

IDENTIFICATION OF A SUBSET OF MURINE  
NATURAL KILLER CELLS THAT MEDIATES REJECTION OF  
*Hh-1<sup>d</sup>* BUT NOT *Hh-1<sup>b</sup>* BONE MARROW GRAFTS

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NK cells are large granular lymphocytes that do not rearrange TCR or Ig genes (1). They kill susceptible tumor or virus-infected cells *in vitro* without any MHC restriction (2, 3), regulate antibody synthesis (4, 5), and mediate rejection of bone marrow cells (BMC)<sup>1</sup> in irradiated mice (6, 7). Whether these functions are performed by all NK cells or by distinct subsets is unknown. Unlike tumor cell lysis, NK-mediated rejection of BMC is highly specific, involving recognition of the *H-2* linked hemopoietic histocompatibility-1 (*Hh-1*) antigen (8). Because of the fine specificity of *Hh-1* recognition, it is possible that NK cell-mediated BMC rejection occurs through distinct subsets of NK cells that have receptors for Hh antigens.

The genes for the major Hh antigens, *Hh-1*, map to the murine MHC (*H-2*) between the *S* and *D* regions (9), but they do not appear to be class I genes (10). There are five *Hh-1* antigens that can be expressed and seven different combinations of the five antigens have been identified in mice (11). Mice reject bone marrow grafts that express incompatible Hh antigens, if the recipient mice have the necessary non-MHC-linked responder genes (12). *Hh-1* expression is recessively inherited (8) presumably because *Hh-1* expression is regulated by dominant *trans*-acting down regulatory genes (13). Consequently, F<sub>1</sub> hybrid mice express only those Hh antigens that are expressed by both of their parents, so they are often able to recognize parental *Hh-1* antigens as foreign and reject parental BMC grafts, a phenomenon known as "hybrid resistance."

In this report, data are presented describing a new mAb, SW5E6, which binds to ~50% of murine NK cells. Administration of SW5E6 antibodies *in vivo* eliminated the ability of recipient mice to reject *Hh-1<sup>d</sup>* but not *Hh-1<sup>b</sup>* BMC. This indicates that the recognition of different *Hh-1* antigens may occur by distinct subsets of NK cells *in vivo*. The characterization of the 5E6<sup>+</sup> subset of NK cells and the possible role of the 5E6 molecule in *Hh-1<sup>d</sup>* recognition is discussed.

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<sup>1</sup> *Abbreviations used in this paper:* BMC, bone marrow cells; BRC, baby rabbit complement; SCID, severe combined immune deficiency.

## Materials and Methods

**Animals.** All animals were bred and maintained in the mouse colony at the University of Texas Southwestern Medical Center, except for the strains 129/J and B10.BR which were purchased from The Jackson Laboratory (Bar Harbor, ME).

**Production of mAbs.**  $1.25 \times 10^6$  purified and rIL-2 (Cetus Corp., Emeryville, CA) propagated NK1.1<sup>+</sup> cells (1) from C57BL/6 mice (*H-2<sup>b</sup>*) were injected into a 129/J mouse (*H-2<sup>b</sup>*) intrasplenically (14). The animal was immunized intrasplenically 8 wk later with  $1.5 \times 10^6$  purified and rIL-2 propagated NK1.1<sup>+</sup> cells from C57BL/6 mice. 3 d after the final injection, the spleen was removed, a cell suspension was made, and the cells were mixed with murine myeloma SP2/0 cells at a ratio of 5:1. The cells were pelleted at 800 *g* for 2 min, and the supernatant was discarded. Polyethelene glycol (0.4 ml PEG 1000, British Drug House, Poole, UK) was added, and the cells were resuspended with a pipette for 50 s. The tube was closed and rolled on the counter top for 40 s. 1 ml of hybridoma media (DME, 10% NCTC 109; Irvine Scientific, Santa Ana, CA), 0.15 g/liter oxalacetic acid, 8.33 mg/liter bovine pancreatic insulin, 13.6 mg/liter hypoxanthine, 3.9 mg/liter thymidine, 0.22 mg/liter glycine, penicillin/streptomycin) containing 20% FCS was added to the cells. The cell suspension was then added, dropwise, to a flask containing 38 ml of media containing 20% FCS and incubated overnight at 37°C in a 5% CO<sub>2</sub>/95% air atmosphere. The following day, the cells were pelleted at 800 *g* for 4 min, the supernatant discarded, and the cells were resuspended in HAT (hybridoma media with 0.16 mg/liter aminopterin) containing media. The cells were placed in 96-well round-bottomed plates. The wells were examined for growth after 10 d, and the supernatants from the wells containing hybridomas were tested for their ability to bind to purified C57BL/6 NK1.1<sup>+</sup> cells but not to C57BL/6 thymocytes by flow cytometry. The hybridomas that produced antibodies that bound to NK cells but not to thymocytes were isolated and cloned twice by limiting dilution for further analysis.

