

## Polymorphic Hh Genes in the HLA-B(C) Region Control Natural Killer Cell Frequency and Activity

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### Summary

We demonstrated earlier that individuals homozygous for conserved major histocompatibility complex (MHC)-extended haplotypes have low natural killer (NK) activity as measured by cytotoxicity of the K562 tumor cell line. In the present study, we investigated the segregation and MHC linkage of NK activity in families in which MHC haplotypes of human histocompatibility leukocyte antigens (HLA)-A, -C, and -B, complotype, and DR specificities are known. In two informative families, low activity was inherited as a recessive trait linked to the MHC. By using individuals homozygous for specific fragments of extended haplotypes or for HLA-B alleles, we found that the HLA-C and -B and not the complotype or HLA-DR region contains genes controlling NK activity. The majority of the unrelated individuals with low NK activity were homozygous or doubly heterozygous for HLA-B7 (Cw7), B8 (Cw7), B44 (Cw5), B18, or B57 (Cw6). Thus, these alleles form one complementation group designated NKB1. Another less frequent group, NKB2, was also identified, and consisted of individuals homozygous for B35 (Cw4). NK activity was correlated with the number of circulating NK (CD16<sup>+</sup>CD56<sup>+</sup>) cells. Individuals homozygous for the NKB complementation groups have fewer circulating NK cells than individuals heterozygous for these alleles and alleles of other complementation groups, possibly explaining the low activity of cells in these subjects. These findings suggest that during the maturation of NK cells there is NK cellular deletion in donors homozygous for NKB genes resulting in low NK cell numbers and activity.

Genetic control of NK cell activity against tumor cells in humans is poorly understood. We had shown earlier that NK cell activity in human MHC homozygotes was significantly lower than in heterozygotes (1). This finding suggested that the genes controlling NK activity may be located on chromosome 6. However, it is not known whether the genes that control the level of NK cell activity in humans are MHC linked nor is it known whether low NK activity is the result of a reduction in NK cell number or per cell specific activity. It has recently been reported that the genes controlling target antigens recognized by allospecific NK clones are linked to the HLA region (2-6). The relationship between the putative MHC genes controlling NK cell level and those determining NK cell targets is not known.

Based on our finding that cell activity in MHC homozygotes was lower than in heterozygotes, we hypothesized that the putative gene(s) controlling NK activity is (are) recessive and in the MHC region, and we suggested that different extended MHC haplotypes may also carry different specific alleles for NK activity (1). The genetic material between class I and class II MHC loci is relatively fixed on these extended haplotypes (7) (see Table 1). Some instances of extended haplo-

types appear to have undergone historical recombinations resulting in apparent fragments of these haplotypes in the population. These fragments have shorter conserved segments than complete extended haplotypes but the shared segments appear to be identical. By using these fragments it has been possible to map chromosomal regions containing specific genes (8-11). Because homozygosity mapping provides a way to localize a recessive gene (12), we used homozygosity for either telomeric or centromeric fragments of MHC-extended haplotypes to localize the gene(s) controlling NK activity. We also investigated the segregation and MHC linkage of NK activity in families in which HLA-A, -C, and -B, complotype, and DR allele haplotypes were known. In addition, we investigated if MHC class I genes affect the development of NK cells in vivo. We found evidence for polymorphic recessive genes controlling NK activity and cell number located within the HLA-(C)B region telomeric of the complement -DR region of the MHC.

### Materials and Methods

**Subjects.** We studied 29 men and 30 women aged 20-55 yr. Five families with 27 members were also studied. Four of these

families were selected because at least one member was homozygous for HLA-B and had low NK activity. The unrelated individuals were previously genotyped for HLA and complotypes, had at least one extended haplotype or a fragment of an extended haplotype, and were available for study. The list of extended haplotypes used in this study is given in Table 1. We have also included NK data on 111 individuals from 33 families for whom complete HLA and complototype data were not available.

**Preparation of Lymphocytes and Cell Cultures.** We used freshly isolated or cryopreserved PBMC as a source of NK cells. PBMC were obtained from heparinized blood by centrifugation over Ficoll-Hypaque density gradients. Cells at the interface were collected and then washed in RPMI-1640 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine. Cells were plated at 37°C to remove adherent macrophages. The nonadherent cells were gently poured off, washed three times with 5 ml of culture medium, and used as NK cells. To minimize the effect of cryopreservation on NK activity, cryopreserved PBMC were incubated overnight at 37°C (1, 13).

**HLA Typing.** Subjects were typed for HLA-A, -B, -C, and -DR antigens by a microcytotoxicity assay (14) and for MHC-encoded complement proteins C4, Bf, and C2 (15-17). Haplotypes were assigned based on family studies.

**Immunofluorescence Assay.** To analyze NK cell subpopulations, we used two-color immunofluorescence. FITC-conjugated mAb Leu-11b (18) (anti-CD16; Becton Dickinson & Co., Mountain View, CA) and PE-conjugated Leu-19 (19) (anti-CD56; Becton Dickinson & Co.) were incubated with 10<sup>6</sup> cells for 30 min on ice and the labeled cells were washed twice. Background fluorescence was determined by incubating cells with nonreactive FITC-conjugated, and PE-conjugated antibodies. The simultaneous determination of green (FITC) and red (PE) fluorescence was obtained from a single laser excitation of both FITC and PE chromophores at 488 nm by using a FACS® (Becton Dickinson & Co.). A total of 10,000 cells were analyzed in each sample and results were displayed as

an orthogonal plot. Cryopreserved cells from five individuals were tested in parallel with fresh cells for NK activity and for the number of cells carrying the CD16 and CD56 markers to test the reproducibility of and the effect of cryopreservation on the determinations.

