Heterogeneous Phenotypes of Expression of the NKB1 Natural Killer Cell Class I Receptor among Individuals of Different Human Histocompatibility Leukocyte Antigens Types Appear Genetically Regulated, but Not Linked to Major Histocompatibility Complex Haplotype

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Summary

Natural killer (NK) cells that express the NKB1 receptor are inhibited from killing target cells that possess human histocompatibility leukocyte antigen (HLA) B molecules bearing the Bw4 serological epitope. To investigate whether NKB1 expression is affected by HLA type, peripheral blood lymphocytes of 203 HLA-typed donors were examined. Most donors had a single population of $NKB1^+$ cells, but some had two populations expressing different cell surface levels of NKB1, and others had no detectable $NKB1^+$ cells. Among the donors expressing NKB1, both the relative abundance of $NKB1^+$ NK cells and their level of cell surface expression varied substantially. The percentage of $NKB1^+$ NK cells ranged from 0 to >75% (mean 14.7%), and the mean fluorescence of the positive population varied over three orders of magnitude. For each donor, the small percentage of T cells expressing NKB1 (usually <2%), had a pattern of expression mirroring that of the NK cells. NKB1 expression by NK and T cells remained stable over the 2-yr period that five donors were tested. Patterns of NKB1 expression were not associated with Bw4 or Bw6 serotype of the donor or with the presence of any individual HLA-A or -B antigens. Cells expressing NKB1 are often found in donors who do not possess an appropriate class I ligand, and can be absent in those who express $Bw4^+$ HLA-B antigens. Family studies further suggested that the phenotype of NKB1 expression is inherited but not HLA linked. Whereas identical twins show matching patterns of NKB1 expression, HLA-identical siblings can differ in NKB1 expression, and conversely, HLA-disparate siblings can be similar. Thus NKB1 expression phenotypes are tightly regulated and extremely heterogenous, but not correlated with HLA type.

NK cells kill a variety of neoplastic and virally infected target cells without prior sensitization (1). Unlike most CTL, lysis of target cells by NK cells does not require presentation of an antigen by class I or II MHC molecules. Instead, target cell lysis is controlled by a number of novel receptors that either stimulate or inhibit cytolytic function (2–5). Cellular ligands recognized by the stimulatory receptors are as yet unidentified. It is clear, however, that the inhibitory receptors recognize polymorphic determinants of MHC class I molecules (6). Thus NK cells kill target cells that lack class I antigens, but expression of certain class I allotypes can protect a target cell from lysis (7, 8). Kärre et al. (9) have postulated that the inhibitory effect of class I molecules on NK cells allows them to complement the role of CTL in immune surveillance, by detecting and eliminating cells with reduced expression of class I due to viral infection or malignant transformation.

Nine different cDNAs encoding human NK cell class I receptors have been identified (3–5). One of these receptors, NKB1, is recognized by the DX9 mAb (10). NK cells that express NKB1 demonstrate reduced lysis of target cells that express HLA-B antigens having the Bw4 serological epitope (11). HLA-B allotypes possess either Bw4 or its alternative, the Bw6 epitope (12). Certain HLA-A allotypes also possess the Bw4 epitope. Thus, each individual is either homozygous or heterozygous with regard to HLA-B

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Bw4 or Bw6 epitopes, and some persons also express one or two $Bw4^+$ HLA-A antigens.

Preliminary analysis showed that the percentage of NK cells that express NKB1 varied among the individuals tested (10). Heterogeneity in expression of murine NK cell class I receptors has been observed among inbred mouse strains of different MHC types (13). Additionally, the expression level of one murine receptor, Ly-49A, was influenced by the presence of a class I MHC antigen that acts as a ligand (14). Thus, in mice it appears that MHC type may influence the types and/or levels of class I receptors expressed by NK cells. The present study was undertaken to investigate the heterogeneity in NKB1 expression among different individuals and its relationship to HLA type.

Materials and Methods

Flow Cytometry and mAbs. The anti-NKB1 mAb, DX9 (10), was conjugated to phycoerythrin. Anti-CD3 (Leu 4) was conjugated to Cy-Chrome (PharMingen, San Diego, CA). Anti-CD56 (L185) was provided generously by Becton Dickinson Immunocytometry Systems (San Jose, CA), and was conjugated to FITC as described (15). For flow cytometric analysis, mononuclear cells from the peripheral blood of healthy donors were isolated by density centrifugation with Ficoll-Hypaque (Pharmacia/LKB, Piscataway, NJ). PBMC were divided into two aliquots and stained with the three antibody conjugates described above, or isotype-matched control antibody conjugates, as described (15). Analysis was performed on a FACScan® flow cytometer (Becton Dickinson). Forward and side light scatter gates were set to identify the lymphocyte population. NKB1 expression, as measured by DX9 mAb staining, was then analyzed separately for NK ($CD56^+$, $CD3^-$) and T cell ($CD3^+$) populations.

Preparation of NK Cell Clones. PBMC from a single healthy adult who possessed a bimodal population of $NKB1^+$ cells were isolated and stained as described above. NK cells ($CD56^+$, $CD3^-$) that expressed either low levels of NKB1, high levels of NKB1, or did not express NKB1, were cloned at one cell per well using a single cell deposition system of the FACStar$^{PLUS®}$ flow cytometer (Becton Dickinson). Clones were established by using culture conditions described previously (16). All clones generated by this procedure had the cell surface phenotype $CD3^-$, $CD56^+$, and mediated NK cytolytic function.

