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NKB1: A Natural Killer Cell Receptor Involved in the Recognition of Polymorphic HLA-B Molecules

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Summary

Natural killer (NK) cells kill normal and transformed hematopoietic cells that lack expression of major histocompatibility complex (MHC) class I antigens. Lysis of HLA-negative Epstein Barr virus–transformed B lymphoblastoid cell lines (B-LCL) by human NK cell clones can be inhibited by transfection of the target cells with certain HLA-A, -B, or -C alleles. NK cell clones established from an individual demonstrate clonal heterogeneity in HLA recognition and a single NK clone can recognize multiple alleles. We describe a potential human NK cell receptor (NKB1) for certain HLA-B alleles (e.g., HLA-B*5101 and-B*5801) identified by the mAb DX9. NKB1 is a 70-kD glycoprotein that is expressed on a subset of NK cells and NK cell clones. DX9 monoclonal antibody (mAb) specifically inhibits the interaction between NK cell clones and B-LCL targets transfected with certain HLA-B alleles, but does not affect recognition of HLA-A or HLA-C antigens. An individual NK cell clone can independently recognize B-LCL targets transfected with HLA-B or HLA-C antigens; however, DX9 mAb only affects interaction with transfectants expressing certain HLA-B alleles. These findings demonstrate the existence of NK cell receptors involved in the recognition of HLA-B and imply the presence of multiple receptors for MHC on an individual NK clone.

NK cells are lymphocytes distinct from T and B cells that mediate cell-mediated cytotoxicity and secrete cytokines after immune stimulation (1). The specific receptors involved in NK cell recognition and activation remain undefined; nonetheless, it is becoming increasingly clear that both triggering and inhibitory molecules dictate their lytic specificity. NK cell effector function presumably involves a positive signal that initiates cytotoxicity and cytokine production. In the case of antibody-dependent cellular cytotoxicity, the positive signal is generated by the interaction between the Ig-coated target and CD16 (FcγRIII) on the NK cell (2). The membrane receptors responsible for initiating lysis of transformed or virus-infected cells in the absence of specific Ig have not been identified.

NK cell–mediated cytotoxicity is also regulated by inhibitory signals. Kärre et al. (3, 4) initially observed that certain murine tumor variants lacking H-2 antigens were more susceptible to NK lysis than the parental tumor cells expressing high levels of H-2. Similarly, when MHC class I molecules were transfected into human HLA class I–deficient B lymphoblastoid cell lines (B-LCL),[1] the transfectants were less susceptible to NK cell lysis than the parental lines (5, 6). Analysis of an extensive panel of NK cell clones revealed that human NK cells have the ability to recognize multiple HLA-A, -B, and -C alleles (7). Two models that could account for this phenomenon have been proposed: (*a*) target interference and (*b*) effector inhibition (3, 4). The target interference model postulates that target cell MHC class I molecules mask antigens that stimulate NK lysis; the effector inhibition model proposes that target cell MHC class I molecules interact with specific receptors on NK cells, thereby transmitting a negative signal that prevents the initiation of cytolysis. Recent findings support the latter model (8).

Karlhofer et al. (8) have identified a class I MHC receptor, Ly-49, that is expressed on a subset of murine NK cells. The interaction between Ly-49 on the NK cell and H-2D^d molecules on target cells prevents NK cell–mediated cytotoxicity. Ly-49 is a type II membrane glycoprotein of the C-type lectin superfamily. *Ly-49* and a family of at least five related genes are on mouse chromosome 6 (9–11). One member of the Ly-49 family, SW5E6, (12) appears to be involved in mediating the rejection of allogeneic bone marrow cells (13). SW5E6 and Ly-49 are expressed as disulfide-linked homodimers; as yet, there is no evidence for heterodimers between members of the Ly-49 family. Human NK cell receptors for HLA have

[1] *Abbreviation used in this paper:* B-LCL, B lymphoblastoid cell lines.

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yet to be characterized fully, although Colonna et al. (14) and Ciccone et al. (15) describe two antigens expressed on NK subsets that appear to correlate with recognition of HLA-C. Our studies, however, have demonstrated that NK clones recognize not only HLA-C, but also HLA-A and HLA-B (7), suggesting the existence of additional receptors. In the present study, we describe an NK cell receptor involved in the recognition of HLA-B.