**Cytotoxicity Assays.** NK cell activity was determined using K562 as a target (1). Briefly, target cells were incubated with 0.1 ml of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 1 h at 37°C followed by three washings at 4°C. The final suspension was made in culture medium. The effector cells were mixed in triplicate in 96-well V-bottom microtiter plates along with a fixed number of labeled target cells. The microtiter plates were spun for 3 min at 200 g. The cell mixtures were incubated for 4 h at 37°C in a 95% air and 5% CO<sub>2</sub> atmosphere. The microtiter plates were spun again for 1 min at 200 g and 120 µl supernatant was collected and counted in a gamma counter. Percent cytotoxicity (specific release) was calculated using the formula: percent specific release = 100 × [(E - SR)/(T - SR)], where E = counts per min (cpm) in the experimental group, SR = spontaneously released cpm, and T = total cpm incorporated. Spontaneous release was obtained from target cells incubated with medium alone and was usually <10% of the total count. Total incorporation of <sup>51</sup>Cr was determined by incubating cells in the presence of 0.1% NP-40. Percent cytotoxicity was determined at E/T ratios of 100, 50, 25, 12.5, and 6.25.

**Data Analysis.** Percent specific release at different E/T ratios was used to evaluate the cytotoxic activity of NK cells, expressed in LU per 10<sup>6</sup> effector cells. One LU was defined as the number of effector cells required to kill 20% of target cells (10,000 cells in the assays used) (20).

The experiments consisted of 11 sets using frozen cells (36 individuals) and 6 sets using fresh cells (23 individuals). The NK activity of the cryopreserved PBMC from three or four unrelated donors used as controls was tested on 11 occasions and fresh cells were tested in parallel with cryopreserved cells on 6 occasions. The geometric mean NK activities of these controls were comparable: 29.5 ± 3.6 LU for fresh and 31.7 ± 2.8 LU for frozen cells. The overall geometric mean lytic activity of fresh and cryopreserved control groups was used to normalize the NK data obtained in different experiments (21).

Centroid analysis was used to determine the cutoff value between low and high NK reactors (1, 22). χ<sup>2</sup> statistics were calculated for analysis of 2 × 2 contingency tables prepared from Tables 2 and 3. Statistical analysis was performed using Student's *t* test for comparison of different groups (23). Family data were analyzed using a method proposed by Smith (24).

## Results

The analysis of NK activity in 59 unrelated individuals showed two groups. In one group, 21 subjects, including three individuals from four of the five families, had low activity (≤7 LU). There was no significant difference in NK activity due to gender.

**Putative NK Genes Map to the HLA-C/B Region.** Table 2 shows 12 individuals with low NK activity who had extended haplotypes or fragments of them. Five of them were homozygous for HLA-B(C) complotype whereas five were homozygous for HLA-B(C) only. Table 2 also shows 10 individuals who were not homozygous for HLA-B but were homozygous for complotype, DR, or both, 9 of whom were high NK reactors. The frequency of low reactor individuals homozygous for HLA-B only, or homozygous for HLA-B

**Table 1.** List of Extended Haplotypes

[Cw7, B7, SC31, DR2]
[Cw7, B8, SC01, DR3]
[Cwx, B14, SC2(1,2) DR1]
[Cw5, B18, F1C30, DR3]
[Cwx, B18, S042, DR2]
[Cw4, B35, FC(3,2)0, DR1]
[Cwx, B38, SC21, DR4]
[Cw4, B44, FC31, DR7]
[Cw5, B44, SC30, DR4]
[Cw6, B57, SC61, DR7]
[Cwx, B60, SC02, DR6]
[Cw3, B62, SC33, DR4]
[Cwx, B62, SB42, DR4]
Provisional extended haplotypes
Cw4, B35, SC31, DR4
Cw3, B60, SC31, DR4

x refers to undetermined generic type.

**Table 2. Homozygosity for Extended Haplotypes or HLA-B Locus-associated Fragments; the NKB1 Homozygous Group and Mapping of NKB1**

Code	Haplotypes	LU	Code	Haplotypes	LU
<u>Homozygotes</u>			<u>Heterozygotes</u>		
BII-1*	[A1, Cw7, B8, SC01, DR3] [A1, Cw7, B8, SC01, DR3]	1	M-11*	[A1, Cw7, B8, SC01, DR3] A1, Cwx, B18, SC01, DR3	4
GT-1	[A1, Cw7, B8, SC01, DR3] [A1, Cw7, B8, SC01, DR3]	1	N-15	[A1, Cw7, B8, SC01, DR3] A33, Cw3, B57, SC01, DR3	90
A-2	[A3, Cw7, B7, SC31, DR2] A3, Cw7, B7, SC31, DR1	2	SBII-2*	A24, Cw4, B35, SC30, DR4 [A2, Cwx, B44, SC30, DR4]	79
BTH-1*	A2, Cwx, B51, SC31, DR6 A2, Cwx, B51, SC31, DR6	4	Br-2	A28, Cwx, B41, SC31, DR7 A2, Cwx, B49, SC31, DR4	15
SBI-2*	A2, Cw4, B35, SC30, DR1 A24, Cw4, B35, SC30, DR4	1	O-5*	[A3, Cw7, B7, SC31, DR2] A2, Cwx, B51, SC31, DR6	14
D-1	[A3, Cw7, B7, SC31, DR2] A2, Cw7, B7, SC31, DR4	3	P-16*	A2, Cwx, B18, SC02, DR5 A3, Cwx, B57, SC61, DR5	18
E-6	[A3, Cwx, B7, SC31, DR2] A2, Cwx, B7, SC31, DR4	3	Fa-M	[A28, Cwx, B44, SC30, DR4] A3, Cwx, B14, FC31, DR4	59
J-4*	A3, Cw4, B35, SC31, DRx A3, Cw4, B35, FC31, DR2	7	Fl-2	A2, Cwx, B44, SC31, DR2 A2, Cw2, B27, SC42, DR2	89
H-5	A11, Cwx, B7, SC31, DR4 A2, Cwx, B7, SC21, DR1	1	Fa-Fo	A2, Cwx, B51, FC30, DR6 [A2, Cw3, B60, SC02, DR6]	99
Q-8	[A2, Cw5, B44, SC30, DR4] A2, Cw2, B44, SC31, DR2	5	Sr-2	A2, Cw1, B51, SC31, DR5 A2, Cw1, B58, FC31, DR5	19
V-9	[A2, Cw5, B44, SC30, DR4] [A23, Cwx, B44, FC31, DR7]	3			
KL-3	[A3, Cw6, B57, SC61, DR7] A3, Cwx, B57, SC31, DR4	4			
K-10	[A2, Cwx, B60, SC02, DR6] A11, Cwx, B60, SC31, DR4	3			

