Cutting Edge: Induction of IFN-γ Production but Not Cytotoxicity by the Killer Cell Ig-Like Receptor KIR2DL4 (CD158d) in Resting NK Cells

Sumati Rajagopalan, Jacqueline Fu and Eric O. Long

*J Immunol* 2001; 167:1877-1881; doi: 10.4049/jimmunol.167.4.1877
http://www.jimmunol.org/content/167/4/1877

**References**
This article cites 23 articles, 10 of which you can access for free at:
http://www.jimmunol.org/content/167/4/1877.full#ref-list-1

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

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

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Activated NK cells lyse tumor cells and virus-infected cells and produce IFN-γ upon contact with sensitive target cells. The regulation of these effector responses in resting NK cells is not well understood. We now describe a receptor, KIR2DL4, that has the unique property of inducing IFN-γ production, but not cytotoxicity, by resting NK cells in the absence of cytokines. In contrast, the NK cell-activation receptors CD16 and 2B4 induced cytotoxicity but not IFN-γ production. The induction by KIR2DL4 of IFN-γ production by resting NK cells was blocked by an inhibitor of the p38 mitogen-activated protein kinase signaling pathway, in contrast to the IL-2-induced IFN-γ secretion that was sensitive to inhibition of the extracellular signal-regulated kinase mitogen-activated protein kinase pathway. These results reveal a functional dichotomy (cytokine production vs cytotoxicity) in the response of resting NK cells, as dictated by the signals of individual receptors. 


The signals that convert a circulating NK cell from a resting to a more activated, cytokine-secreting, and lytic mode in vivo are not fully understood. Although resting NK cells are clearly activated by certain cytokines and chemokines (including IL-2 and IL-12) to become cytotoxic and to secrete cytokines, the regulation of these effector responses in resting NK cells is not well understood. We now describe a receptor, KIR2DL4, that has the unique property of inducing IFN-γ production, but not cytotoxicity, by resting NK cells in the absence of cytokines. In contrast, the NK cell-activation receptors CD16 and 2B4 induced cytotoxicity but not IFN-γ production. The induction by KIR2DL4 of IFN-γ production by resting NK cells was blocked by an inhibitor of the p38 mitogen-activated protein kinase signaling pathway, in contrast to the IL-2-induced IFN-γ secretion that was sensitive to inhibition of the extracellular signal-regulated kinase mitogen-activated protein kinase pathway. These results reveal a functional dichotomy (cytokine production vs cytotoxicity) in the response of resting NK cells, as dictated by the signals of individual receptors.

20 h, culture supernatants were tested for IFN-γ production by ELISA (R&D Systems, Minneapolis, MN). In parallel, NK cells were also tested for cytotoxicity against P815 cells in a 3-h [51Cr] release assay. Purified vaccinia virions were used to infect IL-2-activated NK cells as previously described (9). After infection with 10 PFU/cell of each virus for 1.5 h, cells were washed and either monitored for receptor expression by flow cytometry or plated for standard 3-h [51Cr] release assays using P815 target cells. Treatment of NK cells with the extracellular signal-regulated (ERK) kinase (MEK)1 inhibitor PD098059 (Calbiochem, La Jolla, CA) or the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (Sigma-Aldrich, St. Louis, MO) was for 1 h before addition of Abs or rIL-2 (5 U/ml). Inhibitors were present during stimulation with mAbs. Negative controls contained as much DMSO as the highest concentration of inhibitor. The inhibitors did not interfere with NK cell viability as assessed by trypan blue exclusion. After 20 h, culture supernatants were tested for IFN-γ by ELISA.

Results and Discussion

2DL4 induces cytotoxicity by activated NK cells but not by resting NK cells

The mAbs against 2DL4 were generated to characterize the expression and signaling properties of 2DL4. One IgG (#33) and two IgMs (#36 and #64) were identified that reacted specifically with 2DL4 and not other KIR family members. In an ELISA, mAb 33 bound to 2DL4-1g but not to KIR-Ig fusion proteins of 2DL1, 2DL2, 2DL3, and 3DL2 (Fig. 1A). This mAb also did not bind KIR-Ig fusion proteins of 2DS2 and 2DS4 (data not shown). Similar results were obtained with the mAbs 36 and 64 (data not shown). These mAbs also reacted with cells transfected with 2DL4 but not 2DL5, a closely related KIR (C. Chang and A. King, personal communication).