**Generation of IL-2-activated NK Cells.** Nylon wool nonadherent spleen cells were cultured in 500 U/ml rIL-2. After 2 d, the nonadherent cells were removed, and the adherent cells washed by rinsing the flask with 5 ml of RPMI 1640 media three times. The nonadherent cells were pelleted and the supernatant was added back to the adherent cells in the flask. These adherent cells were cultured for another 5–7 d. Fresh medium containing rIL-2 was added on the fourth day, if necessary. The cells were harvested and analyzed for cell surface phenotype or used for cell sorting. The cells obtained from this culture were routinely 80–100% NK1.1<sup>+</sup>.

**Cell Surface Analysis.** For staining, rIL-2-activated NK cells or nylon wool nonadherent spleen cells were washed three times in the staining buffer, 1% fraction 5 BSA (Sigma Chemical Co., St. Louis, MO) in PBS. Approximately  $5 \times 10^5$  cells were used per sample and were stained in 100  $\mu$ l. Primary incubation was with staining buffer alone (negative control) or the appropriate dilution of specific antibody (positive) for 30 min at 4°C followed by two washes in staining buffer. Primary antibodies included: PK136 (anti-NK1.1), SW3A4, SW5E6, 500A2 (anti-T3; provided by Dr. J. Allison, University of California at Berkeley), and H02.2 (anti-Ly-2). In the secondary incubation, all samples were stained with 50  $\mu$ l of the appropriate species and isotype-specific secondary antibodies for 30 min at 4°C (all from Jackson Laboratories, West Grove, PA). After two further washes in staining buffer, the samples were resuspended in 0.4 ml of staining buffer and analyzed on a FACSTAR flow cytometer (Becton Dickinson & Co., Mountain View, CA).

**Cell Sorting and Propagation.** Cells were stained as described above and sorted on a FACSTAR flow cytometer. The sorted cells were propagated in medium containing 500 U/ml rIL-2 and 2-ME ( $5 \times 10^{-5}$  M) and were plated at 1–2  $\times 10^4$  cells per well in 96-well, round-bottomed microtiter plates, as previously described (1).

**NK Cell Assay.** Spleens were excised from animals, and a cell suspension was made. The red blood cells were lysed by hypotonic shock. Aliquots of  $5 \times 10^6$  cells were treated with either SW3A4 ascites (1:50) and baby rabbit complement (BRC), SW5E6 (1:20) and BRC, or BRC alone. The samples were incubated for 45 min at 37°C. After washing, the cells were assayed for their ability to lyse <sup>51</sup>Cr labeled YAC-1 tumor cells as previously described (15).

**Treatment of Animals with Antibodies.** Animals received a single injection of antibodies intraperitoneally 3 d before lethal irradiation or lung clearance assay, with the exception of anti-asialo GM1 (Wako Chemical Co., Dallas, TX) which was given intravenously 1 d before lethal irradiation. Antibodies were prepared by ammonium sulfate (50%) precipitation of tissue culture supernatants. 1.5 mg of SW5E6 or control antibody, 22B5 (antiarsonate; kindly provided by Dr. Donald Capra, University of Texas Southwestern Medical Center, Dallas, TX), preparations were given to each recipient in the treated groups, and 1 mg of anti-NK1.1 was given to each animal in the anti-NK1.1-treated group.

**Stem Cell Growth Assay.** All mice were kept in conventional facilities but were given acidified drinking water containing neomycin to prevent sepsis after whole-body irradiation. The animals were exposed to 8.6 Gy of  $^{137}\text{Cs}$  gamma irradiation (Gamma Cell 40; Atomic Energy Ltd., Ottawa, Ontario). Within 6 h, the mice were infused with BMC in a lateral tail vein. Growth of donor-derived cells was assessed 5 d after the transfer of marrow cells. 60 min after each mouse was given  $10^{-7}$  M fluoro-deoxyuridine (0.15 ml i.p.) to inhibit endogenous deoxythymidic acid synthesis, each mouse was injected with 0.5  $\mu\text{Ci}$  5-iodo- $^{125}\text{I}$ -2'-deoxyuridine (IUdR), a thymidine analogue and specific DNA precursor. The spleens were removed 16 h after IUdR injection, and the  $^{125}\text{I}$  radioactivity was measured. The geometric mean (95% confidence limits) splenic uptake (%) values of injected IUdR were calculated for each group (15). All values have been normalized to the syngeneic control uptake value (100%).