\* Also listed in Figs. 1A, 1C, 1D, and Tables 3 and 4.

Shaded areas represent homozygous regions of HLA haplotypes. Lightly shaded areas represent HLA-C locus with incomplete typing information. Bracketed alleles denote conserved extended haplotypes, and fragments of conserved haplotypes are underlined.

and complotype, was significantly higher than that for those homozygous for DR and complotype ( $P < 0.005$  in each case).

**Low NK Activity Is a Recessive Trait.** Our initial study consisted of the analysis of NK cell activity of 136 individuals belonging to 37 families with two parents and at least one child. There were nine families in which at least one child

was a low reactor. In five of the nine families, one parent had low NK activity and in rest of the four families, both parents had high NK activity. The observed number of low reactors, 10, in this sample was not significantly different from that expected on the basis of mendelian segregation ( $\chi^2 = 0.49, P > 0.1$ ). This suggests that the gene(s) responsible for

low activity is (are) recessively inherited. All 13 homozygotes (12 for extended haplotypes or HLA-B regions and one for HLA-B51) had low NK activity. 11 out of 43 individuals heterozygous for HLA-B (Cw) alleles had low NK activity. Again, these data are consistent with recessive genes controlling low NK activity and suggest that homozygosity for an MHC gene is necessary to confer low NK activity.

*Polymorphism of NK Activity.* Based on our NK data, we categorized individuals in four groups based primarily upon

homozygosity or heterozygosity for specific haplotypes or fragments thereof (Tables 3, 4, and 5). Table 3 shows the NK activity of individuals heterozygous for extended haplotypes [HLA-Cw7, B8, SC01, DR3], [HLA-Cw7, B7, SC31, DR2], [HLA-Cw5, B44, SC30, DR4] or their fragments. The first group contains the majority of the low NK reactors in the MHC heterozygous group with two haplotypes or fragments of them containing HLA-B7 (Cw7), B8 (Cw7), B18, B44 (Cw5), or B57 (Cw6). These B(C) alleles were assigned to

**Table 3.** NK Activity of Heterozygous Subjects Sharing Alleles of Two Extended Haplotypes (NKB1 Homozygous), or Fragments of Extended Haplotypes Marked by HLA-B Alleles: B7, B8, B18, B44, and B57

Code	Haplotypes	LU	Code	Haplotypes	LU
Group A (HLA-B heterozygous : NKB1 homozygous)			Group B (HLA-B heterozygous : NKB1 heterozygous)		
He-2	[A1, Cw7, B8, SC01, DR3] [A3, Cw7, B7, SC31, DR2]	3	FN-5	A2, Cwx, <u>B7, SC31</u> , DR9 A11, Cw4, B53, <u>SC31</u> , DR1	47
P-1	[A1, Cw7, B8, SC01, DR3] [A3, Cwy, B7, SC31, DR2]	4	Fa-M	[A28, Cwx, B44, SC30, DR4] A3, Cwx, B14, <u>FC31</u> , DR4	59
BI-1*	[A1, Cw7, B8, SC01, DR3] [A2, Cw5, B44, SC30, DR4]	6	Mo-P	A2, <u>Cw5, B44, SC31, DR2</u> A2, <u>Cw3, B62, SB42, DRx</u>	63
BI-2*	[A1, Cw7, B8, SC31, DR3] [A23, Cw4, B44, FC31, DR7]	5	LuR	A3, Cwx, <u>B18, SC01, DR5</u> A3, Cwx, B49, <u>SC31, DR4</u>	46
BII-2*	[A1, Cw7, B8, SC01, DR3] [A2, Cw5, B44, SC30, DR4]	2	Ryg	A3, Cwx, <u>B18, SC01, DR5</u> A1, Cwx, B13, <u>SC33, DR7</u>	89
M-11	[A1, Cw7, B8, SC01, DR3] A1, Cwx, <u>B18, SC01, DR3</u>	4	DJ	A1, Cwx, <u>B18, SC31, DR5</u> A3, Cwx, B39, <u>SC30, DR2</u>	78
Wa-3	[A3, Cwx, B7, SC31, DR2] A2, Cwx, <u>B57, FC30, DR4</u>	5	AN	A24, Cwx, <u>B18, SC01, DR1</u> A2, Cwx, B50, <u>S1C21, DR7</u>	83
D-Bo	A2, Cwx, <u>B7, SC31, DR1</u> A25, Cwx, <u>B18, SC02, DR5</u>	1	CA	A2, Cwx, <u>B18, SC31, DR5</u> A1, Cwx, <u>B14, SC02, DRx</u>	33
Sd-4	A3, Cwx, <u>B44, SC31, DR3</u> A2, Cwx, <u>B57, SC60, DR4</u>	1	Fi-5	A2, Cw2, <u>B44, SC31, DR1</u> A2, Cwx, B37, <u>FC30, DR2</u>	69
Ma-Ro	[A23, Cwx, B7, SC31, DR2] Ax, Cwx, <u>B18, SC02, DR5</u>	23	Fl-2	A2, Cwx, <u>B44, SC31, DR2</u> A2, Cw2, B27, <u>SC42, DR2</u>	89
M-15	[A1, Cw7, B8, SC01, DR3] A33, Cw3, <u>B57, SC01, DR3</u>	90	A-31*	[A2, Cwx, B8, SC01, DR3] A2, <u>Cw3, B62, SC33, DR1</u>	17
P-16	A2, Cwx, <u>B18, SC02, DR5</u> A3, Cwx, <u>B57, SC61, DR5</u>	18	O-5*	[A3, Cw7, B7, SC31, DR2] A2, Cwx, B51, <u>SC31, DR6</u>	14
Ug-4	[A1, Cw7, B8, SC01, DR3] A26, Cwx, <u>B44, SC31, DR5</u>	20	Bu-1	A26, Cw1, B51, <u>SC31, DR6</u> A33, Cw3, <u>B57, SC30, DR3</u>	58
P-4	[A1, Cwx, B8, SC01, DR3] A2, Cwx, <u>B7, SC31, DR4</u>	78			