Lysates of 293T/17 cells transfected with a cDNA encoding an epitope-tagged 2DL4 (HA-2DL4) were immunoprecipitated with mAb 33. Western blotting for the HA epitope revealed a broad 45-kD band similar to that seen by blotting of the total cell lysate (Fig. 1C). The KIR-Ig fusion proteins of 2DL2 and 2DL3 (data not shown). These mAbs also reacted with cells transfected with 2DL4 but not 2DL5, a closely related KIR (C. Chang and A. King, personal communication).

Lysates of 293T/17 cells transfected with a cDNA encoding an epitope-tagged 2DL4 (HA-2DL4) were immunoprecipitated with mAb 33. Western blotting for the HA epitope revealed a broad 45-kD band similar to that seen by blotting of the total cell lysate (Fig. 1B). All three anti-2DL4 mAbs bound to the NK cell line NK3.3, but not to the B cell line 271,221 and the T cell line Jurkat as shown by Western blotting with anti-HA Abs (Covance), followed by goat anti-rabbit IgG peroxidase (Amersham, Arlington Heights, IL) and Super Signal substrate (Pierce, Rockford, IL). Western blotting was done as previously described (10), and blots were developed using rabbit anti-HA Abs (Covance), followed by goat anti-rabbit IgG peroxidase (Amersham, Arlington Heights, IL) and Super Signal substrate (Pierce, Rockford, IL).

Functional assays with NK cells

NK cells (5 × 10^5/well) were cocultured with or without P815 cells (1 × 10^5/well) for 20 h with mAbs as indicated. Culture supernatants were tested for IFN-γ production by ELISA (R&D Systems, Minneapolis, MN). In parallel, NK cells were also tested for cytotoxicity against P815 cells in a 3-h [51Cr] release assay. Purified vaccinia virions were used to infect IL-2-activated NK cells as previously described (9). After infection with 10 PFU/cell of each virus for 1.5 h, cells were washed and either monitored for receptor expression by flow cytometry or plated for standard 3-h [51Cr] release assays using P815 target cells. Treatment of NK cells with the extracellular signal-regulated (ERK) kinase (MEK)1 inhibitor PD098059 (Calbiochem, La Jolla, CA) or the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (Sigma-Aldrich, St. Louis, MO) was for 1–2 h at 37°C before addition of Abs or rIL-2 (5 U/ml). Inhibitors were present during stimulation with mAbs. Negative controls contained as much DMSO as the highest concentration of inhibitor. The inhibitors did not interfere with NK cell viability as assessed by trypan blue exclusion. After 20 h, culture supernatants were tested for IFN-γ by ELISA.

mAb 33 was also obtained with several NK cell clones and with the cell lines NK92, NKL, and NK3.3 (data not shown). We conclude that 2DL4 has properties of an activation receptor.

To test the outcome of 2DL4 coligation with an inhibitory receptor, NK cells that express mainly the CD94/NKG2A inhibitory receptor were selected among different donors (7). Coligation of CD94/NKG2A inhibited the lysis induced by 2DL4, 2B4, and CD16 receptors. The NK cell lines NKL and NK92 displayed low levels of cell surface 2DL4. NK cells isolated from peripheral blood of different donors varied from low (similar to that seen with NK92) to intermediate (as shown in Fig. 1C) reactivity with the different anti-2DL4 mAbs.

The 2DL4-specific mAb 33 was used to test whether 2DL4 induces activation or inhibition of lysis. The FcR-positive P815 cells by IL-2-activated NK cells in the presence of mAb 33 was comparable to that obtained with mAbs to the activation receptors 2B4 and CD16 (Fig. 2A). Lysis of P815 cells induced by 2DL4 was also obtained with several NK cell clones and with the cell lines NK92, NKL, and NK3.3 (data not shown). We conclude that 2DL4 has properties of an activation receptor.