**Lung Clearance Assay.** YAC-1 tumor cells ( $30 \times 10^6$ ) were incubated in 2 ml complete medium with 25  $\mu\text{g}/\text{ml}$  5-fluorodeoxyuridine for 15 min and then 30  $\mu\text{Ci}$  of  $^{125}\text{I}$  IUdR was added, and the cells were incubated for an additional 75 min at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Cells were washed three times in HBSS and adjusted to  $2 \times 10^6/\text{ml}$ . In a volume of 0.5 ml,  $10^6$  YAC-1 cells ( $3\text{--}5 \times 10^5$  cpm) were injected into a lateral tail vein of individual mice. At 3 h after injection the mice were killed, their lungs were removed, and the  $^{125}\text{I}$  radioactivity was measured. Results are expressed as the geometric mean of the percentage recovery of the injected radioactivity.

**Lactoperoxidase-catalyzed Radioiodination.** Nylon wool nonadherent spleen cells cultured in IL-2, as described above, were harvested and washed in PBS. The cells were labeled with  $\text{Na}^{125}\text{I}$  (Amersham Corp., Arlington Heights, IL) as described (16). Labeled cells were washed in PBS, were lysed in 0.5% NP-40 (Accurate Chemical and Scientific Corp., Hicksville, NY), and were centrifuged at 2,500 g to remove nuclei. 5% FCS was added to inhibit proteolysis.

**Immunoprecipitation and SDS-PAGE.** Lysates from radioiodinated cells were incubated with Staphylococcus aureus coated Pansorbin Cells (Calbiochem-Behring Corp., La Jolla, CA) and rabbit antisera to mouse immunoglobulins (RAMIGs) (Dako Corp., Santa Barbara, CA) for 30 min at  $4^\circ\text{C}$  to remove nonspecific binding components. Aliquots of the precleared cells were incubated with specific antibodies for 2 h at  $4^\circ\text{C}$  and Pansorbin cells, coated with RAMIGs, were added for an additional 40 min. The cells were pelleted and washed three times with Tris-buffered saline (pH 8) containing 0.5% NP-40, 0.2% sodium deoxycholate, and 0.1% SDS. The bound proteins were eluted from the cells by boiling them in 2% SDS buffered with Tris (pH 6.8). Eluates were reduced with 10% 2-ME and were analyzed by SDS-PAGE according to the method of Laemmli (17). Slab gels were dried and were exposed to x-ray film overnight at  $-70^\circ\text{C}$ .

**Statistics.** Control samples of 1:100 dilution of the preparation of IUdR were counted. The purity of the IUdR was 90%. The formula used to calculate percent IUdR incorporation was:  $[\text{I}^{125}\text{ spleen or lung (cpm)}]/[\text{standard (cpm)} \times \text{purity (0.9)}]$ . The computed values were converted to  $\log_{10}$  numbers to calculate values of mean, SD, and SEM for each group. The geometric means (95% confidence limits) were calculated. Here the arithmetic mean IUdR uptake (%) values of the syngeneic groups (for BMC transplant experiments) were considered 100% growth, and each individual value was calculated as percent growth, which is  $[\text{experimental}/\text{mean syngeneic}] \times 100$ . The data are represented on an arithmetic scale and mean  $\pm$  SEM values are shown. There were five to seven animals per group, and parametric, nonparametric, and Student's *t* test were performed to determine if the mean values were significantly different ( $p < 0.05$ ).