\* Also listed in Table 4 and Fig. 1 D.

complementation group NKB1, which is composed of a subgroup of low responders (group A) and high responders (group B). Group A individuals were doubly heterozygous for HLA-B(C) alleles of NKB1, whereas group B individuals were heterozygous for one NKB1 and one non-NKB1 allele. Individuals heterozygous for HLA-B18 (Cw3) and HLA-B57 (Cw6) were also included in this group even though the status of these alleles is less clear. 9 of the 14 individuals denoted as homozygous for NKB1 had low NK activity (group A, Table 3). The degree of association between homozygosity at NKB1 and low NK activity is 0.68 ( $\chi^2 = 12.5, P < 0.001$ ). The second group is made up of two individuals homozygous for HLA-B35 (Cw4) and one heterozygous for HLA-B62 (Cw3)/HLA-B35 (Cw4), all of whom showed low activity. These alleles were placed in group A of the NKB2 complementation group. In eleven individuals who were heterozygous for these haplotypes and haplotypes with non-NKB1 alleles who had high NK activity, the non-NKB1/MHC alleles were

placed in the group NKB2 (Table 4). Confirmation of this group came from the results of family BT (Fig. 1 C). Three members of this family exhibiting low NK activity were homozygous for HLA-B35 (Cw4), and 9 of 12 independent examples of heterozygous B35 (Cw4) donors had high NK activity and were presumably heterozygous for the putative NKB1 and NKB2 complementation groups. Because one HLA-B35/B62 individual, NK activity was low (Table 4), and two individuals with B62 together with a haplotype associated with the first complementation group (B8 or B44) had high NK activity, this haplotype was tentatively assigned to the complementation group NKB2 even though one HLA-B15 (?62)/B35 subjects had high activity. Table 5 shows individuals who did not have HLA-B alleles of the two defined complementation groups. 9 of 10 had high NK activity. The one individual exhibiting low NK activity carried HLA-B14/B38 haplotypes. HLA-B14-carrying individuals could not be part of the NKB1 complementation group because

**Table 4.** NK Activity of Donors with Extended Haplotypes or Fragments Associated with B35 Alleles

Code	Haplotypes	LU	Code	Haplotypes	LU
<b>Group A (NKB2 homozygous)</b>			<b>Group C (HLA-B heterozygous: NKB2/NKBX)</b>		
J-4	A3, <u>Cw4</u> , B35, SC31, DRx A3, <u>Cw4</u> , B35, FC31, DR2	7	AW	A3, <u>Cw4</u> , B35, FC30, DR1 A24, Cwx, B39, SC42, DR8	22
SBI-2*	A2, <u>Cw4</u> , B35, SC30, DR1 A24, <u>Cw4</u> , B35, SC30 DR4	1	Tr-5	A28, <u>Cw4</u> , B35, SC31, DR8 A3, Cwx, B51, FC30, DR6	13
<b>Group B (HLA-B heterozygous: NKB1/NKB2)</b>			Bat-2	A1, <u>Cw4</u> , B35, SC01, DR5 A24, Cw3, B15, FC31, DR4	43
Q-7	A28, <u>Cw4</u> , B35, SC31, DR5 [A1, Cw7, B8, SC01, DR3]	25	Y-1	[A2, <u>Cw4</u> , B35, SC31, DR4] A2, <u>Cw3</u> , B62, SC32, DR1	4
T-8	A28, <u>Cw4</u> , B35, SC31, DR8 [A1, Cwx, B8, SC01, DR3]	35			
L-Co	A11, Cw1, <u>B35</u> , SC31, DR5 [A30, Cwx, B18, F1C30, DR3]	80			
Fa-R	A32, <u>Cw4</u> , B35, SC31, DR5 A24, Cwx, <u>B18</u> , FC31, DR2	56			
BA-4	A1, <u>Cw4</u> , B35, SC31, DR5 [A23, Cw4, B44, FC31, DR7]	61			
SBII-2*	A24, <u>Cw4</u> , B35, SC30, DR4 [A2, Cwx, B44, SC30, DR4]	79			
Mo-F	A24, Cwx, B35, SC31, DR4 A2, <u>Cw5</u> , B44, SC30, DR2	14			
Ja-R	A32, <u>Cw4</u> , B35, SC31, DR5 A24, Cwx, <u>B18</u> , FC31, DR2	21			

\* Also listed in Fig. 1 A.