Materials and Methods

NK Clones. Peripheral blood from healthy individuals was purchased from the Stanford Blood Bank (Stanford, CA). NK cell clones ($CD3^-$, $CD56^+$) were established using the culture conditions described by Yssel et al. (16).

mAbs and Flow Cytometry. mAbs were provided generously by Becton Dickinson Immunocytometry Systems (Mountain View, CA). Cy-chrome™ conjugated anti-CD3 was purchased from PharMingen (San Diego, CA). The DX9 (IgG1) hybridoma was generated by immunizing BALB/c mice with the human NK clone VL186-1 ($CD3^-$, $CD16^+$, $CD56^+$) and fusing their splenocytes with the Sp2/0 myeloma cell line. Fab and $F(ab')_2$ fragments were prepared by digesting DX9 IgG with immobilized papain (5 mg IgG in 5 ml of 20 mM NaH_2PO_4, 20 mM cysteine-HCl buffer, pH 7.0, with 1 ml immobilized papain for 7 h at 37°C) or immobilized pepsin (10 mg IgG in 10 ml of 0.2 M sodium citrate, 0.15 M NaCl buffer, pH 3.5, with 2.5 ml immobilized pepsin for 2 h at 37°) (Pierce, Rockford, IL), respectively, and afterwards removing residual intact mAb by protein A affinity chromatography. Purity of the $F(ab')_2$ and Fab fragments was determined by SDS-PAGE. Methods of immunofluorescent staining and flow cytometry have been described previously (17).

Biochemistry. Cells were labeled with ^{125}I using lactoperoxidase/glucose oxidase or with [^{32}P]orthophosphate (Amersham Corp., Arlington Heights, IL) (18). Cells were lysed in Tris-buffered saline (50 mM Tris, 15 mM NaCl, pH 8.0) containing 1% NP-40 and protease inhibitors (1 mM PMSF and 20 Kallikrein inhibitor U/ml aprotinin) and phosphatase inhibitors (1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 0.1 mM β-glycerophosphate, 1 mM Na_3VO_4) or 20 mM triethanolamine/150 mM NaCl buffer (pH 7.8) containing 1% digitonin (CalBiochem-Novabiochem Corp., La Jolla, CA) and 0.12% Triton X-100 (Sigma Chemical Co., St. Louis, MO) with protease inhibitors. NKB1 antigen was immunoprecipitated using the method described previously (18). NKB1 glycoprotein was treated with neuraminidase (Sigma Chemical Co.), O-glycanase (Genzyme, Boston, MA), and *N*-glycosidase F (Boehringer Mannheim Corp., Indianapolis, IN) using the conditions recommended by the manufacturers. Samples were analyzed by SDS-PAGE.

Cytotoxicity Assays. NK cell-mediated cytotoxicity was measured using a 4-h ^{51}Cr radioisotope release assay (2). Assays were performed using an effector/target ratio of 6:1 in the presence and absence of DX9 mAb (5 μg/ml). C1R B-LCL (19, 20) (HLA-A^-, -B^*3503^{LOW}, -Cw^*0401^+) and C1R transfectants expressing HLA-A*0201, -A*0301, -A*6801, -B*3701, and -B*5801 were provided generously by Dr. Peter Cresswell (Yale University, New Haven, CT) and Dr. Jeffrey Dawson (Duke University, Durham, NC). 721.221 (HLA-A^-, -B^-, -C^-) and 721.221.B*5101 and 721.221.Cw*0301 transfectants were provided generously by Dr. Robert DeMars (University of Wisconsin, Madison, WI) (21). 721.221 transfectants expressing HLA-A*0201, -A*0211, -A*3601, -B*3505, -B*4801, -B*5401, -B*5501, -B*5801, -Cw*0102, -Cw*0304, -Cw*0401, -Cw*0801, and -Cw*1503 were generated as described previously (7).

Results and Discussion

Generation and Characterization of DX9 mAb. Whereas many NK cell clones and polyclonal NK populations kill the MHC class I–deficient B-LCL C1R and 721.221, transfection of these cell lines with certain alleles of HLA-A, -B, or -C results in protection from NK cell lysis (5, 6, 7). In particular, the products of certain HLA-B alleles (e.g., HLA-B*5101, -B*5801) frequently protected against lysis by NK clones (7). To identify NK cell receptors for HLA, mAbs were generated against an NK clone (VL186-1) that was strongly inhibited by the expression of HLA-B*5801 on the surface of the C1R B-LCL target (7). mAbs were therefore selected for their capacity to permit lysis of the HLA-B*5801 C1R transfectant and in this manner DX9 mAb was isolated. Whereas NK clone VL186-1 failed to kill HLA-B*5801–transfected C1R (0% cytotoxicity), in the presence of mAb DX9 64% lysis of HLA-B*5801–transfected C1R was observed. The DX9 mAb reacted with a molecule on the surface of NK clone VL186-1, but failed to stain the HLA-B*5801 transfected C1R, indicating that the effect was due to interaction with the NK effector cell and not the target.