Preparation of $F(ab')_2$ and Fab Fragments of DX9 mAb, and NKB1 Binding Experiments. $F(ab')_2$ and Fab fragments were prepared by digesting DX9 IgG with immobilized pepsin or papain, respectively (10 mg/ml 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°C) (Pierce Chemical Co., Rockford, IL). Fc-containing species were then removed by protein A affinity chromatography. $F(ab')_2$ and Fab fragment preparations were determined to be pure by SDS-PAGE analysis. To compare the affinity of DX9 for the molecules expressed by NKB1 bright and dim clones, 10^6 cells of four NKB1 bright and four NKB1 dim NK cell clones were incubated on ice for 45 min with 10 nmol DX9 $F(ab')_2$ and Fab fragments, in a volume of 100 μl. The samples were then washed with 3 ml PBS containing 0.01% NaN_3. The cells were stained with $F(ab')_2$ of goat Ig, specific for the $F(ab')_2$ fragment of mouse Ig, conjugated to fluorescein (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), washed with 3 ml PBS/NaN_3 containing propidium iodide, and resuspended in 1 ml PBS/NaN_3. Samples were incubated on ice until analysis using a FACScan® flow cytometer. Thereafter, the cells were incubated at room temperature, and reanalyzed at the specified time intervals after initial analysis. For titration of DX9 mAb binding to NKB1 bright and dim clones, 10^6 cells were incubated with the following quantities of DX9 $F(ab')_2$: 500, 250, 125, 62.5, 31.3, 15.6, and 0 ng in 100 μl PBS/NaN_3. Staining was carried out as described above, and the samples were analyzed immediately by flow cytometry.

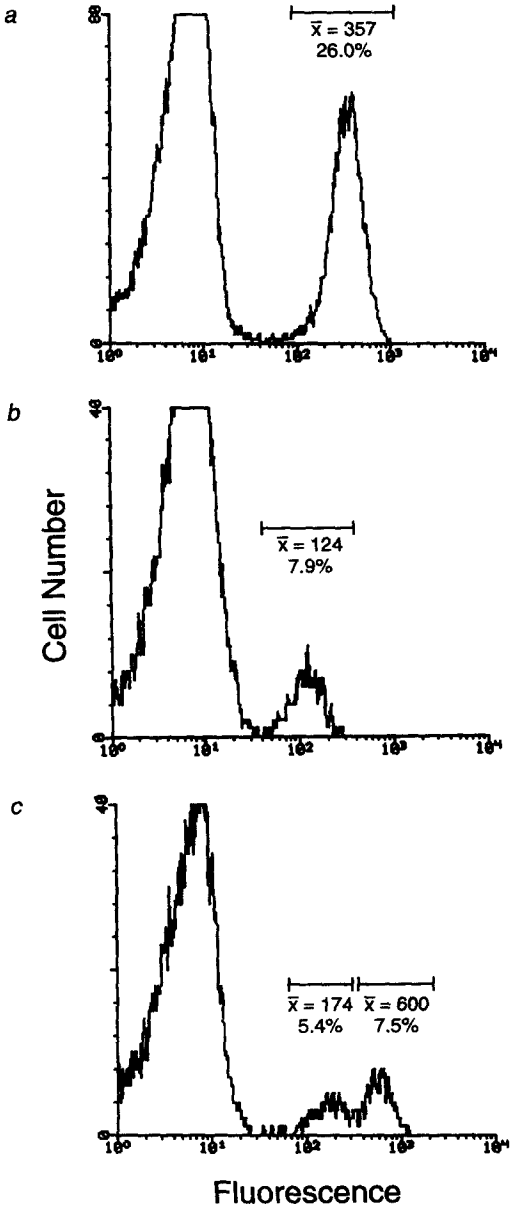


Figure 1. FACS® histograms showing DX9 antibody binding to NK cells of three peripheral blood donors. The lymphocyte population was isolated by setting forward and sidelight scatter gates, then gates were set to display DX9 staining of NK cells ($CD3^-$, $CD56^+$). Peaks under the bars represent the populations of cells that stain positively for NKB1. Unmarked peaks (left) are NK cells that do not stain for NKB1. The mean fluorescence intensity of the positive populations is given in arbitrary fluorescence units. Percentage values indicate the proportion of the total NK cell population contained within the $NKB1^+$ population.

Immunoprecipitation and SDS-PAGE Analysis. Two NKB1 bright and two NKB1 dim NK cell clones were expanded to 40–100 × 10^6 cells, and labeled with ^{125}I using lactoperoxidase/glucose oxidase. 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 U/ml aprotinin). NKB1 antigen was immunoprecipitated as described previously (10). Samples were analyzed by SDS-PAGE on a 10% gel.

Cell-mediated Cytotoxicity. Cell killing was measured by using a standard 4-h ^{51}Cr radioisotope-release assay (17). Percent lysis was calculated by the formula: [(Experimental cpm − spontaneous cpm)/(Total cpm − spontaneous cpm)] × 100 = % lysis. The HLA class I–negative lymphoblastoid cell line 721.221 (18) and transfectants of this line expressing individual class I allotypes, were used as target cells. Transfectants were generated as described (11). The DX9 mAb was added where specified to a final concentration of 6.7 μg/ml. Assays were performed in duplicate or triplicate, and were repeated two to three times for each clone.

Serological Class I HLA Typing. Serologic typing for all HLA class I antigens was performed using a complement-dependent, fluorochromasia, microcytotoxicity technique. Frozen PBL were used directly after relayering over a Ficoll-Hypaque gradient and staining with carboxy-fluorescein diacetate (C-FDA; Molecular Probes Inc., Eugene, OR) yielding original viabilities of 99%. Sera and cells were incubated at 22°C for 30 min; rabbit complement was then added and the trays were incubated an additional 90 min. 288 sera obtained by international exchange and covering all specificities with the exception of A43 (an antigen found only in South African Blacks) were used for each typing.

Results

Heterogeneity in NKB1 Expression. To assess NKB1 expression by PBL, we used flow cytometric analysis. Anti-CD3 and anti-CD56 antibodies were used to differentiate the three types of lymphocytes (T, NK, and B cells). Thus, T cells stained positively for CD3, NK cells were $CD3^-$ and $CD56^+$, and B cells were double negative. The DX9 mAb was used to investigate NKB1 expression by each type of lymphocyte. Only NK and T cell populations stained specifically for NKB1. Three patterns of NKB1 expression were observed. Some donors had a unimodal distribution of $NKB1^+$ cells (Fig. 1, *a* and *b*), others had a bimodal distribution consisting of two distinct populations differing in fluorescence intensity (Fig. 1 *c*), and the rest had no detectable $NKB1^+$ cells (data not shown).