### Results

**SW5E6 Antibodies Bind to ~40–50% of Murine NK Cells.** To determine whether rejection of Hh incompatible BMC was mediated by subsets of NK cells, mAbs against NK cells were produced by immunizing 129/J (*H-2<sup>b</sup>*) mice with purified NK1.1<sup>+</sup> cells from C57BL/6 (*H-2<sup>b</sup>*) mice. NK1.1 is a pan NK-specific antigen present on murine NK cells (18). Several clones were isolated that produced antibodies that bound exclusively to NK cells but not to other lymphocytes (data not shown). None of the antibodies stained the tumor cell lines EL4 (thymoma), RAW 309.F.1.1 (macrophage), P815 (mastocytoma), CTLL-2 (CTL), YAC-1 (thymoma), or 70Z (pre-B cell) (data not shown). The staining profiles for two of these mAbs on C57BL/6 NK1.1<sup>+</sup> cells are shown in Fig. 1. SW3A4 is an IgM antibody that binds to all NK cells (Sentman, C. L., J. Hackett, V. Kumar, and M. Bennett, manuscript in preparation). SW5E6, on the other hand, is an IgG2a antibody that binds to ~50% of purified IL-2-propagated NK1.1<sup>+</sup> cells. In experiments not shown, SW5E6 was found to bind ~50% of fresh, sorted splenic NK1.1<sup>+</sup> cells as well. 5E6<sup>+</sup> cells were purified by cell sorting, and a summary of those experiments is shown in Table I. When sorted cells were cultured in rIL-2 and analyzed for cell surface antigen expression, the 5E6<sup>+</sup> cells obtained all expressed 5E6 and NK1.1 but not T3 (CD3), and their phenotype remained stable after in vitro propagation in IL-2. The 5E6<sup>-</sup> cells obtained consisted of 5E6<sup>-</sup> NK cells and T3<sup>+</sup> T cells. Although most of the 5E6<sup>-</sup> cells were T cells, the 5E6<sup>-</sup> NK cells proliferated much better in 500 U/ml IL-2 than the T cells and accounted for almost 50% of the final population on day 7. Thus the 5E6<sup>+</sup> cells form a distinct and stable subset of murine NK cells.

**SW5E6 and Complement Treatment of Spleen Cells Results in a 50% Reduction in NK Cell Lytic Activity.** The data in Fig. 2 demonstrate that treatment of spleen cells with SW3A4 (which binds to all NK cells) and complement removed all NK lysis of YAC-1 tumor cells. In contrast, SW5E6 and complement removed some but not all of the NK activity in every mouse strain tested, C57BL/6 (*Hh-1<sup>b</sup>*), B10.A(3R) (*Hh-1<sup>d</sup>*),

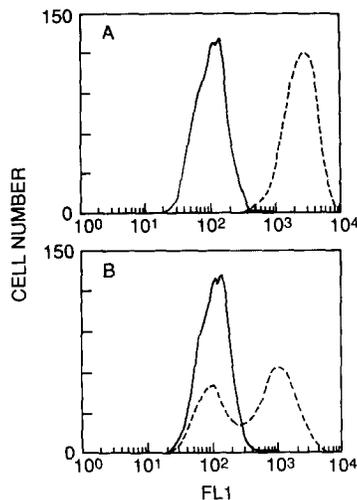


FIGURE 1. Fluorescence histograms of NK1.1<sup>+</sup> cells stained with (A) SW3A4 or (B) SW5E6 mAbs. The solid line in each histogram represents fluorescence with FITC labeled secondary alone, and the dotted line represents the fluorescence with SW3A4 or SW5E6 followed by the FITC secondary, respectively.

TABLE I  
Analysis of Sorted 5E6<sup>+</sup> Spleen Cells

| Exp. | Antibodies         | Sorted nylon wool nonadherent spleen cells* |                    |
|------|--------------------|---|--------------------|
|      |                    | 5E6 <sup>+</sup>                            | 5E6 <sup>-</sup>   |
| 1    | PK136 (anti-NK1.1) | 97  | 47                 |
|      | SW5E6              | 97  | 3.8                |
|      | SW3A4              | 96  | 47                 |
|      | 500A2 (anti-CD3)   | 3.0   | 53                 |
| 2    |                    | 5E6 <sup>+</sup>                            | NK1.1 <sup>+</sup> |
|      | PK136 (anti-NK1.1) | 100   | 100                |
|      | SW5E6              | 99  | 60                 |

C57BL/6 nylon wool nonadherent spleen cells were sorted into 5E6<sup>+</sup> and 5E6<sup>-</sup> fractions (Exp. 1) or 5E6<sup>+</sup> and NK1.1<sup>+</sup> fractions (Exp. 2). Sorted cells were placed in microtiter wells at 1-2 × 10<sup>4</sup> cells per well. The cells were stained with the various antibodies indicated after 6-7 days in 500 U/ml of rIL-2.

\* The numbers indicate the percent of sorted and cultured cells that stained positive with various antibodies.