The antigen recognized by DX9 mAb, designated NKB1, was found to be expressed on the surface of a subset of

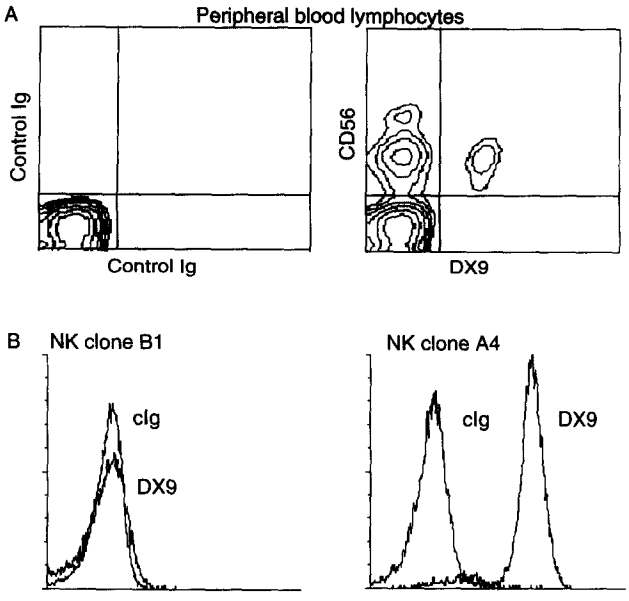

Figure 1. Expression of NKB1 on adult peripheral blood NK cells and NK cell clones. (*A*) Peripheral blood mononuclear cells were stained with FITC-conjugated anti-CD56 and PE-conjugated DX9 mAb or fluorochrome-conjugated control Ig. Samples were analyzed by flow cytometry. Lymphocytes were identified by their characteristic forward and side angle light scatter properties. Data are displayed as contour plots (4 decade log scales). (*B*) $NKB1^+$ and $NKB1^-$ NK cell clones were established by single cell cloning using flow cytometry. Representative $NKB1^-$ and $NKB1^+$ NK clones were stained with PE-conjugated DX9 mAb or PE-conjugated control Ig (cIg). Histograms of DX9 mAb-stained cells are superimposed over histograms of Ig control-stained cells (nearest the ordinate).

$CD3^-$, $CD56^+$ NK cells isolated from peripheral blood (Fig. 1), but was not detected on the surface of granulocytes, monocytes, thymocytes, or B lymphocytes (not shown). Analysis of peripheral blood from 18 normal adult donors indicated that NKB1 is present from <0.1 to 61% (mean = 14%) of $CD3^-$, $CD56^+$ NK cells. The significance of this considerable heterogeneity is unknown. NKB1 was observed infrequently on adult peripheral blood $CD3^+$ lymphocytes, although a minor subset of $NKB1^+$ T cells (<0.1–3%) could be detected in certain individuals (not shown). A panel of $NKB1^-$ and $NKB1^+$ NK clones (all of these were $CD3^-$, $CD56^+$ cells) were established by single cell cloning using flow cytometry for further analysis. In all cases, the NKB1 phenotype of the clones remained stable (representative clones are shown in Fig. 1). We have never observed acquisition of NKB1 by NK clones initially isolated as being $NKB1^-$ or the loss of NKB1 from $NKB1^+$ NK clones.

HLA Specificity of NKB1. NK clones were established from two donors on the basis of NKB1 expression ($CD3^-$, $CD56^+$, $NKB1^+$ and $CD3^-$, $CD56^+$, $NKB1^-$). Of the 29 $NKB1^+$ NK clones examined, all lysed HLA-deficient EBV transformed B-LCL C1R and demonstrated diminished lytic activity against HLA-B*5801 C1R transfectants. Cytolysis by $NKB1^+$ NK clones against the HLA-B*5801 transfectant was augmented in the presence of DX9 mAb (Fig. 2 and Table 1). By contrast, killing of the untransfected HLA-deficient EBV transformed B-LCL cell lines 721.221 and C1R by $NKB1^+$ NK clones was neither augmented nor inhibited by DX9 mAb (Fig. 2). As shown in Fig. 3, $F(ab')_2$ and Fab fragments of the DX9 mAb, like the intact antibody, were able to permit lysis of the HLA-B*5801–transfected 721.221 target cell.