To test the outcome of 2DL4 coligation with an inhibitory receptor, NK cells that express mainly the CD94/NKG2A inhibitory receptor were selected among different donors (7). Coligation of CD94/NKG2A inhibited the lysis induced by 2DL4, 2B4, and CD16 receptors. The activation signal delivered by 2DL4 in activated NK cells is typical of NK activation receptors that are sensitive to inhibition by ITIM-containing receptors.

Resting NK cells were incubated with P815 cells in the presence of mAbs for 2DL4, CD16, and 2B4 (Fig. 2B). Enhancement of lysis by CD16 and by 2B4 was similar to that obtained with activated NK cells, except for the weaker lytic potential of freshly isolated NK cells. In contrast, 2DL4 did not enhance cytotoxic activity in resting NK cells. Thus, 2DL4 induction of killing was restricted to activated NK cells, distinguishing its activity from that
of CD16 and 2B4. Coligation of 2B4 or CD16 with CD94 did not decrease the level of lysis (Fig. 2B and data not shown), even though resting NK populations were chosen that yielded activated NK cells in which CD94 was inhibitory. Time course (6–72 h) and titration studies with 0.1–16 μg/ml of the Abs against 2DL4, 2B4, and CD16 were done to ensure that the unique induction of IFN-γ but not cytotoxicity upon 2DL4 activation was not a function of the intensity of mAb triggering (data not shown).

The transmembrane region of 2DL4 is necessary for the activation signal

Recombinant vaccinia viruses encoding 2DL4 and a mutated 2DL4 in which the amino acids arginine-tyrosine in the transmembrane region were replaced by the glycine-threonine (mutant RY-GT) conserved in all other KIR receptors were generated. HA-2DL4 receptor by anti-HA mAb resulted in activation of lysis of P815 cells (Fig. 3B). In contrast, ligation of the RY-GT mutant did not result in activation despite higher expression of mutant RY-GT than wild-type HA-2DL4 and even though P815 lysis was induced by ligation of 2B4 in the same infected cells. The small reduction in lysis of P815 cells as compared with control Abs may be a function of the inhibitory effect mediated by the ITIM in the cytoplasmic tail. Mutation of the tyrosine in the ITIM to a phenylalanine did not diminish the ability of 2DL4 to inhibit lysis and IFN-γ secretion (Fig. 4); RY-GT mutant/control Ig = 0.56 ± 0.03 (n = 4).

2DL4 induces strong IFN-γ production by resting NK cells

IFN-γ secretion in both activated and resting NK cells was measured after coculture with P815 cells in the presence of mAbs to NK receptors. In activated NK cells, the ability of 2DL4 to induce IFN-γ was greater than that of the activation receptors CD16 and 2B4 (Fig. 4A). As seen in cytotoxicity assays (Fig. 2A), coligation of 2DL4 with CD94 resulted in inhibition of IFN-γ production (Fig. 4A). Activation of IFN-γ production by 2DL4 cross-linking was even more striking in resting NK cells (Fig. 4B). By comparison, ligation of 2B4 and CD16 induced a much smaller response. Coligation of 2DL4 with CD16 had no additional effect on IFN-γ production by resting NK cells (Fig. 4A). Therefore, in resting NK cells, 2DL4 is unique in its ability to induce efficient IFN-γ production in the absence of any discernible lytic function. Intracellular staining of IFN-γ in both activated and resting NK cells stimulated with 2DL4 revealed that expression occurred in the bulk of the NK cell population (data not shown) in contrast to the selective expression of IFN-γ by CD56bright NK cells in response to IL-12 (13).

Co-cross-linking of CD94 with 2DL4 on activated NK cells inhibited lysis and IFN-γ production (Figs. 2 and 4A). In contrast, similar co-cross-linking on resting NK cells did not inhibit IFN-γ secretion induced by 2DL4 (Fig. 4B) or lysis induced by 2B4 (Fig. 2B). Expansion of NK cells from this resting NK population yielded cells with inhibitory CD94 (data not shown). The lack of 2DL4 sensitivity to CD94-mediated inhibition in resting cells could be due to different repertoires of CD94/NKG2 on resting vs
activated NK cells. Alternatively, the ITIM-based inhibitory pathway may not function in resting NK cells. In any case, it is significant that activation through 2DL4 in resting NK cells is not inhibited by the receptor for HLA-E.