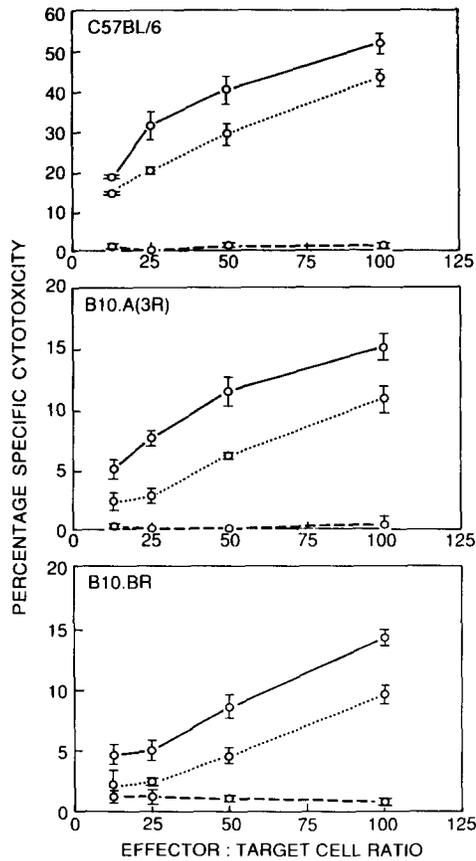


FIGURE 2. Lytic activity of 5E6<sup>-</sup> spleen cells against YAC-1. (· · · ·) Spleen cells treated with SW5E6 and complement, (- - -) spleen cells treated with SW3A4 and complement, and (—) spleen cells treated with complement alone.

and B10.BR (*Hh-1<sup>k</sup>*). Based on these data, it is evident that both 5E6<sup>+</sup> and 5E6<sup>-</sup> NK cells are capable of lysing YAC-1 tumor cells. The decrease in NK activity was ~50%, as assessed in lytic units, a finding that is consistent with the observation that approximately one-half of NK cells are 5E6<sup>+</sup>.

*Administration of SW5E6 Antibodies Inhibits Rejection of Hh-1<sup>d</sup> BALB/c BMC but not Hh-1<sup>b</sup> C57BL/6 BMC.* We next determined the role of 5E6<sup>+</sup> NK cells in the rejection of BMC grafts. The data in Fig. 3 demonstrate that animals given anti-NK1.1 (PK136), which has been previously shown to remove all NK cell activity in vivo

