005. IL-18-induced $CD83^+CCR7^+$ NK helper cells. *J. Exp. Med.* 202: 941–953.
35. Rogge, L., A. Papi, D. H. Presky, M. Biffi, L. J. Minetti, D. Miotto, C. Agostini, G. Semenzato, L. M. Fabbri, and F. Sinigaglia. 1999. Antibodies to the IL-12 receptor β2 chain mark human Th1 but not Th2 cells in vitro and in vivo. *J. Immunol.* 162: 3926–3932.
36. Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington. 12007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol.* 25: 821–852.
37. Wu, C.-Y., X. Wang, M. Gadina, J. J. O'Shea, D. H. Presky, and J. Magram. 2000. IL-12 receptor β2 (IL-12Rβ2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J. Immunol.* 165: 6221–6228.
38. Yoshimoto, T., M. Furuhata, S. Kamiya, M. Hisada, H. Miyaji, Y. Magami, K. Yamamoto, H. Fujiwara, and J. Mizuguchi. 2003. Positive modulation of IL-12 signaling by sphingosine kinase 2 associating with the IL-12 receptor β1 cytoplasmic region. *J. Immunol.* 171: 1352–1359.
39. Fehniger, T. A., M. A. Cooper, G. J. Nuovo, M. Cella, F. Facchetti, M. Colonna, and M. A. Caligiuri. 2003. $CD56^{bright}$ natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* 101: 3052–3057.
40. Ferlazzo, G., D. Thomas, S. L. Lin, K. Goodman, B. Morandi, W. A. Muller, A. Moretta, and C. Münz. 2004. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *J. Immunol.* 172: 1455–1462.
41. Pisegna, S., G. Pirozzi, M. Piccoli, L. Frati, A. Santoni, and G. Palmieri. 2004. p38 MAPK activation controls the TLR3-mediated up-regulation of cytotoxicity and cytokine production in human NK cells. *Blood* 104: 4157–4164.
42. Billadeau, D. D., J. L. Upshaw, R. A. Schoon, C. J. Dick, and P. J. Leibson. 2003. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat. Immunol.* 4: 557–564.
43. Bryceson, Y. T., M. E. March, H.-G. Ljunggren, and E. O. Long. 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* 107: 159–166.
44. Wu, J., H. Cherwinski, T. Spies, J. H. Phillips, and L. L. Lanier. 2000. DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. *J. Exp. Med.* 192: 1059–1068.
45. Upshaw, J. L., and P. J. Leibson. 2006. NKG2D-mediated activation of cytotoxic lymphocytes: unique signaling pathways and distinct functional outcomes. *Semin. Immunol.* 18: 167–175.
46. Rajagopalan, S., J. Fu, and E. O. Long. 2001. Cutting edge: induction of IFN-γ production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. *J. Immunol.* 167: 1877–1881.
47. Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-γ for T_H1 priming. *Nat. Immunol.* 5: 1260–1265.
48. Ferlazzo, G., M. Pack, D. Thomas, C. Paludan, D. Schmid, T. Strowig, G. Bougras, W. A. Muller, L. Moretta, and C. Münz. 2004. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proc. Natl. Acad. Sci. USA* 101: 16606–16611.
49. Pure, E., J. P. Allison, and R. D. Schreiber. 2005. Breaking down the barriers to cancer immunotherapy. *Nat. Immunol.* 6: 1207–1210.