Examination of a panel of transfectants expressing different class I HLA genes indicated that DX9 mAb permitted cyto-

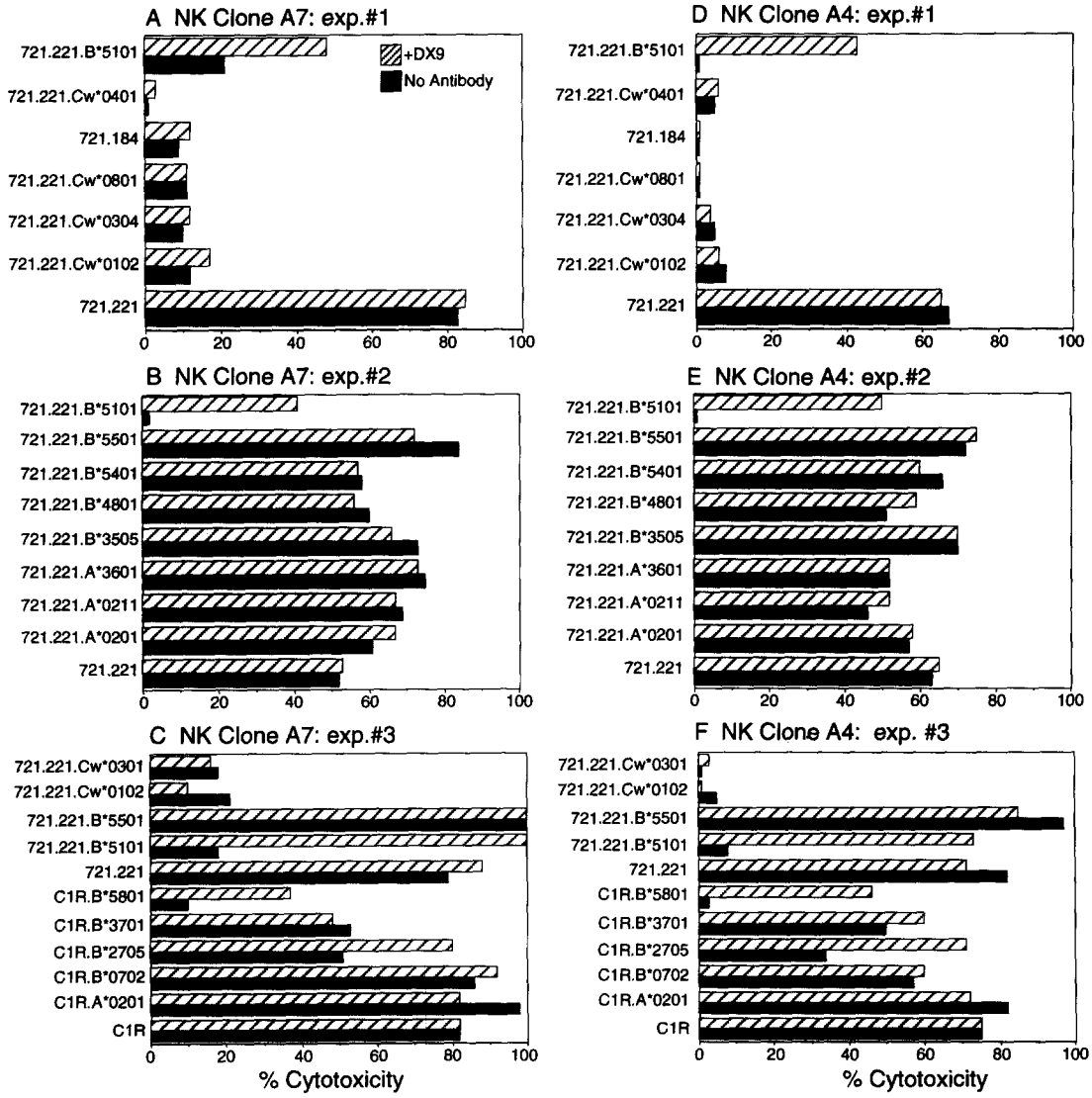

Figure 2. The effect of DX9 mAb on the ability of NK clones to lyse HLA-deficient or HLA-transfected B-LCL target cells. Two representative $NKB1^+$ NK clones A4 and 47 were assayed for cytotoxicity against 721.221, C1R, or the indicated HLA transfectants in the presence (*striped bars*) or absence (*solid bars*) of DX9 mAb (5 μg/ml). The E/T ratio was 6:1.