To investigate the stability of NKB1 expression by PBL, we analyzed frozen and fresh PBMC samples drawn from five donors over a 2-yr period (Table 1). For each donor, the percentage of NK cells that expressed NKB1 and their mean fluorescence intensity remained remarkably constant. Donors who possessed either a unimodal or bimodal distribution of $NKB1^+$ cells maintained the phenotype. To examine further the stability of NKB1 expression, NK cells were cloned from the bright, dim, and NKB1-negative cell populations of the bimodal donor (Fig. 1 *c*). NK cells that did not express NKB1 at the time of sorting remained negative in culture, and those with dim or bright NKB1 staining maintained these phenotypes when assayed repeatedly during 2 mo of growth in culture (data not shown).

The differences in NKB1 staining intensity among individuals could be due to variations in the level of expression of NKB1 at the cell surface, or to expression of isoforms of NKB1 with different affinities for the DX9 mAb. To address this question, the rates of dissociation of DX9 $F(ab')_2$ and Fab fragments bound to cells of the dim and bright

Table 1. *Stability of NK and T Cell NKB1 Expression*

Donor	Sample	Date	Percent $NKB1^+$ NK cells	MFI	Percent $NKB1^+$ T cells	MFI
1	Frozen PBMC	19/2/93	51.1	756	13.1	475
1	Fresh PBMC	5/1/95	47.5	781	8.0	483
2	Frozen PBMC	19/2/93	16.5	325	2.3	233
2	Fresh PBMC	5/1/95	17.7	342	0.8	267
3	Frozen PBMC	9/9/94	7.9	118	0.4	109
3	Frozen PBMC	16/9/94	7.8	124	0.2	88
3	Frozen PBMC	29/9/94	7.2	119	0.3	127
3	Frozen PBMC	30/9/94	7.3	125	0.3	105
4	Frozen PBMC	19/2/93	5.0, 6.0	159, 538	2.9	136, 402
4	Fresh PBMC	5/1/95	5.4, 7.5	174, 600	1.4	164, 503
5	Frozen PBMC	19/2/93	23.1	335	1.1	272
5	Frozen PBMC	23/6/94	26.0	357	0.8	293
5	Frozen PBMC	7/7/94	23.9	352	0.5	297

Results of flow cytometric analysis of NKB1 expression by lymphocytes from peripheral blood samples collected over a 2-yr period. Percent $NKB1^+$ NK and $NKB1^+$ T cells represent the percentage of the total NK or T cell population that stained positively for NKB1. Mean fluorescence staining intensity (MFI) of the positive population is given in arbitrary fluorescence units. Two percentage and fluorescence values are shown for donors with a bimodal distribution of $NKB1^+$ cells, representing the dim and bright populations. All samples were analyzed in the same experiment.

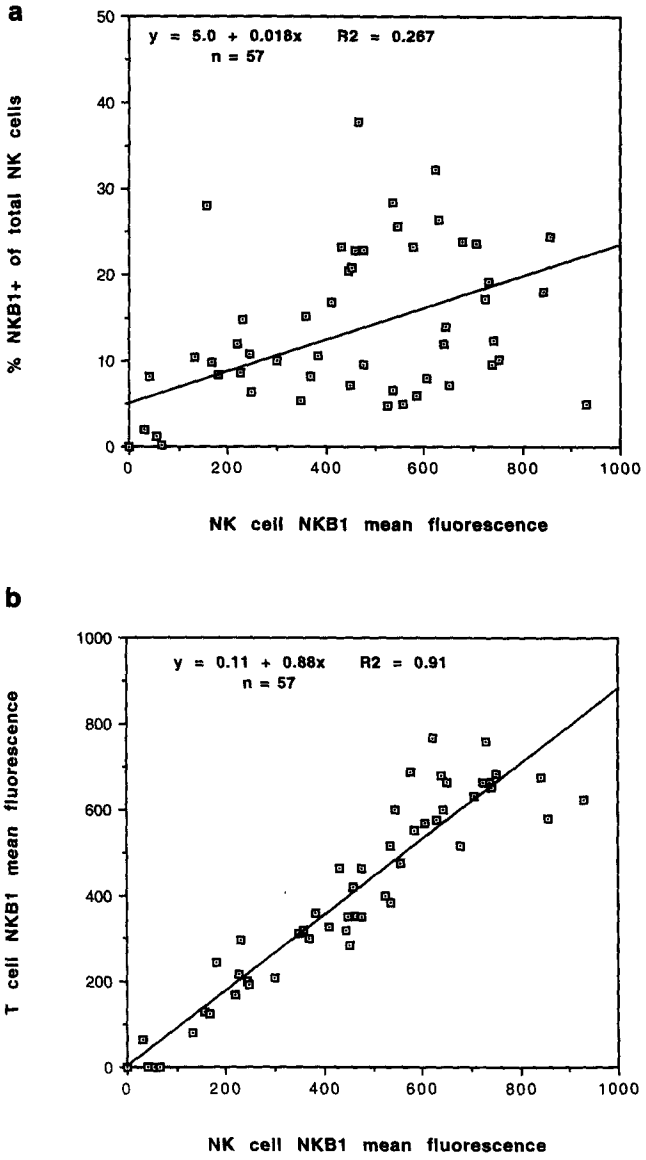


Figure 2. (*a*) The percentage of NK cells expressing NKB1 is not correlated with its expression level among unrelated donors. The NKB1 expression levels and the relative frequency of NKB1 expression both form broad distributions of phenotypes, without evidence of clustering. (*b*) NK and T cell NKB1 expression levels correlate well for each donor. Each plot represents a set of peripheral blood samples from unrelated donors, analyzed in a single experiment. Samples displaying a bimodal phenotype of NKB1 expression were excluded from the analyses. Other sets of samples gave similar distributions.

clonal cultures were compared. Were the difference in staining intensity of the two types of clones due to DX9 affinity differences, then the monovalent Fab fragment would dissociate faster than the divalent $F(ab')_2$ fragment. This was not observed; $F(ab')_2$ and Fab fragments dissociated with similar kinetics from clones staining brightly or dimly for NKB1 (data not shown). Furthermore, titration of DX9 $F(ab')_2$ binding to NKB1 dim and bright clonal cultures demonstrated nearly identical curves, consistent with a similar avidity of DX9 binding for both types of $NKB1^+$ cell (data not shown). NKB1 immunoprecipitated from radiolabeled NKB1 bright and dim clones had matching mobility by SDS-PAGE, indicating they are the same molecular size (data not shown). Thus, differences in the fluorescence staining intensity of NK cell populations with the DX9 mAb appear to result from quantitative differences in the level of cell surface expression of NKB1 molecules.

