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Evidence That the Cellular Ligand for the Human NK Cell Activation Receptor NKp30 Is Not a Heparan Sulfate Glycosaminoglycan

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NKp30 (NCR3, CD337) is a natural cytotoxicity receptor, expressed on subsets of human peripheral blood NK cells, involved in NK cell killing of tumor cells and immature dendritic cells. The cellular ligand for NKp30 has remained elusive, although evidence that membrane-associated heparan sulfate (HS) proteoglycans are involved in the recognition of cellular targets by NKp30 was recently reported. The data presented in this report show conclusively that HS glycosaminoglycans (GAG) are not ligands for NKp30. We show that removing HS completely from the cell surface of human 293-EBNA cells with mammalian heparanase does not affect binding of rNKp30/human IgG1 Fc chimera complexes or binding of multimeric liposome-rNKp30 complexes. Removing HS from 293-EBNA cells, culture-generated DC, MM-170 malignant melanoma cells, or HeLa cells does not affect the NKp30-dependent killing of these cells by NK cells. We show further that the GAG-deficient hamster pgsA-745 cells that lack HS and the GAG-expressing parent CHO-K1 cells are both killed by NK cells, with killing of both cell lines inhibited to the same extent by anti-NKp30 mAb. From these results we conclude that HS GAG are not ligands for NKp30, leaving open the question as to the nature of the cellular ligand for this important NK cell activation receptor. The Journal of Immunology, 2005, 175: 207–212.

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Abbreviations used in this paper: DC, dendritic cell; NCR, natural cytotoxicity receptor; GAG, glycosaminoglycan; HS, heparan sulfate; CHO, Chinese hamster ovary.

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from American Type and Culture Collection (ATCC) CRL-1573, transformed primary human embryonic kidney fibroblast) was obtained from Dr. B. Loveland (Austin Research Institute, Heidelberg, Victoria, Australia). Other cell lines were HeLa cells obtained from S. Ford (John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory, Australia), and MM-170 malignant melanoma cells (17) obtained from Dr. R. Whitehead (Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia). Cells lines were cultured in RPMI 1640 containing 10% heat-inactivated FCS, and the CHO-K1 cells and CHO-A549 cells were grown in F12 and DMEM with 10% heat-inactivated FCS. Adherent cell cultures were released with culture flasks after washing in PBS and incubation at 37°C for 2-5 min in PBS containing 0.09 m EDTA. Polyclonal NK cells were generated from purified peripheral blood NK cells by culture with gamma-irradiated MM-170 malignant melanoma cells and rIL-2 (18). The cultured cells were entirely NK cells as they lacked cell surface CD3 and expressed CD16 and/or CD56 and/or CD94. DC were generated from plastic adherent PBMC by culture for 7 days with rGM-CSF and rIL-4 (19). The cultured cells lacked CD14, and expressed CD1c and high levels of HLA-DR, consistent with an immature DC phenotype. The procedures for obtaining peripheral blood were approved by the Human Ethics Committees of the Australian National University and the Australian Capital Territory Department of Health and Community Care.

Abs and enzymes

The anti-HS mAbs F58-10E4 (cat. no. 370255-1) and Hepss-1 (cat. no. 270426-1) were obtained from Seikagaku Kogyo. The rNKp30/human IgG1 Fc chimera (cat. no. 1849-NK-025) and the anti-NKp30 mAb (clone 2160465, 1:502a) were purchased from R&D Systems. Human IgG was purchased from the Commonwealth Serum Laboratories. PE- and FITC-conjugated mAbs were anti-NKp30 PE (cat. no. IM3709) and anti-CD94 PE (cat. no. IM2276) from Beckman Coulter; anti-HLA-DR PE (cat. no. 347367). Simultest (anti-CD3 FITC/anti-CD16 PE/anti-CD56 PE, cat. no. 340042) and anti-CD14 FITC (cat. no. 347493) from BD Biosciences; and anti-CD1c PE (anti-BDCA-1, cat. no. 130-090-508) from Miltenyi Biotec. mAb to class I HLA (DX17, IgG1) was a gift from Prof. L. Lanier (University of California, San Francisco, CA). mAb to NKG2D (clone 149804, IgG2a) was purchased from R&D Systems. Human IgG1 Fc chimera (cat. no. H-2519; Sigma-Aldrich) was used at a concentration of 3 U/ml.