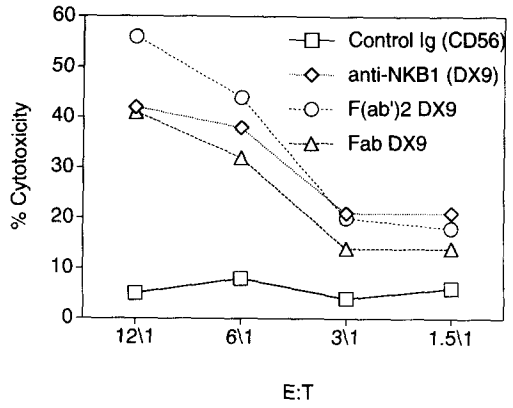

Figure 3. Effect of $F(ab')_2$ and Fab fragments of DX9 mAb on the ability of NKB1$^+$ NK clone A13 to lyse HLA-B*5801–transfected 721.221 target cells. NKB1$^+$ NK clone A13 was assayed for cytotoxicity against HLA-B*5801–transfected 721.221 target cells in the presence of intact DX9 mAb (*diamonds*), DX9 $F(ab')_2$ fragments (*circles*), and DX9 Fab fragments (*triangles*) as indicated. Anti-CD56 (Leu 19) mAb (*squares*) was used as the control Ig. Leu 19 is a murine IgG1 mAb that binds to all NK cells, but does not affect NK cell function. All mAb were used at 5 μg/ml and were added to the NK cells immediately before addition of the target cells.

toxicity against certain HLA-B alleles, in addition to HLA-B*5801, but did not affect cytotoxicity against any of the HLA-C transfectants examined. Representative data obtained from experiments with two NKB1$^+$ clones are shown in Fig. 2 and the results obtained from several NKB1$^-$ and NKB1$^+$ NK clones are summarized in Table 1. Whereas NKB1$^+$ NK clones recognized and therefore failed to lyse transfectants expressing certain HLA-C alleles, including -Cw*0102, Cw*0301, -Cw*0304, -Cw*0401, -Cw*0801, and -Cw*1503, the presence of DX9 mAb had no effect on the target protection conferred by these molecules. This observation demonstrates that binding DX9 mAb to the NK clones need not interfere with the recognition of all HLA molecules, but only that of specific alleles.

Individual NK clones have the ability to recognize multiple alleles of HLA-B (7). Therefore, we examined the effect of DX9 mAb on NK cell–mediated lysis of B-LCL transfectants. The DX9 mAb and its $F(ab')_2$ and Fab fragments did not affect NK cell-mediated lysis of the HLA-negative 721.221 EBV-LCL target cells (Fig. 2 and not shown).

Table 1. *Lysis of HLA Transfected Target Cells in the Presence and Absence of DX9 mAb*

	C1R transfectants				721.221 transfectants							
	B*3701		B*5801		B*5101		Cw*0301		Cw*0304		Cw*0401	
NK clone	No mAb	DX9	No mAb	DX9	No mAb	DX9	No mAb	DX9	No mAb	DX9	No mAb	DX9
					DX9$^+$ NK clones							
A4	+*	+	−‡	+	−	+	−	−	−	−	−	−
A6	+	+	−	+	−	+	+	+	nd	nd	nd§	nd
A7	+	+	−	+	−	+	−	−	−	−	−	−
A9	−	−	−	+	−	+	+	+	+	+	−	−
A21	+	+	−	+	−	+	+	+	+	+	−	−
A23	+	+	−	+	−	+	−	−	−	−	+	+
A26	+	+	−	+	−	+	−	−	−	−	−	−
A36	−	−	−	+	−	+	−	−	nd	nd	nd	nd
					DX9$^-$ NK clones							
B1	+	+	−	−	+	+	−	−	−	−	−	−
B2	−	−	−	−	+	+	+	+	+	+	−	−
B5	+	+	−	−	−	−	−	−	−	−	−	−
B6	+	+	−	−	+	+	+	+	nd	nd	nd	nd
B12	+	+	−	−	+	+	+	+	+	+	−	−
B19	+	+	−	−	+	+	+	+	nd	nd	nd	nd