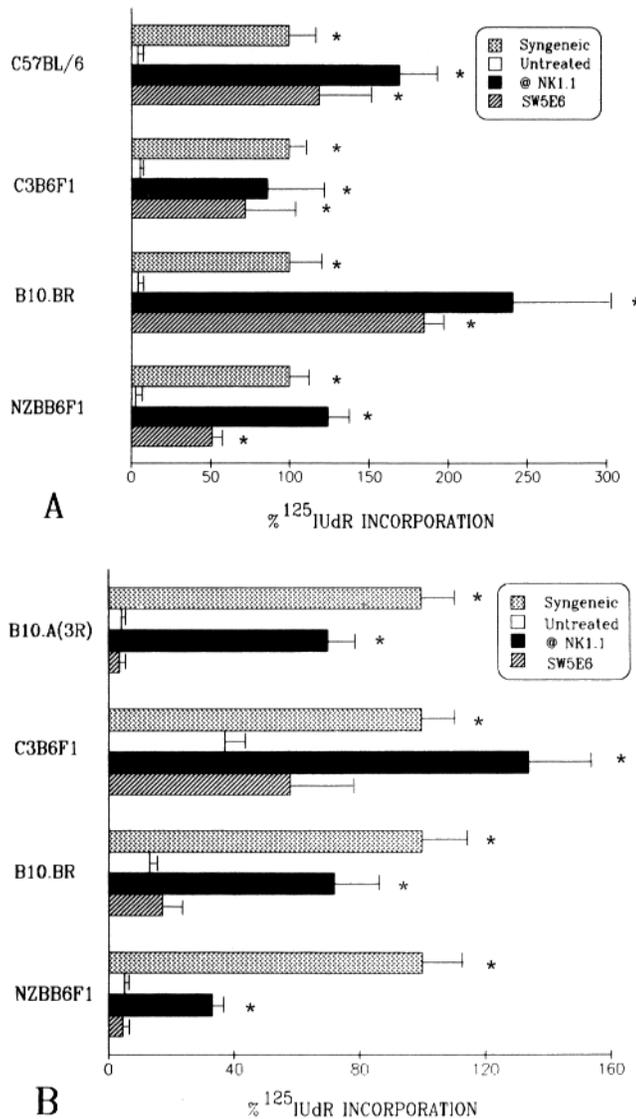


FIGURE 3. Effect of SW5E6 on the rejection of bone marrow cells. Percent <sup>125</sup>IUDR incorporation in mice challenged with either (A) BALB/c (*Hh-1<sup>d</sup>*) or (B) C57BL/6 (*Hh-1<sup>b</sup>*) BMC. All recipient mice received 5 × 10<sup>6</sup> BMC, except C57BL/6 and B10.A(3R), which received 3 × 10<sup>6</sup> BMC. Recipient mice were syngeneic BALB/c (A) or C57BL/6 (B), or *Hh-1* incompatible recipients indicated in the figures which were untreated, treated with anti-NK1.1 or treated with SW5E6 3 d before lethal irradiation and bone marrow cell infusion. Data have been normalized to the syngeneic control group values for each BMC transplant. An asterisk indicates groups that are significantly different than the untreated control group.

(19), were no longer able to reject  $H-2^d/Hh-1^d$  BALB/c or  $H-2^b/Hh-1^b$  C57BL/6 BMC. In contrast, treatment of irradiated C57BL/6, (C3H  $\times$  B6) $F_1$ , B10.BR, and (NZB  $\times$  B6) $F_1$  recipient mice with SW5E6 antibody prevented rejection of  $Hh-1^d$  BALB/c BMC (Fig. 3 A) but had no detectable effect upon the ability of B10.A(3R), (C3H  $\times$  B6) $F_1$ , B10.BR, and (NZB  $\times$  B6) $F_1$  hosts to reject  $Hh-1^b$  C57BL/6 BMC (Fig. 3 B). Since the last three sets of recipients are capable of rejecting both  $Hh-1^d$  and  $Hh-1^b$  BMC grafts, these data force the conclusion that  $5E6^+$  NK cells are necessary for rejection of  $Hh-1^d$  but not  $Hh-1^b$  BMC grafts.

*SW5E6 Does Not Mediate its Effects by Interacting with Stem Cells.* It was possible that the SW5E6 antibodies were interacting with  $Hh-1^d$  BALB/c stem cells, but not the  $Hh-1^b$  C57BL/6 stem cells, and in some way protecting them from host NK cells. To test this possibility, BALB/c BMC were treated with SW5E6 and complement in vitro, then transplanted into syngeneic BALB/c hosts and the stem cell activity was measured 5 d later. The results from this experiment are shown in Fig. 4. Treatment of BMC with SW3A4 (pan-NK) or SW5E6 and complement had no effect on the stem cell activity of BALB/c BMC compared with those treated with complement alone. However, treatment with anti- $H-2D^d$  antibodies did reduce stem cell activity. These results indicate that SW5E6 does not interact with stem cells to mediate its effect in vivo.

*SW5E6 Treatment Partially Inhibits the Ability of Mice to Lyse YAC-1 Tumor Cells In Vivo.* It was of interest to determine the effect of SW5E6 treatment on in vivo NK activity. The ability of an animal to clear tumor cells from their lungs is considered to be a measurement of in vivo NK activity (20). The data is shown in Fig. 5. When SW5E6 was injected into C57BL/6 ( $Hh-1^b$ ), B10.BR ( $Hh-1^b$ ), or B10.A(3R) ( $Hh-1^d$ ) mice, 3 d before assay, it resulted in a partial loss of their ability to clear YAC-1 tumor cells from their lungs. The groups treated with control antibodies 22B5 (antiarsenate) had between 0.8 and 1.4% retention of  $^{125}I$  in their lungs. Groups treated with the pan-NK antibody, anti-NK1.1, had the largest retention of  $^{125}I$ -labeled YAC-1 cells in their lungs, between 17 and 36%. The groups treated with SW5E6 had between 3.7 and 8.0% retention of radiolabeled YAC-1 cells. SW5E6 had the least effect on lung clearance in the B10.A(3R) (which express  $Hh-1^d$ ) recipients and a greater effect in the strains C57BL/6 and B10.BR which do not express  $Hh-1^d$ .  $5E6^+$  NK cells are