Robust IFN-γ production was obtained by incubation with the IgG1 33 or either one of the two IgM mAbs in the absence of further cross-linking (Fig. 4C). The use of the two IgM mAbs, 36 and 64, excluded a role of FcγR in the activation by 2DL4. Therefore, the signal transduced by 2DL4 alone induces IFN-γ production by resting NK cells in the absence of cytokines or signals from other receptors. In contrast, engaging the receptors CD16 and 2B4 with soluble mAbs is not sufficient, and additional accessory interactions are needed to trigger IFN-γ release (Refs. 10 and 14 and data not shown).

The IFN-γ secretion by NK cells induced by IL-2 depends on an ERK mitogen-activated protein kinase pathway (15). The ERK-dependent IFN-γ secretion was also observed upon cross-linking of CD16 or β2 integrin on activated NK cells or by mixing NK cells with the sensitive target cell line K562 (16, 17). Inhibitors of MEK1 (PD98059) and of p38 MAPK (SB203580) were added to resting NK cells during stimulation with anti-2DL4 mAbs to test whether 2DL4-induced production of IFN-γ required MAPK (Fig. 4D, top). Complete inhibition occurred at 1 μM of the p38 inhibitor SB203580. The ERK pathway inhibitor, PD98059, had only a partial inhibitory effect. In contrast, the IL-2-mediated induction of IFN-γ production was severely inhibited by 50 μM PD98059 and unaffected by SB203580 (Fig. 4D, bottom), as previously reported (15). Thus, the p38 MAPK-dependent 2DL4 signal for IFN-γ secretion is different from signals delivered to activated NK cells by CD16 and β2 integrin and to resting NK cells by the IL-2R, which are all sensitive to an ERK MAPK inhibitor. Further biochemical analysis of the 2DL4 signaling pathway has been hampered thus far by the limited number of freshly isolated resting NK cells available. Polarization of cytotoxic granules in NK cells, leading to target cell killing, is also erk dependent (18). The use of a p38 rather than an ERK pathway for activation of resting NK cells by 2DL4 may be designed to avoid cytotoxicity while maintaining the IFN-γ response. In this regard, IFN-γ induction by 2DL4 is similar to IFN-γ production by Th1 cells and to IFN-γ gene transcription in T cells stimulated by IL-12 and IL-18 that are also regulated by p38 (19, 20).

To fully understand the physiological relevance of 2DL4-mediated IFN-γ production, the ligands that recognize this receptor need to be identified. One ligand that interacts with 2DL4 is HLA-G, because 2DL4 binds to cells expressing HLA-G (8, 9, 21). HLA-G is expressed by fetal trophoblast cells that invade maternal decidua, where they encounter NK cells during early pregnancy (22). Uterine NK cells are a major source of IFN-γ in pregnant mice, in which IFN-γ has an important role in vascularization at the implantation site (23). We have observed enhanced IFN-γ production by resting NK cells in the presence of HLA-G-expressing cells (S. Rajagopalan and E. O. Long, unpublished observations). The potential role of 2DL4 in stimulating IFN-γ production during pregnancy warrants further study with decidual NK cells and trophoblast cells.

This study has identified 2DL4 as a receptor with the unique and autonomous ability to induce rapid IFN-γ secretion but not cytotoxicity by resting NK cells in the absence of cytokines. Signals that can activate resting NK cells have physiological relevance to the in vivo induction of NK responses. Furthermore, we show that resting NK cells behave differently than activated NK cells in terms of the outcomes of receptor activation. This is noteworthy because most studies on NK receptor function are conducted using IL-2-activated NK cell populations or clones. The polarized response of resting NK cells to signals received from 2DL4 vs other activation receptors is analogous to the distinct NK responses induced by different cytokines and reveals another facet in the complex regulation of these effector cells.

Acknowledgments

We thank M. Sandusky and M. Weston for technical assistance; D. Barber, Y. Bryceson, M. Faure, and C. Watzl for help with NK cell isolation; L. Lanier for Abs; W. Mahana for advice on mAb production; and S. Pierce and H. Young for comments on the manuscript.

References