* + Indicates that the percent specific lysis of the HLA-transfected target cell was comparable with that of the parental cell line or substantially increased in the presence of DX9 mAb. In all cases the lysis of the parental cell lines was 30% or greater. The data are a composite of several experiments.
‡ − Indicates that the percent specific lysis of the HLA-transfected target cell was 50% or less than that of the parental cell line, i.e., the NK clone recognizes that particular HLA allele.
§ nd, not determined.

fectants expressing B*0702, -B*2705, -B*3505, -B*3701, -B*4801, -B*5101, -B*5401, or -B*5501. We observed that DX9 mAb reversed the protection conferred by HLA-B*5101 consistently, in addition to HLA-B*5801 (Fig. 2 and Table 1). The DX9 mAb also affected recognition of HLA-B*2705. However, the protection conferred by -B*2705 was less than with -B*5101 or -B*5801 (Fig. 2). Consistent with previous observations (7), NK clones recognizing -B*0702, -B*3505, -B*3701, -B*4801, -B*5401, or -B*5501 and the HLA-A alleles (-A*0201, -A*0211, -A*0301, -A*3601, -A*6801) were less frequent than NK clones recognizing -B*5801; however, preliminary studies indicate that DX9 mAb has little effect upon the lysis of the latter transfectants (not shown). Further analysis of NK clones that recognize these less frequently protective HLA-A and HLA-B alleles is required to exclude reactivity with these molecules.

Certain NKB1$^-$ NK cell clones recognized HLA-B*5801 and -B*5101, although as expected DX9 mAb failed to affect lysis of these transfectants. Therefore, whereas the results are compatible with the possibility that NKB1 is a receptor for HLA-B*5801 and -B*5101, they also suggest that additional receptors with this specificity are also present on NKB1$^-$ NK clones. NKB1$^-$ NK clones may express a variant of the NKB1 molecule not recognized by the DX9 mAb or a distinct receptor that also recognizes HLA-B*5801 and -B*5101. There was no apparent bias in the recognition of HLA-A or HLA-C alleles by clones derived from either the NKB1$^-$ or NKB1$^+$ NK subsets, suggesting the existence of independent receptors for HLA-A and HLA-C.

Structure of NKB1 Antigen. The DX9 mAb immunoprecipitates a 70-kD glycoprotein from ^{125}I-labeled NKB1$^+$ NK cell clones that on SDS-PAGE gels migrates as a single species under both reducing and nonreducing conditions (Fig. 4). Identical results were obtained using 1% NP-40 or 1% digitonin detergent for cell disruption. Treatment with neuraminidase slightly decreased the mobility of NKB1, indicating that the protein is sialated. Digestion of the NKB1 protein with *N*-glycanase reduced the apparent molecule weight to ~50 kD, demonstrating the presence of complex *N*-linked oligosaccharides. In contrast, endo-H and *O*-glycanase had no effect upon the mobility of the NKB1 protein. Immunoprecipitation from NK cell clones metabolically labeled with [^{32}P]orthophosphate indicated that NKB1 is phosphorylated. Stimulation with PMA neither increased nor decreased phosphorylation of NKB1.

Conclusions. Analysis of the specificity of NK clones against a panel of target cells transfected with class I MHC molecules argues strongly that distinct receptors for individual HLA alleles or groups of HLA alleles are expressed by human NK cells. Our results support the possibility that NKB1 represents one such receptor that is specific for certain HLA-B alleles, although formal proof requires evidence for the direct interaction of NKB1 and HLA-B molecules. Several observations suggest that NK cell recognition of HLA is complex. An individual NK clone can recognize HLA-A, -B, and -C molecules. Whereas NKB1$^+$ NK clones all recognize HLA-B*5801, these clones demonstrate heterogeneous recognition of different HLA-C molecules, implying the existence of multiple NK receptors. Some NKB1$^-$ NK clones were also functionally inhibited by HLA-B*5801, indicating the presence of another HLA-B*5801 receptor on the NKB1$^-$ subset. Moreover, NKB1 appears to recognize HLA-B*5801, -B*5101, and possibly -B*2705.