Cytolysis by NK Cell Clones Expressing Low or High Levels of NKB1. NK cells cloned from the NKB1 dim and bright populations of a donor with the bimodal NKB1 phenotype were tested for their ability to kill 721.221 target cells expressing HLA-B*5101 or B*5801 (both $Bw4^+$) as the only class I antigen. Both NKB1 dim and bright clones showed diminished lysis of the B*5101 and B*5801 transfected target cells, in comparison with the untransfected 721.221 cell line (data not shown). In each case, addition of the DX9 mAb prevented the inhibitory effect of B*5101 and B*5801 expression, demonstrating that the inhibition was due to an interaction with NKB1. Thus, NK cells with different NKB1 expression levels nevertheless have similar inhibitory specificity for $Bw4^+$ HLA-B antigens.

Relationship of NKB1 Expression to HLA Type. To investigate the relationship between NKB1 expression and HLA type, blood samples from 164 unrelated donors, and 39 members of nine families were examined. All donors were serologically typed for HLA-A, -B, and -C antigens, and haplotypes were assigned for the family members. In the first sampling of 69 donors, comparable numbers of HLA-B Bw4 homozygous (n = 21), Bw4/6 heterozygous (n = 27), and Bw6 homozygous (n = 21) individuals were chosen. Within this population, 47 donors (68.1%) had a unimodal distribution of $NKB1^+$ NK cells, 12 donors (17.4%) had a bimodal phenotype, and 10 donors (14.5%) had no detectable NKB1 expression.

The level of NKB1 cell surface expression and the relative abundance of NK cells expressing NKB1 appeared to be unrelated traits. Fluorescence intensity of NKB1 staining showed no correlation with the percentage of $NKB1^+$ NK cells present in the sample (Fig. 2 *a*). Both traits showed significant polymorphism, varying as an essentially continuous gradation that did not cluster into distinct subpopulations (Fig. 2 *a*). The NKB1 fluorescence intensity of NK cells correlated well with that of $NKB1^+$ T cells for each donor (Fig. 2 *b*). However, no correlation of the percentage of $NKB1^+$ NK cells with that of T cells expressing NKB1 was observed (data not shown).

No association of Bw4/Bw6 serotype with either the percentage of NK cells expressing NKB1, or with NKB1 expression level, was found for the donors in this sampling. An equally broad distribution of $NKB1^+$ percentages and NKB1 staining intensities was seen for Bw4 homozygotes, Bw4/6 heterozygotes, and Bw6 homozygotes (Fig. 3, *a* and *b*). Samples with the unimodal, bimodal, and undetectable phenotypes of NKB1 expression were also evenly distributed among the three serotypes (data not shown).

Polymorphisms at positions 77-83 of the class I heavy chain determine Bw4 and Bw6 serological reactivity (12).