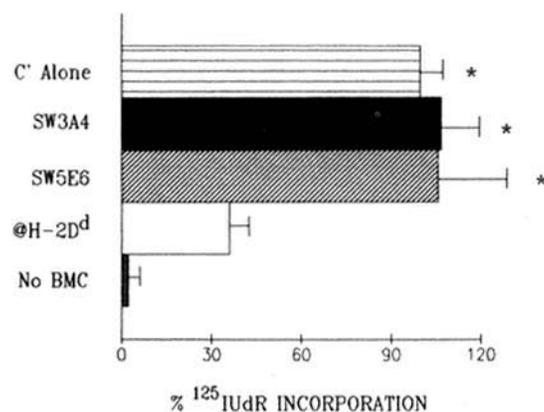


FIGURE 4. Effect of SW5E6 on BALB/c ( $Hh-1^d$ ) stem cell activity. Percent splenic  $^{125}IUdR$  incorporation in lethally irradiated BALB/c mice infused with  $2.5 \times 10^6$  syngeneic BALB/c BMC. The BALB/c BMC were treated in vitro, before infusion, with either baby rabbit complement alone (▨), SW3A4 and complement (■), SW5E6 and complement (▩), or anti- $H-2D^d$  and complement (□). One group received no BMC (▣). Data have been normalized to the complement alone control group ( $^{125}IUdR$  uptake was 0.57%). An asterisk indicates that groups are significantly different than the group that received no BMC.

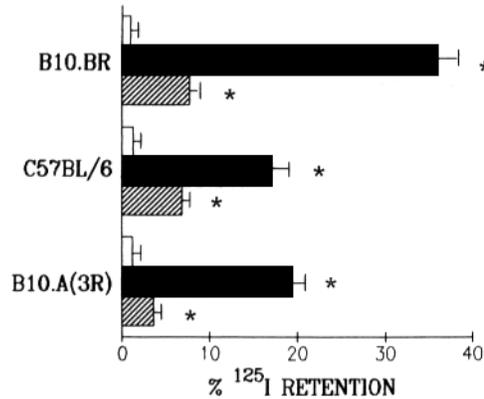


FIGURE 5. Effect of SW5E6 on lung clearance activity. Percent  $^{125}\text{I}$  retention in the lungs 3 h after intravenous infusion of  $^{125}\text{IUdR}$  labeled YAC-1 tumor cells. Recipient mice were treated with either control (22B5) ( $\square$ ), anti-NK1.1 ( $\blacksquare$ ), or SW5E6 antibodies ( $\text{▨}$ ) 3 d before assay. An asterisk indicates that groups are significantly different than the control group treated with 22B5.

present in similar numbers in strains of mice which do and do not express  $Hh-1^d$  (data not shown). These data indicate that both  $5E6^+$  and  $5E6^-$  NK cells are capable of lysing YAC-1 tumor cells in vivo.

*SW5E6 Treatment Differentially Inhibits the Ability of Mice to Lyse YAC-1 Tumor Cells In Vivo after Lethal Irradiation.* Transplantation of BMC is done in lethally irradiated recipients. The effect of SW5E6 treatment on in vivo NK activity in lethally irradiated mice may be different than that in unirradiated mice. Therefore the effect of SW5E6 on the ability of mice to clear YAC-1 cells from their lungs was measured in animals 1 d after lethal irradiation. The results for mice whose BMC do not express  $Hh-1^d$  are shown in Fig. 6 A and those for mice whose BMC do express  $Hh-1^d$  in Fig. 6 B. In all strains there was a partial loss of NK activity in vivo after lethal