**Table 5.** NK Activity of Individuals without NKB1 or NKB2 Complementation Groups

Code	Haplotypes	LU
FM-1	A25, Cw2, B51, FC30, DR5 [A11, Cw4, B60, SC31, DR4]	53
Fa-Fo	A2, Cwx, B51, FC30, DR6 [A2, Cw3, B60, SC02, DR6]	99
E-Fo	A2, Cwx, B51, FC30, DR6 A3, Cw1, B27, SC31, DR4	24
St-Tr	A2, Cw1, B27, SC42, DR7 A28, Cwx, B37, FC30, DR4	35
Bi-3	A2, Cw4, B38, SC31, DR6 A29, Cw1, B53, SC30, DR7	21
Br-2	A28, Cwx, B41, SC31, DR7 A2, Cwx, B49, SC31, DR4	15
RS	A2, Cwx, B14, SC02, DR1 A1, Cwx, B58, SC21, DR4	34
IM	A11, CW1, B27, SC32, DR1 A33, Cwx, B14, SC42, DR8	95
Nu-3	A29, Cwx, B14, FC31, DR1 A3, Cwx, B38, SC30, DR6	1
Sr-2	A2, Cw1, B51, SC31, DR5 A2, Cw1, B58, FC31, DR5	19
Mo-R	A24, Cw3, B62, FC31, DR2 A25, Cwx, B39, SC31, DR5	40

we found two cases (B14/B18 and B14/B44) with high NK activity (see Table 3, group B). With the information available, these individuals could not be assigned to any of the two complementation groups and the alleles were therefore assigned to NKBx.

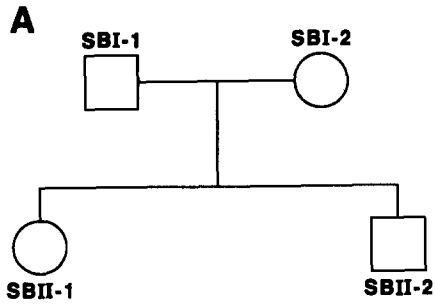
**Family Studies of Individuals with Low NK Activity.** Four of five families studied were informative for inheritance and segregation of low NK activity with the MHC and are shown in Fig. 1. In the fifth family, the parents and two children had high NK activity and none of the members were homozygous for HLA or for the three putative NKB complementation groups (data not shown). In Fig. 1 A, a low reactor, SBI-2, was homozygous for NKB2 (Cw4, B35, SC30). The siblings, SBII-1 and SBII-2, carried the NKB2 (HLA-Cw4, B35, SC30, DR3) haplotype from the mother (SBI-2) and the extended haplotype NKB1 [HLA-Cwx, B44, SC30, DR4] from the father (SBI-1). As expected on the basis of recessive inheritance, the two children heterozygous for NKB, each inheriting NKB1 and NKB2 haplotypes from their parents, showed high NK activity. In Fig. 1 B, the mating between

parents heterozygous for NKB resulted in three out of the four children being homozygous for the NKB2 haplotype. Two out of these three children were low reactors. The third sibling homozygous at NKB (CII-4) had high NK activity which could be due to either recombination between HLA-(C)B and the putative NKB allele or due to a gene outside the MHC influencing NK activity. Further proof of the segregation of low NK activity with homozygosity at HLA-(C)B or NKBx was obtained in Fig. 1 C in which all three siblings who were homozygous for HLA-B51, SC31, DR6 also had low activity. The gene(s) controlling low activity is (are) in a region close to HLA-(C)B on chromosome 6. Fig. 1 D gives an interesting example of a family in which homozygosity at NKB and not HLA-(C)B leads to low NK activity. The two parents, BI-1 and BI-2, are homozygous for the putative NKB1 haplotype but are heterozygous at the HLA-B locus. As expected, all siblings who were homozygous for the NKB1 haplotype but who were heterozygous for HLA-(C)B alleles showed low NK activity. These examples thus provide further evidence that (a) low NK activity segregates with homozygosity for NKB alleles and (b) the gene(s) controlling low NK is (are) located in the MHC.

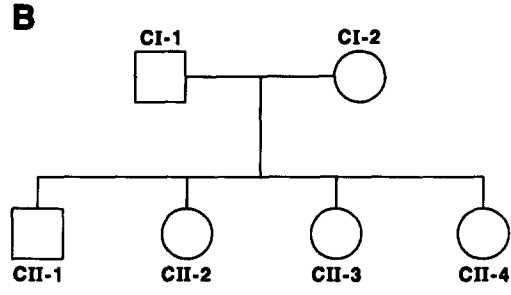
**Surface Marker Analysis of NK Cells.** Because the majority (>90%) of NK activity resides in the CD16<sup>+</sup>CD56<sup>+</sup> cell population (19), we used two-color immunofluorescence to define the NK cell population in PBMC from 41 individuals using mAbs specific for those determinants. The frequency of the CD16<sup>+</sup>CD56<sup>+</sup> cells in our population ranged between 3.8 and 20.5% of mononuclear cells. Examples of two individuals, one with a low number of CD16<sup>+</sup>CD56<sup>+</sup> cells (9.5%) and homozygous for NKB1, and the other with a high number of CD16<sup>+</sup>CD56<sup>+</sup> cells (19.1%) and heterozygous for NKB1, are shown in Fig. 2. The mean value of CD16<sup>+</sup>CD56<sup>+</sup> of NKB1 homozygotes was  $8.8 \pm 3.2\%$  ( $n = 8$ ) and for NKB2 homozygotes was  $7.9 \pm 3.0\%$  ( $n = 6$ ). Fig. 3 shows that individuals heterozygous for NKB1, NKB2, and NKBx groups have a higher cell number,  $14.4 \pm 0.7\%$  ( $n = 27$ ), than individuals homozygous for NKB1 or NKB2 (Student's *t* test, both  $P < 0.01$ ).