Cytotoxicity assays

Cells were incubated with 31Cr by incubation with Na235CrO4 (25 μCi/0.5 × 107 cells/0.1 ml volume) for 90 min, with and without heparanase, as previously detailed. After washing, 5000 31Cr-labeled target cells were combined with NK-92 cells or polyclonal NK cells (at E:T cell ratios between 1:1 and 4:1) in a final volume of 0.1 ml of HBSS containing 10% heat-inactivated FCS, and analyzed by flow cytometry. To ensure binding was specific, the rNKp30-30 liposome complexes were incubated with an anti-NKp30 mAb (10 μg/ml) or a murine isotype control (10 μg/ml) before incubating with the cells.

Results

293-EBNA cells express a ligand for NKp30, which is not HS

In initial experiments we showed that 293-EBNA cells bind the extracellular domain of NKp30 (Fig. 1). For these experiments two different rNKp30 preparations were used, a commercially prepared rNKp30/human IgG1 Fc chimera and a 6-His rNKp30 prepared in our laboratory. Preliminary studies showed that binding of rNKp30/human IgG1 Fc chimera to cells, detected with FITC-conjugated anti-human IgG, is weak (2-fold above background). However when soluble complexes of the rNKp30/human IgG1 Fc chimera are prepared with FITC-conjugated anti-human IgG before incubation with the cells, the efficacy of binding is substantially increased, presumably due to a more multivalent structure. This binding is specific because preincubation of the soluble complexes with anti-NKp30 mAb before incubation with the cells inhibits binding by 95% (Fig. 1). In the case of the 6-His rNKp30, the protein was attached via nitrotriacyclic acid groups to fluorescent liposomes to achieve a multimeric complex (22, 23). These
liposome-rNKp30 complexes also bound specifically to 293-EBNA cells in that preincubation of the liposome-rNKp30 with anti-NKp30 mAb before incubation with cells abolished binding (Fig. 1). These experiments show that NKp30 ligands can be detected on 293-EBNA cells using multimeric complexes of NKp30.

To evaluate HS as a ligand for NKp30, 293-EBNA cells were treated with the mammalian HS degrading endoglycosidase, heparanase (20). The data in Fig. 2A show that treatment with heparanase totally removes cell surface HS detected with two anti-HS-specific mAbs, F58-10E, and HepSS-1. F58-10E reacts with an epitope present in many types of HS, which include N-sulfated glucosamine residues (24), whereas HepSS-1 recognizes a HS-specific epitope containing O-sulfated and N-acetylated glucosamine residues (25). Treatment of 293-EBNA cells with bacterial heparinase-1 only partially removed HS (data not shown), so this enzyme was not used in further studies. Having established the efficacy of mammalian heparanase treatment of 293-EBNA cells, the treated and untreated cells were compared for their ability to bind rNKp30. Data in Fig. 2B show that binding of the liposome-rNKp30 complexes and the rNKp30/human IgG1 Fc/anti-human IgG complexes to heparanase-treated 293-EBNA cells is equivalent to their binding to untreated cells. In all cases binding was inhibited by >95% by preincubation of the complexes with anti-NKp30 mAb (data not shown). These data show that NKp30 does not bind to HS on 293-EBNA cells.

To further substantiate that HS is not a ligand for NKp30, we tested the ability of NK cells to kill untreated and mammalian heparanase-treated 293-EBNA cells (Fig. 2C). These experiments

![FIGURE 1. Multimeric complexes of NKp30 detect NKp30 ligands on 293-EBNA cells. The filled histograms show binding of preformed complexes of rNKp30/human IgG1 Fc chimera and FITC-conjugated anti-human IgG (rNKp30/human IgG1 Fc/anti-human IgG), or fluorescent liposome-rNKp30 complexes, to 293-EBNA cells. Binding in the presence of an anti-NKp30 mAb (dotted line histogram) and in the presence of control IgG2a mAb (filled histogram) coincident with the histograms shown were obtained. The line histogram shows cells incubated with a control human IgG/anti-human IgG complex (left) or cells incubated with liposomes without added recombinant protein (right). FL1, Fluorescence intensity channel 1.](http://www.jimmunol.org/)