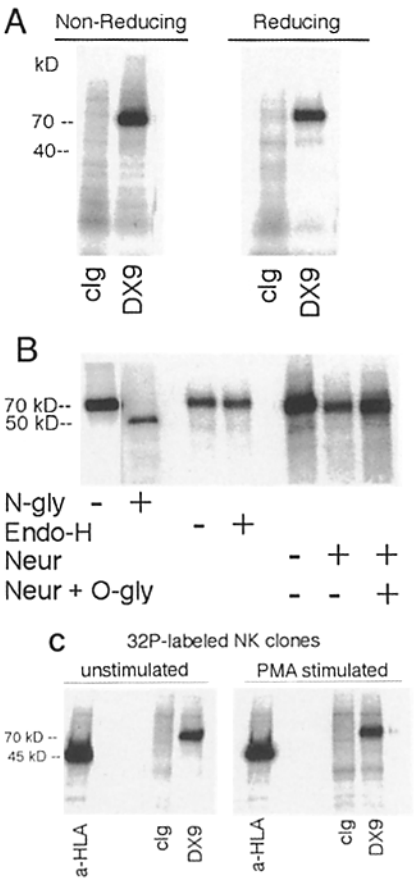

Figure 4. Structure of NKB1. (*A*) NKB1$^+$ NK clones were ^{125}I-labeled and lysed in 1% NP-40. Lysates were immunoprecipitated with control Ig or DX9 mAb and samples analyzed by SDS-PAGE in the presence or absence of 2-mercaptoethanol. (*B*) ^{125}I NKB1 antigen was treated with neuraminidase, *O*-glycanase, endo H, or *N*-glycanase, as indicated, and analyzed by SDS-PAGE in the presence of 2-mercaptoethanol. (*C*) NKB1$^+$ NK clones were metabolically labeled with [^{32}P]orthophosphate and were then stimulated or not with 1 ng/ml PMA for 5 min. Cells were lysed in 1% NP-40, lysates were immunoprecipitated with control Ig, anti-HLA-A, -B, -C mAbs (positive control) or DX9 mAb and samples analyzed by SDS-PAGE in the presence of 2-mercaptoethanol.

It should be noted that NKB1$^+$ NK clones shown in Fig. 2 were derived from an HLA-B*5801$^+$ donor. Thus, both the NKB1 receptor and its putative ligand HLA-B*5801 can be expressed by the same NK clone. Moreover, NKB1$^+$ NK clones from an HLA-B*5801$^+$ donor efficiently lysed the HLA-deficient B-LCL C1R, whereas no lysis of C1R.B*5801 was observed. These results indicate that if a "negative signal" is induced it is directional and target specific (i.e., the potential interaction of NKB1 with HLA-B on either the same or an adjacent NK clone does not prevent the lysis of a sus-

ceptible target lacking HLA class I molecules). Therefore, the putative inhibitory signal sent through the NKB1 receptor does not irreversibly inactivate the NK cell. Recent studies using NK cell lines reactive with HLA-C also support the concept that bystander cells expressing inhibitory HLA alleles are unable to affect the interaction between an NK cell and a susceptible target (22).

Further heterogeneity is introduced by the expression of NKB1 on only a subset of NK cells and the variable size of this subset in different individuals. A similar situation exists with expression of Ly-49 and 5E6, which are expressed only on a subset of NK cells in certain mouse strains (9, 13). Similarly, the EB6 and GL183 antigens implicated in recognition of HLA-C are expressed only on partially overlapping subsets of human NK cells (23).

Previous studies by Colonna et al. (14) have demonstrated a correlation between expression of EB6 and GL183 and target cell protection by HLA-C. From family studies and analysis of HLA-C transfectants, two NK target specificities (designated group 1 and group 2) corresponding to a dimorphic sequence motif at amino acids 77 and 80 of HLA-C alleles have been defined (14, 22, 24). The NKB1 molecule is distinct both in class I HLA specificity and structure from EB6 and GL183. Certain NKB1$^+$ NK clones have the ability to recognize HLA-C alleles of the group 1 or 2 specificities. EB6/GL183 and NKB1 are all structurally different from the murine Ly-49 receptors, which are disulfide-linked homodimers (9). Furthermore, the 70-kD NKB1 glycoprotein appears larger on SDS-PAGE than the EB6 and GL183 glycoproteins, which migrate at 58 kD. Further understanding of the relationship between these various NK receptors will come from comparison of the sequences of the cloned genes. Nonetheless, the results are compatible with the existence of distinct NK receptors for HLA-B and HLA-C on a single NK clone that function independently.

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