## Discussion

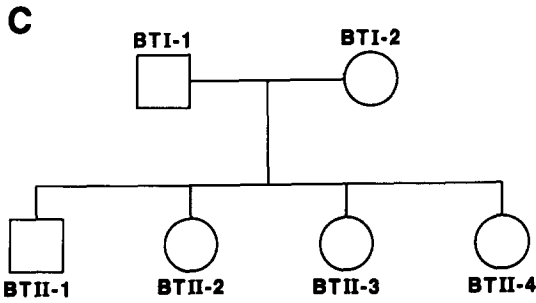
Our studies were undertaken to investigate the relationship between antitumor NK activity and homozygosity for extended haplotypes or fragments of them to (a) determine whether or not genes in the MHC control NK cell activity; (b) to localize the region containing the NK effector gene(s); and (c) to determine whether low NK activity was the result of low NK cell number. We have confirmed the relationship between homozygosity for extended haplotypes and low NK activity and extended our findings to show that fragments heterozygous for some specific extended haplotypes may also carry the low NK phenotype. Although our family studies are preliminary, the results suggest that recessive gene(s) controlling NK activity are localized in a region close to HLA-(C)B and they may act by specifying low NK cell number or/and activity. We found that high responders are primarily in the MHC heterozygous group. The low responders were



Code	Haplotypes	LU
SBI-1	[A2, Cwx, B44, SC30, DR4] A32, Cwx, B14, <u>SC31</u> , DR7	68
SBI-2	A2, <u>Cw4, B35, SC30</u> , DR1 [A24, Cw4, B35, SC30, DR4]	1
SBII-1	[A2, Cwx, B44, SC30, DR4] A2, <u>Cw4, B35, SC30</u> , DR1	76
SBII-2	[A2, Cwx, B44, SC30, DR4] A24, <u>Cw4, B35, SC30</u> , DR4	79

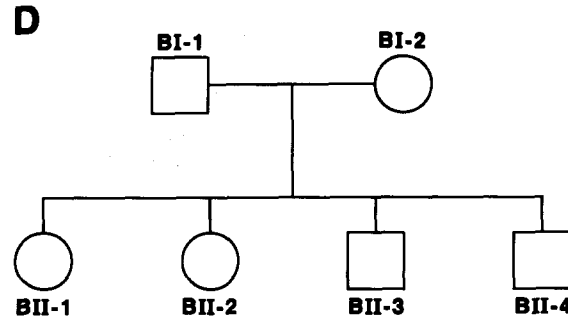


Code	Haplotype	LU
CI-1	A2, Cwx, B49, <u>SC01</u> , DR4 A3, <u>Cw4, B35, SC31</u> , DRx	36
CI-2	A3, Cwx, B40, SC0x, DR8 A3, <u>Cw4, B35, FC31</u> , DR2	89
CII-1	A2, Cwx, B49, <u>SC01</u> , DR4 A3, Cwx, B40, <u>SC01</u> , DR8	33
CII-2	A3, <u>Cw4, B35, SC31</u> , DRx A3, <u>Cw4, B35, FC31</u> , DR2	4
CII-3	A3, <u>Cw4, B35, SC31</u> , DRx A3, <u>Cw4, B35, FC31</u> , DR2	5
CII-4	A3, <u>Cw4, B35, SC31</u> , DRx A3, <u>Cw4, B35, FC31</u> , DR2	71



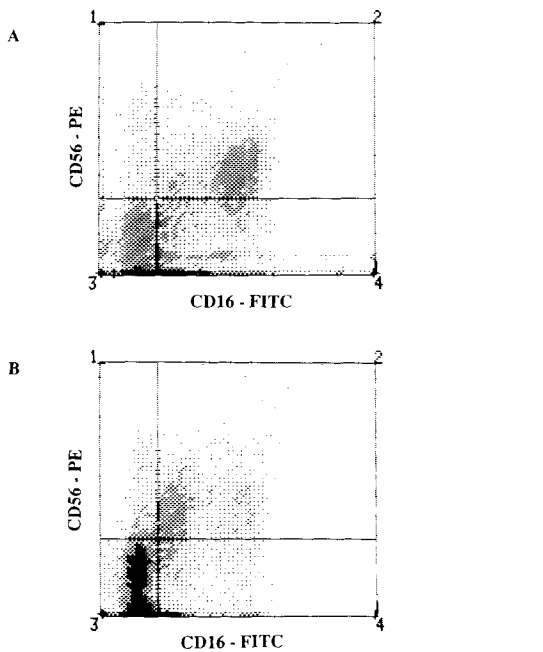
Code	Haplotypes	LU
BTI-1	A2, Cwx, B51, <u>SC31</u> , DR6 A24, Cwx, B49, FC01, DR4	NT
BTI-2	A2, Cwx, B51, <u>SC31</u> , DR6 A25, Cwx, B18, <u>SB42</u> , DR2	NT
BTII-1	A2, Cwx, B51, <u>SC31</u> , DR6 A2, Cwx, B51, <u>SC31</u> , DR6	4
BTII-2	A2, Cwx, B51, <u>SC31</u> , DR6 A2, Cwx, B51, <u>SC31</u> , DR6	1
BTII-3	A2, Cwx, B51, <u>SC31</u> , DR6 A2, Cwx, B51, <u>SC31</u> , DR6	3
BTII-4	A24, Cwx, B49, FC01, DR4 A2, Cwx, B51, <u>SC31</u> , DR6	17
BTII-5*	A2, Cwx, B51, <u>SC31</u> , DR6 A25, Cwx, B18, <u>SB42</u> , DR2	22