![FIGURE 2. HS is not a NKp30 ligand on 293-EBNA cells. A, 293-EBNA cells were treated with mammalian heparanase and then tested for their reactivity with the anti-HS mAbs F58-10E4 and HepSS-1, mAb binding being detected with FITC-conjugated anti-mouse Ig (filled histograms). The line histogram shows staining with the FITC-conjugated secondary reagent alone. B, Binding of NKp30 complexes to 293-EBNA cells before and after treatment with mammalian heparanase (filled histograms). Data for the rNKp30/human IgG1 Fc/anti-human IgG complexes and liposome-rNKp30 complexes are shown. The line histograms show cells incubated with a control human IgG-anti-human IgG complex or incubated with liposomes without added recombinant protein. C, Killing of 293-EBNA cells by NK-92 cells or polyclonal NK cells without a mAb (Medium), with a control anti-CD56 mAb or with an anti-NKp30 mAb, as indicated. The E:T cell ratio was 4:1. The lower limit in the assays was 1.3 (untreated cells) and 1.6% (heparanase-treated cells) determined at 2 SD above the spontaneous release of ⁵¹Cr from target cells in the absence of NK cells. FL1 and FL1-H, Fluorescence intensity channel 1.](http://www.jimmunol.org/)
used the NK-92 cell line (16) and polyclonal NK cells established from human peripheral blood (18). The NK-92 cell line and the polyclonal NK cells are both effective at killing 293-EBNA cells. The NK-92 cells express 6-fold higher levels of NKp30 compared with the polyclonal NK cells as assessed by anti-NKp30 mAb staining (data not shown). NK-92 is a convenient cell line in cytotoxicity experiments with human target cells as it lacks inhibitory receptors for MHC class I (16) and has only weak expression of the NKG2D activation receptor (H. S. Warren, unpublished observation). By contrast the polyclonal NK cells express a range of class I HLA inhibitory receptors (KIR, CD94/NKG2A, ILT2) and a fully functional NKG2D activation receptor (data not shown). For polyclonal NK cells, blocking class I HLA mAb and blocking NKG2D on NK cells with an anti-NKG2D mAb was necessary to demonstrate NKp30-dependent killing of 293-EBNA cells. The results in Fig. 2C show that NK-92 cells and polyclonal NK cells kill 293-EBNA cells and that killing is unaffected following treatment of 293-EBNA cells with mammalian heparanase. NK-92 killing of both untreated and mammalian heparanse-treated 293-EBNA cells was almost totally inhibited by anti-NKp30 mAb. In the case of polyclonal NK cells, killing was partially inhibited in the presence of anti-NKp30 mAb, with the extent of inhibition being similar for both the untreated and heparanse-treated cells. As a control in this experiment we showed that killing was not inhibited by a mAb to CD56, a receptor highly expressed by NK-92 cells. It is also of interest that both the NKp30-dependent and the residual NKp30-independent killing of 293-EBNA cells was not affected by removal of HS. Therefore ligands on 293-EBNA cells for other NK cell activation receptors on polyclonal NK cells are also not HS. In these experiments we established, by mAb staining, that HS was not regenerated on the cell surface during the 4 h incubation used for the cytotoxicity assay (data not shown). Therefore 293-EBNA cells are killed by NK cells through NKp30, and the NKp30 ligand is not HS.

Other human cells lines express a ligand for NKp30, which also is not HS

We next showed that HS is not a ligand for NKp30 on culture-generated DC, MM-170 malignant melanoma cells, and HeLa cells (Fig. 3). All cells express HS, although at different levels, and treatment with mammalian heparanase removed HS from the cell surface (Fig. 3A). The data in Fig. 3B show that killing of DC, MM-170, and HeLa cells by polyclonal NK cells is unaffected by heparanase treatment, and that killing of both untreated and heparanase-treated cells is partially inhibited by NKp30 mAb. In these experiments we verified that HS was not regenerated on the heparanase-treated cells during the 4 h cytotoxicity assay (data not shown). Therefore a number of different human cell lines in addition to 293-EBNA are killed by NK cells through NKp30, and the NKp30 ligands on these cells are also not HS.

An NKp30 ligand is present on CHO-K1 cells and the GAG-deficient pgsA-745 cells

To definitively establish that HS is not a ligand for NKp30 we tested CHO-K1 cells and the mutant GAG-deficient pgsA-745 cells derived from the CHO-K1 cell line, for their ability to bind multimeric NKp30 complexes and to be killed by NK cells. We first established that CHO-K1 cells express cell surface HS detected with the F58-10E mAb (Fig. 4A) and HepSS-1 mAb (data not shown), and that the GAG-deficient mutant lacks cell surface HS (Fig. 4A). We next showed that both CHO-K1 and pgsA-745 cells bind NKp30 complexes (Fig. 4B). In this case we were unable to measure binding of the liposome-rNKp30 complexes as these cells exhibited very high background binding of liposomes lacking attached recombinant protein. However we were able to detect weak and specific binding of rNKp30/human IgG1 Fc/human Ig complexes, provided that biotinylated anti-human IgG was used to make the preformed complexes. In this case bound complex was detected with streptavidin-PE. This detection system is more sensitive compared with complexes prepared with FITC-conjugated anti-human IgG. Both CHO-K1 and pgsA-745 cells bind the NKp30 complexes. We next compared the ability of CHO-K1 with pgsA-745 cells to be killed by NK92 cells and polyclonal NK cells. The extent of NK cell killing of CHO-K1 and pgsA-745 cells was similar and importantly, killing of both cell lines was inhibited to a similar extent by anti-NKp30 mAb and was not inhibited by the control anti-CD56 mAb.