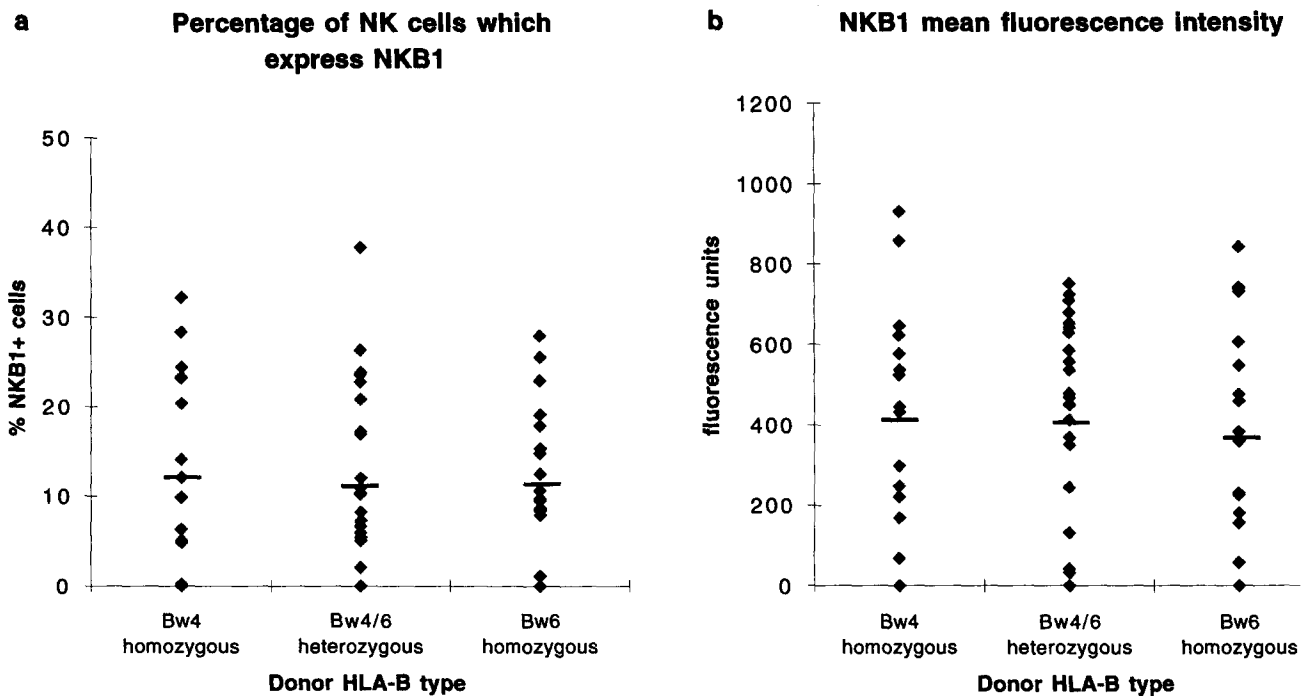


Figure 3. Phenotypes of NKB1 expression do not correlate with HLA-Bw4/Bw6 serotype. (*a*) The percentage of NK cells expressing NKB1 in a population of unrelated peripheral blood donors, divided according to Bw4/Bw6 serotype. For each group of donors, the mean and standard deviation is as follows: Bw4 homozygous 13.4 ± 10.6; Bw4/6 heterozygous 12.0 ± 9.7; Bw6 homozygous 12.5 ± 8.4. (*b*) The mean fluorescence intensity of NK cell NKB1 staining of samples from a population of unrelated peripheral blood donors, divided according to Bw4/Bw6 serotype. The mean of each data set is shown as a horizontal bar. Mean and standard deviation values are as follows: Bw4 homozygous 411 ± 285; Bw4/6 heterozygous 412 ± 256; Bw6 homozygous 397 ± 276. Mean fluorescence intensity is given in arbitrary fluorescence units. Samples displaying a bimodal phenotype of NKB1 expression are not included in these analyses.

$Bw4^+$ class I allotypes possess one of five related sequence motifs within this region. Cella et al. (19) have found that certain NK cells are more effectively inhibited by HLA-B antigens possessing a Bw4 motif with isoleucine at position 80. To determine whether NKB1 expression correlates with individual Bw4 motifs, we analyzed our data according to the amino acid motifs likely to be present at the Bw4 or Bw6 serological epitopes of the HLA-B antigens expressed by each donor. No correlation of NKB1 expression with the presence or absence of any Bw4 motif was found. Likewise, the percentage of $NKB1^+$ cells and NKB1 expression level showed no association with any individual HLA-B or -A antigen expressed in this population of donors (data not shown).

HLA allotypes for which an inhibitory effect on $NKB1^+$ NK cells has been demonstrated include B*1513, B*2705, B*5101, and B*5801 (11). Whether HLA-B antigens with an alternative Bw4 motif such as B44, and $Bw4^+$ HLA-A antigens such as A24 and A25, mediate inhibition by NKB1 is less clear (11, 19). To investigate the effect of the presence of potential class I ligands on NKB1 expression, a set of 90 donors who possessed a single $Bw4^+$ HLA-A or -B allotype was selected, and the expression of NKB1 by their NK and T cells examined. The test antigens expressed by donors in this group were HLA-A24, A32, B38, B44, B51, and B58. Again, the number of cells expressing NKB1 and their levels of expression showed a wide-ranging and continuous distribution. The heterogeneity of both parameters was similar to that observed within the set of Bw4/Bw6–serotyped donors (Fig. 3, *a* and *b*). None of the three phenotypes of NKB1 expression (unimodal, bimodal, and undetectable) appeared to be preferentially associated with any of the test antigens. From these analyses, NKB1 expression cannot readily be correlated with Bw4/Bw6 serotype or with any individual HLA-A or -B antigens.

NKB1 Expression by Related Individuals. To investigate the genetics of NKB1 expression, we analyzed samples from 39 members of nine families. The samples included 4 pairs of monozygotic identical twins, 6 pairs of class I HLA–identical siblings, and 13 pairs of siblings who had unrelated class I HLA haplotypes (Table 2). The pairs of twins all had the unimodal phenotype of NKB1 expression. For each twin both the percentage of $NKB1^+$ NK cells and the mean fluorescence intensity values were within 20% of the average of the two samples. In contrast, only one of the six pairs of HLA-identical siblings showed such similar patterns of NKB1 expression. For the five other pairs, four consisted of one unimodal and one bimodal sibling, and in the remaining pair, one sibling lacked $NKB1^+$ NK cells whereas the other had a detectable unimodal population (Table 2). Among the 13 pairs of HLA-disparate siblings, 4 pairs had similar NKB1 fluorescence intensities, but only 2

Table 2. *NKB1 Expression by Pairs of Identical Twins, Class I HLA–identical and –disparate Siblings*

Relationship	Sibling pair	Sample	Percent NKB1$^+$ NK cells	NK cell NKB1 MFI	Percent NKB1$^+$ T cells	T cell NKB1 MFI
Identical twins	1	C1	11.5	252	0.3	220
		C2	9.1	232	0.4	218
	2	G1	17.4	750	0.2	633
		G2	12.4	865	0.3	831
	3	H1	8.8	77	0.1	61
		H2	6.5	66	0.1	67
	4	K2	10.1	684	0.2	490
		K3	14.4	448	1.4	384
HLA-identical siblings	1	A3	18.8	526	2.1	366
		A5	4.9, 9.4	108, 421	2.1	96, 375
	2	B1	3.4, 6.4	115, 594	1.0	110, 575
		B4	2.2	491	0.2	312
	3	B2	7.7	92	0.1	70
		B5	5.7	83	0.1	54
	4	D3	0	0	0	0
		D4	5.0	311	0.3	371
	5	F2	2.5, 4.5	72, 415	1.0	58, 300
		F4	4.5	113	0.4	93
	6	F5	7.4, 9.9	155, 502	0.2	177, 348
		F7	14.0	516	<0.1	ND
HLA-disparate siblings	1	A3	18.8	526	2.1	366
		A4	34.2	469	0.9	315
	2	A4	34.2	469	0.9	315
		A5	4.9, 9.4	108, 421	2.1	96, 375
	3	B1	3.4, 6.4	115, 594	1.0	110, 575
		B2	7.7	92	0.1	70
	4	B1	3.4, 6.4	115, 594	1.0	110, 575
		B5	5.7	83	0.1	54
	5	B2	7.7	92	0.1	70
		B4	2.2	491	0.2	312
	6	B4	2.2	491	0.2	312
		B5	5.7	83	0.1	54
	7	E1	38.9	585	2.3	364
		E3	34.8	522	1.4	317
	8	F5	7.4, 9.9	155, 502	0.2	177, 348
		F6	0	0	0	0
	9	F6	0	0	0	0
		F7	14.0	516	<0.1	ND
	10	H1	8.8	77	0.1	61
		H4	0	0	<0.1	ND
	11	H2	6.5	66	0.1	67
		H4	0	0	<0.1	ND
	12	K1	16.8	450	7.2	305
		K2	10.1	684	0.2	490
	13	K1	16.8	450	7.2	305
		K3	14.4	448	1.4	384