\* Cousin



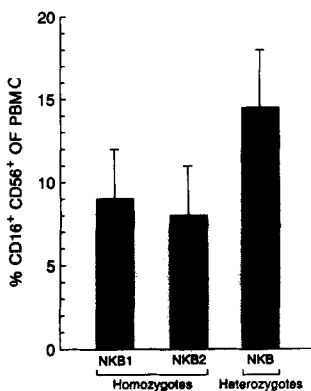
Code	Haplotypes	LU
BI-1	[A1, Cw7, B8, SC01, DR3] [A2, Cw5, B44, SC30, DR4]	6
BI-2	[A1, Cw7, B8, SC01, DR3] [A23, Cwx, B44, FC31, DR7]	5
BII-1	[A1, Cw7, B8, SC01, DR3] [A1, Cw7, B8, SC01, DR3]	1
BII-2	[A2, Cw5, B44, SC30, DR4] [A23, Cwx, B44, FC31, DR7]	3
BII-3	[A2, Cw5, B44, SC30, DR4] [A1, Cw7, B8, SC01, DR3]	3
BII-4	[A1, Cw7, B8, SC01, DR3] [A23, Cwx, B44, FC31, DR7]	7

**Figure 1.** (A-D) Family trees. HLA markers on each haplotype are listed below the tree. Extended haplotypes are shown in brackets. Fragments of extended haplotypes are underlined. Cytotoxic activity is given in terms of LU as defined in the text.



**Figure 2.** Two-color immunofluorescence analysis of PBMC. Cells were analyzed for simultaneous expression of CD16 and CD56 antigens by a direct immunofluorescence method. Directly conjugated mAbs anti-Leu 11b-FITC and anti-Leu 19-PE were used in this assay. Background fluorescence was determined by incubating cells with a nonreactive FITC-conjugated antibody and a nonreactive PE-conjugated antibody. The x-axis is green fluorescence and the y-axis is red fluorescence. 10,000 cells per sample were analyzed. The four quadrants (Qa) represent Qa-1, CD16<sup>-</sup>CD56<sup>+</sup>; Qa-2, CD16<sup>+</sup>CD56<sup>+</sup>; Qa-3, CD16<sup>-</sup>CD56<sup>-</sup>; and Qa-4, CD16<sup>+</sup>CD56<sup>-</sup>. Actual values as percentage of total PBMC with CD16 and CD56 are (A) Qa-1: 1.6, Qa-2: 19.1, and Qa-4: 4.6; (B) Qa-1: 4.7, Qa-2: 9.5, and Qa-4: 4.3.

found with higher frequency among HLA-(C)B homozygotes. Based on NK activity of unrelated individuals, at least three complementation groups: NKB1, NKB2, and NKBx were defined. From the frequencies of HLA-B fragments of different extended haplotypes, we estimate that no more than 30% of individuals in the Caucasian population have low antitumor NK activity.



**Figure 3.** Phenotypic analysis of NK cell subset (CD16<sup>+</sup>CD56<sup>+</sup>) in PBMC of individuals homozygous for the putative NK genes, NKB1 and NKB2, and heterozygous for these alleles. The results are presented as the mean percentage of positive cells  $\pm$  SD for each group as identified by simultaneous two-color immunofluorescence analysis of 10,000 cells. The mean values of CD16<sup>+</sup>CD56<sup>+</sup> cells as percentage of PBMC for homozygotes and heterozygotes for NKB1 and NKB2 are  $8.8 \pm 3.2$  ( $n = 8$ ),  $7.9 \pm 3.0$  ( $n = 6$ ), and  $14.4 \pm 3.5$  ( $n = 27$ ), respectively.

Much of the information on the genetics of NK activity against tumor cells comes from studies in the murine system (25–28). The NK cell population is heterogeneous (29, 30); high NK activity is inherited in a dominant fashion and is linked to the H-2D end of the MHC region (27, 28). NK cells are the effectors of hybrid resistance (26, 31). Hybrid resistance in mice is under the control of Hh genes which are inherited recessively (32–34). NK cells also recognize an antigen present on hematopoietic cells (32, 34). Although the nature of this target antigen is still unknown, it has been suggested that the Hh genes control the expression of the target antigens (35). Specific NK cell subsets recognize allele-specific Hh-1 antigens on hematopoietic cells (36, 37). These antigens may be responsible for the genetic specificity observed in hybrid resistance. The Hh genes are located close to the H-2D and S regions of MHC and are polymorphic (28, 33–35). These genes appear to be distinct from the H-2D/L alleles (38), although evidence from studies of transgenic mice suggests that the gene controlling hybrid resistance in some strains may itself be the H-2D allele (39).