Discussion

The conclusion from this study that HS is not a ligand for NKp30 is based on data using cells that were either replete or deficient in...
NKp30 complexes bound strongly to 293-EBNA cells irrespec-
tive of whether cell surface HS was present or absent. Similarly,
NKp30 complexes bound equally well to GAG-expressing
CHO-K1 cells and GAG-deficient pgsA-745 cells. In all cases
binding was specific in that it was inhibited by an anti-NKp30
mAb. The NKp30 complexes were generated in two ways. The
first was a complex prepared by preincubating rNKp30/human
IgG1 Fc chimera with FITC-conjugated anti-human IgG, using
commercially available reagents. The second was a fluorescent li-
posome-rNKp30 complex prepared using fluorescent liposomes
(23) and a 6-His-tagged rNKp30 prepared in our laboratory. Both
complexes gave equivalent and specific binding to 293-EBNA
cells. In the case of the CHO-K1 and the pgsA-745 cells, only
weak binding was detected and only with the rNKp30/human IgG1
Fc/anti-human IgG complexes.

NK cell killing of 293-EBNA cells, culture-generated DC, MM-
170 cells, and HeLa cells was not dependent on the presence of cell
surface HS. Similarly, killing of GAG-expressing CHO-K1 cells
was equivalent to killing of the GAG-deficient pgsA-745 cells. In
all cases killing was inhibited by an anti-NKp30 mAb, the extent
of inhibition presumably related to the presence or absence of
other activating NK cell receptor/ligand interactions involved in
cytotoxicity. Killing of 293-EBNA cells by the NK-92 cells was
almost entirely NKp30-dependent, whereas killing of the various
human cell lines by polyclonal NK cells was only partly NKp30-
dependent. The fact that the HS-replete and HS-depleted human
cell lines are killed to the same extent by polyclonal NK cells,
and that the NKp30-dependent killing was similar shows that HS is not
involved in other activating NK cell receptor/ligand interactions
involved in NK cell cytotoxicity. Interestingly, killing of CHO-K1
and pgsA-745 cells is mostly NKp30-dependent, and this was the
case using either NK-92 cells or polyclonal NK cells. These data
show that a limited number of human NK cell activation receptors
are involved in NK cell killing of these hamster cell lines. These
studies show conclusively for a range of cell lines that the ligand
for NKp30 is not HS.

In other studies (our unpublished observations) we have es-
ablished that treatment of 293-EBNA cells with neuraminidase does
not affect binding of NKp30 complexes or their ability to be killed
by NK cells. However treatment of 293-EBNA cells with trypsin
abolishes binding of NKp30 complexes. Therefore we conclude
that the ligand for NKp30 is a protein, and that ligand binding is
not dependent on sialic acid or HS moieties.

Our conclusion that HS is not a ligand for NKp30 contrasts with
that of Bloushtain et al. (15). In studies with CHO-K1 cells and the
mutant pgsA-745 cells, this group showed that binding of an
rNKp30/human IgG1 Fc-biotinylated anti-human IgG were incubated with the cells,
and binding was detected with streptavidin-PE (FL2). Binding of the com-
plexes after preincubation with control mouse IgG2a (filled histogram) and
after their preincubation with anti-NKp30 mAb (dotted line histogram) are
shown. The line histogram shows cells alone. C, Killing of CHO-K1 and
mutant pgsA-745 cells by NK-92 cells and polyclonal NK cells, without a
mAb (Medium), with a control anti-CD56 mAb, or with an anti-NKp30
mAb. The E:T cell ratio was 1:1 for NK-92 cells and 4:1 for polyclonal NK
cells. The lower limit in the assays, which was 2 SD above the spontaneous
release of $^{51}$Cr from target cells in the absence of NK cells, was 0.6 (CHO-
K1) and 1.3% (pgsA-745). FL1-H and FL2, Fluorescence intensity chan-
nels 1 and 2.
binding can be achieved using multimeric arrays of recombinant proteins, as shown with studies on the interaction of CD2 (27, 28) and CD4 (23) with their low affinity ligands. The use of complexes of recombinant proteins as in this study, or the use of trimeric isoleucine zipper-fusion proteins described recently (14), should facilitate the isolation of ligands for NK cell activation receptors.

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Disclosures

The authors have no financial conflict of interest.

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