Results of flow cytometric analysis of NKB1 expression by lymphocytes from peripheral blood samples of related donors. Present NKB1$^+$ NK and NKB1$^+$ T cells represent the percentage of the total NK or T cell population that stained positively for NKB1. Mean fluorescence staining intensity (MFI) of the positive population is given in arbitrary fluorescence units. Two percentage and fluorescence values are shown for donors with a bimodal distribution of NKB1$^+$ cells, representing the dim and bright populations. All samples were analyzed in the same experiment.

of these pairs were also similar in the percentage of NKB1$^+$ cells. These observations suggest that NKB1 expression phenotypes are determined genetically, but are not MHC linked.

Evidence from four informative families further indicates inheritance of NKB1 phenotype. The frequency of NKB1$^+$ NK cells and their cell surface expression levels for MHC-haplotyped family members are shown in Fig. 4, *a–d*. The percentage of cells expressing NKB1 segregates independently from NKB1 expression level (Fig. 4, *a–c*). Children usually have NKB1 expression levels similar to one parent, however the percentage of NKB1$^+$ NK cells can be intermediate between those of the parents, or greater than either parent (Fig. 4, *a–c*). Neither the percentage of NKB1$^+$ cells, nor the cell surface expression level of NKB1 segregates with MHC haplotype (Fig. 4, *a–d*).

Discussion

Kärre and co-workers (9, 20) have postulated that NK cells specifically lyse target cells that lack self–class I antigens. According to this hypothesis, the repertoire of receptors expressed by NK cells should be biased towards those recognizing determinants presented by self–class I antigens. NK cells might therefore undergo a selection process in which only developing cells expressing receptors with affinity for self-antigens are allowed to mature, as is postulated for T cells. This study was undertaken to investigate whether heterogeneity in expression of NKB1, an inhibitory class I receptor of known HLA specificity, showed evidence of such an NK cell "education" process relating to class I antigens.

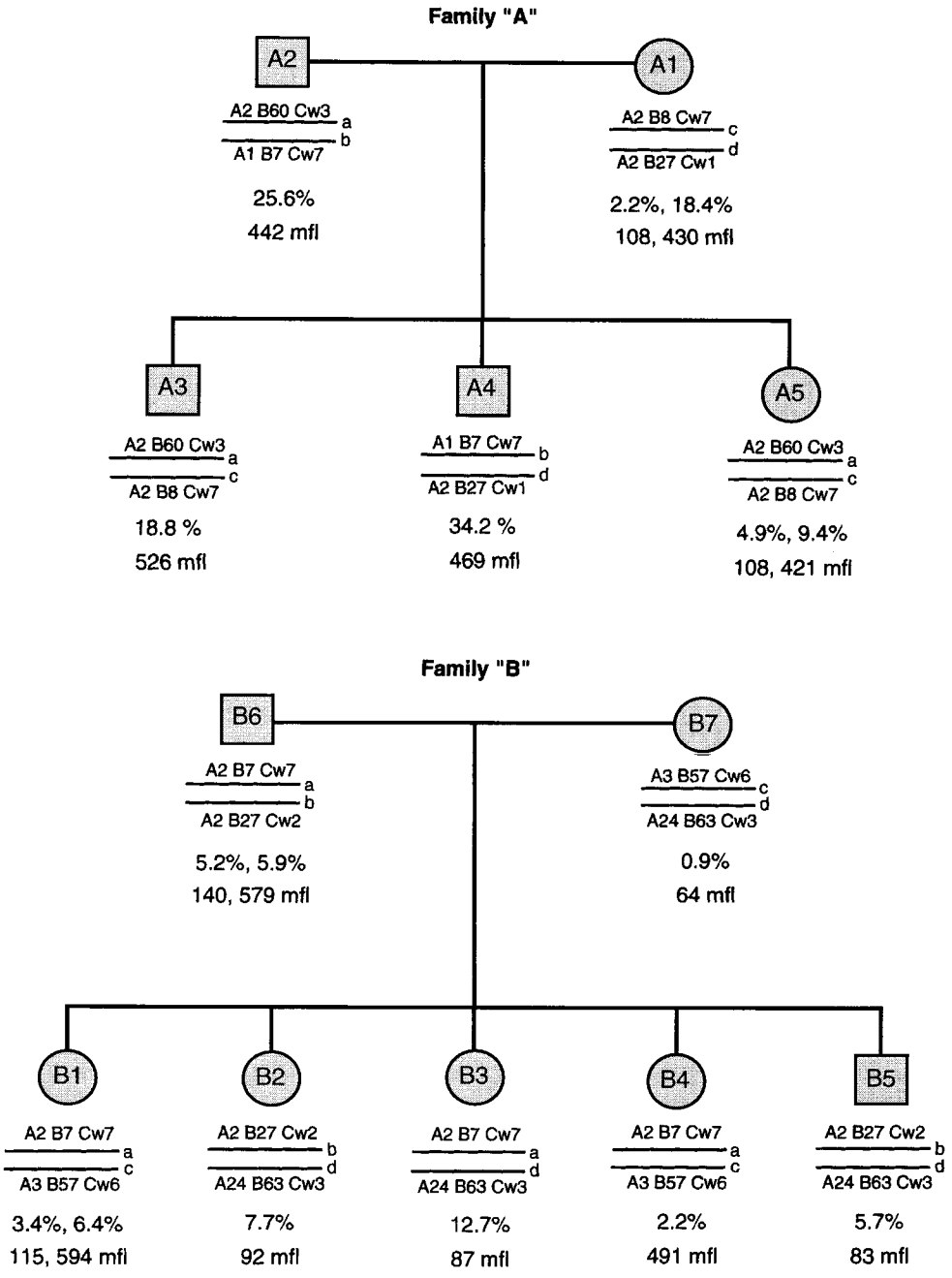
NKB1 recognizes HLA-B antigens that express the Bw4 serological epitope. In our population of unrelated donors, neither the percentage of NKB1$^+$ cells, nor NKB1 expression levels, was associated with Bw4 or Bw6 serotype, or with expression of any individual HLA-A or -B antigens. Furthermore, in family studies, no linkage of NKB1 phenotype with MHC haplotype was observed. Cells expressing NKB1 were often found in donors who do not possess a known class I ligand, and could be absent in those who express Bw4$^+$ HLA-B antigens. Thus we find no evidence for determination of NKB1 expression by donor HLA type.