The existence of hybrid resistance in humans is not known. Allogeneic reaction to hematopoietic cells by NK clones has been used as an in vitro model to study the genetic control of NK cell-hematopoietic cell interactions (2). These studies revealed that the “susceptibility to lysis” phenotype of PHA blasts of normal cells is inherited recessively and the susceptibility or resistance to lysis phenotype segregated with HLA haplotypes (2, 3). Based on our observation that low activity is associated with homozygosity at the HLA, -(C)B loci, suggesting recessive inheritance, we hypothesize that, as in mice, individuals homozygous for NKB alleles will express NK target determinants and will be sensitive to lysis by NK clones, and that individuals heterozygous for different NKB alleles will lack expression of NK target determinants and hence will be resistant to lysis by NK cells. When we reanalyzed the HLA data of the family reported earlier by Ciccone et al. (2), we observed that the T cells from 16 out of the 17 individuals resistant to lysis by NK clones were heterozygous for the NKB1, NKB2, or NKBx complementation groups. This is in keeping with the hypothesis that heterozygosity for these complementation groups that produce high NK activity also determine or mark resistance to lysis. The data reported by Ciccone et al. (2) also show that homozygosity for HLA-B and not DR alleles leads to the expression of NK target antigens, again suggesting that the NK target-determining genes map to the same HLA-(C)B region as NK cell number-determining genes. Our estimate of the upper limit of the frequency of individuals with low NK activity is close to that of the frequency of individuals carrying the phenotype for susceptibility to lysis as reported by Ciccone et al. (2). Furthermore, it has been shown that a subset of NK cells recognize target cells homozygous for HLA-B7, B8, B18, B44, B57, and B60 alleles corresponding to the NKB1 complementation group (40). Thus, the pattern of resistance or susceptibility to lysis phenotypes in these studies fits the predictions of our genetic model of NK polymorphism, and provides independent evidence for the existence of NKB complementation groups determining NK cell number and activity



as shown by the present studies. Whether or not these genes for different activities not only colocalize but are the same remains to be shown.

Another important finding in our current study is that individuals homozygous for NKB complementation groups have a lower NK cell number which may account for low activity. In a study using mice homozygous for a mutant  $\beta_2$ -microglobulin allele, it was observed that there was a significantly reduced cytotoxic activity of splenic NK cells against tumor cells as well as class I-deficient T cell blasts (41). The mechanism responsible for this reduced activity of NK cells is not known. The low activity and reduced frequency of NK cells in homozygous individuals in our study could be due to either partial deletion or a defect in differentiation, or both, during development of NK (CD16<sup>+</sup> CD56<sup>+</sup>) cells in vivo. In NKB homozygous individuals, the development of the class I-related component of NK activity may be defective, resulting in the lower frequency of NK cells or to a decrease in lytic activity per cell. Since the killing of target cells involves recognition of and binding to the target antigens and subsequent release of lytic molecules by effector cells, the signal for the release of lytic molecules may be under the control of class I-linked Hh genes. Better understanding of structure and function of NK receptors and associated molecules involved in signal transduction will help in understanding the mechanisms of class I-related regulation of NK activity. Because recognition of self-MHC class I alleles provides a negative signal to NK cells, it has been suggested that a positive signal selection step during NK cell development may be responsible for defining self-MHC class I (41). Recent studies showed that the expression of the Ly-49 antigen, a molecule expressed on a subset of NK cells in mice (42), was strongly downregulated in B10 (H-2<sup>b</sup>) NK cells obtained from mixed allogeneic chimeras, suggesting that differentiation of NK cells is indeed determined by the MHC (43).

To explain the recessive inheritance of Hh antigen expression, the existence of codominant transacting regulatory genes has been proposed (34, 35, 44). According to this hypothesis, the expression of the Hh-1 hybrid resistance target pheno-

type is governed by the presence or absence of the down-regulatory genes. One possible candidate molecule as a target subjected to downregulatory gene action could be MHC class I antigens. Tumor cells or class I-deficient cells are highly susceptible to NK-induced lysis (41, 45-48). When these class I-deficient cells were transfected with class I genes, the transfectants became resistant to lysis, suggesting that the expression of class I antigens on target cells causes resistance to lysis. Using site-directed mutagenesis, it was shown that the presence of the residue Asp at position 74 within the peptide-binding groove of a class I molecule renders the target cells resistant to lysis by NK cells (47). Also, it was reported that, in mice, Ly-49<sup>+</sup> cells bind the MHC class I alloantigen H-2D<sup>d</sup> but not other class I antigens and protect from lysis (49). More recently, it was reported that the presence of amino acids at positions 77 (Ser) and 80 (Asn) of HLA-Cw3 confers resistance to lysis by some alloreactive NK clones which belong to a subpopulation of NK cells (50). This suggests that NK cells possess receptors which specifically recognize and bind peptides that are part of the class I molecules and protect the target cells from lysis. To explain the protection of target cells from NK-induced lysis, it has been suggested that the binding of some NK cells to a class I-associated target structure induces a negative signal which protects cells from NK-induced lysis. This hypothesis may apply to at least some human NK cells, as it was observed that a mAb, anti-p58, which recognizes a subset of NK cells which specifically binds a determinant present on HLA-C antigen or associated with it, can block or abrogate the resistance to NK cell-induced lysis of the appropriate target cells (51, 52). Further studies demonstrated that the presence of HLA-B and HLA-C alleles confer more protection than HLA-A molecules (53). It is likely that some, but not all, alleles of the class I loci may act in the protection of targets from NK cell-induced lysis.

We suggest that the information in this paper will enable us to further investigate the genetics of NK cell activity in humans. This, it is hoped, will lead to an understanding of Hh genetics in humans. Such findings could be of importance in bone marrow transplantation.

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