This contrasts with studies demonstrating an influence of MHC class I antigens on expression of murine NK cell class I receptors (14, 21, 22). Ly-49A is an inhibitory receptor with specificity for H-2D^d and H-2D^k allotypes (23–25). Experiments with bone marrow chimeras and transgenic mice provide evidence for a role of class I MHC antigens in modulating Ly-49 expression (14, 21). Compared with nonchimeric and nontransgenic mice, those in which a potential class I ligand for the Ly-49A receptor had been introduced showed reduced levels of Ly-49A cell surface expression, but the percentage of NK cells bearing Ly-49A was unchanged (14, 21). In our analysis, there is no significant difference in the mean fluorescence intensity of NKB1 staining among Bw4 homozygous or heterozygous donors compared with Bw6 homozygous donors. Thus, we see no simple correlation of NKB1 expression level with the presence of a potential class I ligand. However, further studies will be required to assess whether the substantial heterogeneity of NKB1 expression levels within donor populations, and the presence of cells with two distinct levels of NKB1 expression in certain individuals, are related to functional differences in the degree of inhibition mediated by NKB1.

Similar to our finding that Bw6 homozygous donors can express NKB1, mouse strains that do not express H-2D^d or H-2D^k can express significant numbers of Ly-49A$^+$ NK cells (13, 22). Therefore, in both species, expression of receptors for which there is no class I ligand is permitted. One explanation for this might be that the receptors recognize other ligand(s) in addition to class I molecules, and selection is based on these alternative interactions. However, our findings are also consistent with the possibility that inhibition of NK cells by self-MHC antigens is governed by a stochastic process, whereby a developing NK cell must express at least one receptor that mediates inhibition by a self–class I allotype, but expression of other receptors is not prohibited. Selection of NK cells may therefore be based on the constellation of receptors expressed, rather than on expression of any individual receptor. Testing this possibility will require functional and molecular assessment of all class I receptors expressed by NK cells from individuals of known HLA type.

Our results suggest NKB1 expression by NK and T cells is genetically controlled. Among unrelated individuals, two parameters of NKB1 expression vary: the relative abundance of cells bearing NKB1, and the level of NKB1 cell surface expression. In our population of unrelated donors, substantial heterogeneity was present in both NKB1 frequency and expression level, but no correlation between the traits was seen within the donor population, suggesting they are independent parameters. Within families, the two characteristics segregated independently, further indicating that they are separate traits. Within an individual, both parameters remained very stable over time, and identical twins had similar phenotypes for both, whereas both HLA-identical and -disparate siblings were usually dissimilar in NKB1 expression phenotype. This suggests the phenotype is probably genetically, rather than environmentally determined. We also saw no correlation of NKB1 expression pattern with the sex of the donor. Thus, the level and frequency of NKB1 expression appear to be independent traits, likely governed by autosomal genes.

The degree of heterogeneity observed for both traits within the population of unrelated donors is surprising. The diversity of phenotypes could be explained by polymorphism of the molecules reactive with the DX9 mAb. Human NK cell class I receptors are members of the Ig superfamily. Two types of human inhibitory class I receptors have been identified: the p58 family, which has two extracellular Ig domains, and the p70 molecules, which has three Ig domains (3, 4). NKB1 belongs to this latter group (5). Complementary DNA encoding four p70 molecules and six members of the p58 family has been identified (3, 4,



26). The two- and three-Ig domain-type receptors appear to be products of different genes (3–5), however it is not clear whether different cDNA sequences within each family are allelic products of a single locus or derive from distinct gene loci. The DX9 mAb reactivity of PBL samples may therefore appear heterogenous because different individuals may express different numbers of related receptors that share the DX9 epitope.

However, dim and bright NKB1$^+$ NK cell clones from a single individual analyzed in this study, and NKB1$^+$ NK cell clones from unrelated donors tested in previous investigations (10, 11), have similar functional specificities for Bw4$^+$ HLA-B antigens. Furthermore, investigation of DX9 Fab and F(ab')$_2$ binding to NKB1 molecules of dim and bright NK cell clonal populations revealed no affinity differences, and immunoprecipitated protein had identical mobility by SDS-PAGE. Therefore the phenotypic heterogeneity is likely to indicate true variations in the level of expression of one or a few NKB1-like receptors. If so, the diversity might be explained by regulation of NKB1 genes by other genetic or epigenetic factors, which produce subtle variations in the frequency and level of NKB1 expression. Phenotypic heterogeneity observed among individuals in expression of the complement regulatory pro-

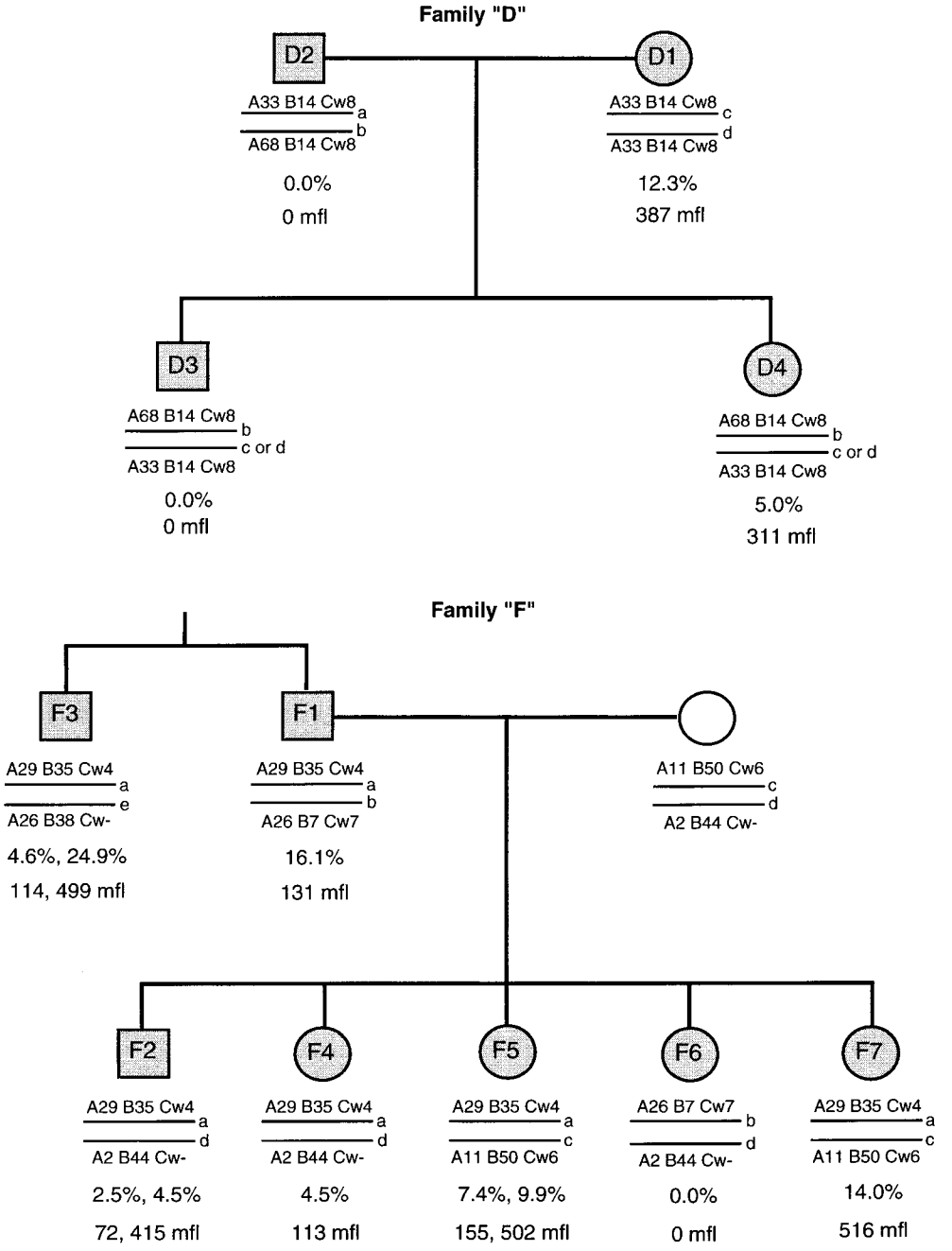


Figure 4. *a–d.* Pedigrees showing the class I HLA haplotypes and phenotypes of NKB1 expression. HLA class I haplotypes are represented by labeled parallel bars (*a–d*). Percent values indicate the proportion of the total NK cell population that expresses NKB1. Mean fluorescence intensity of NK cell NKB1 staining in arbitrary fluorescence units is given below these values. Single values indicate a unimodal distribution of NKB1 staining, whereas two values indicate a bimodal distribution.

tein, C4b-binding protein, may be determined in such a manner (27).

Also unexpected was the finding that two distinct populations of NKB1$^+$ cells could be present in a single individual. Studies of murine NK cell class I receptors indicate that expression of a given receptor may be influenced by the presence of another allele at the same locus. Held et al. (28) have found evidence for mutual exclusion of alleles for two members of the murine Ly-49 family, Ly-49A and Ly-49C. Thus the number of receptors expressed by individual murine NK cells may be limited, and expression of distinct receptors regulated coordinately within the cell. Such a process could explain our observation that certain individuals possess a bimodal distribution of NKB1$^+$ NK cells, if NKB1 expression level can be affected by the presence of other class I receptors expressed by a proportion of the NKB1$^+$ cells.

On the basis of our family data, inheritance of NKB1 expression phenotypes is complex, which further suggests it is controlled by several factors. The frequency of NKB1$^+$ cells observed in parents and children is not readily explained by simple patterns of Mendelian inheritance, since children can have values matching, intermediate between, or greater than those of the parents. Inheritance of NKB1

cell surface expression level might be explained by a multiallelic codominant system that includes a null allele. Two samples (B1 and F2) are not completely consistent with this hypothesis, because the fluorescence intensity of the children is not equivalent to that of the contributing parent. Hence, further analysis will be required to clarify how NKB1 expression phenotypes are inherited.

Molecules recognized by the DX9 mAb are biochemically and antigenically similar, and NK cells with different levels of NKB1 expression have yet to be distinguished functionally. Yet, both NKB1 frequency and expression level were stable over 2 yr, and NKB1$^+$ NK and T cells from the same individual have similar patterns of expression, indicating NKB1 expression is tightly regulated within an individual. The extraordinary heterogeneity of NKB1 expression among individuals combined with its stability is therefore puzzling. Important questions concern the influence of NKB1 expression on NK cell function in vivo, and whether expression patterns are maintained during viral or bacterial infections. If NKB1 influences the functional role of cells activated by infections by interacting with class I molecules, the presence or absence of potential class I ligands for NKB1 and individual phenotypes of expression could be of signifance for NK and T cell responses.

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