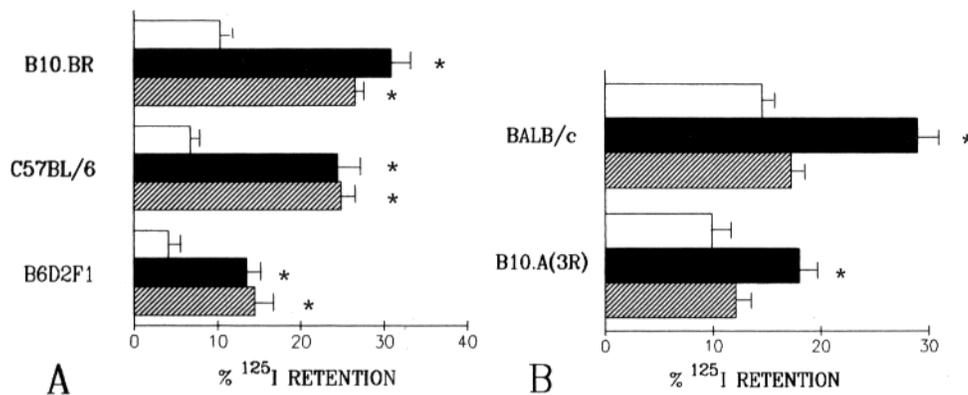


FIGURE 6. Effect of SW5E6 on lung clearance activity 1 day after lethal irradiation. Percent  $^{125}\text{I}$  retention in the lungs 3 h after intravenous infusion of  $^{125}\text{IUdR}$ -labeled YAC-1 tumor cells. Recipient mice were treated with either control (22B5) ( $\square$ ), anti-NK1.1 ( $\blacksquare$ , except BALB/c recipients, which were given anti-asialo GM1 2 d before assay), or SW5E6 antibodies ( $\text{▨}$ ) 4 d before assay. All mice received 800 rad 1 d before assay. Recipient mice in A are all non- $Hh-1^d$  strains, and recipient mice in B express  $Hh-1^d$ . An asterisk indicates that groups are significantly different than the control group treated with 22B5.

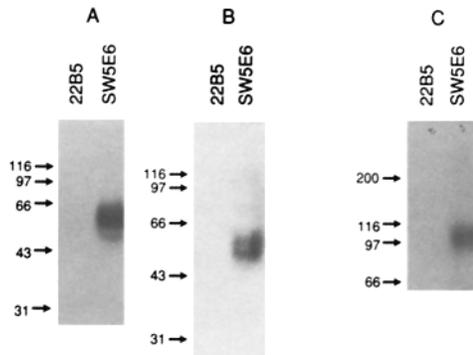


FIGURE 7. Immunoprecipitation of the molecule recognized by the SW5E6 antibodies from IL-2-activated NK cells. Immunoprecipitation of  $^{125}\text{I}$ -labeled NK cells using either 22B5 control antibody or SW5E6. The immunoprecipitable material was obtained from (A) C57BL/6 ( $Hh-1^b$ ) NK cells or (B) NZB ( $Hh-1^d$ ) NK cells and run by SDS-PAGE under *reducing* conditions. The data in C represent immunoprecipitable material from C57BL/6 NK cells run by SDS-PAGE under non-reducing conditions.

irradiation. The injection of anti-NK1.1 resulted in significant additional impairment in the ability to clear radiolabeled YAC-1 cells. However, the effects of SW5E6 antibodies differed in mice based upon the expression of  $Hh-1^d$ . In BALB/c and B10.A(3R) mice, which express  $Hh-1^d$ , administration of SW5E6 did not impair *in vivo* NK activity above that which resulted from irradiation alone. In contrast, SW5E6 antibodies had a significant additional effect on lung clearance ability, equivalent to that produced by treatment with anti-NK1.1 antibodies, in irradiated C57BL/6 ( $Hh-1^b$ ), B10.BR ( $Hh-1^k$ ), and B6D2F1 ( $Hh-1^{null}$ ) mice (which do not express  $Hh-1^d$ ).  $5E6^+$  NK cells are present both in mice that express  $Hh-1^d$  and mice that do not express  $Hh-1^d$  one day after lethal irradiation (data not shown). Thus it appears that  $5E6^+$  NK cells are not capable of lysing YAC-1 tumor cells after lethal irradiation in mice whose BMC express  $Hh-1^d$ .

*The 5E6 Molecule Exists on the Cell Surface as a Homodimer Consisting of Two 54,000 Mol Wt Subunits.* The data in Fig. 7 represent immunoprecipitation of the 5E6 molecule from C57BL/6 ( $Hh-1^b$ ) NK cells (Fig. 7, A and C) or NZB ( $Hh-1^d$ ) NK cells (7 B) and separated on SDS-PAGE under reducing (A and B) and nonreducing (C) conditions. The SW5E6 antibodies precipitate a protein of apparent molecular weight of 54,000 under reducing conditions in all strains tested, including C3H, (C3H  $\times$  C57BL/6) $F_1$ , and (NZB  $\times$  C57BL/6) $F_1$  mice (data not shown). Interestingly, under nonreducing conditions, the SW5E6 antibodies immunoprecipitate a molecule of 108–110  $\times 10^3$  mol wt, suggesting that the 5E6 molecule exists as a homodimer on the cell surface.

### Discussion

NK cells do not express B or T cell receptors, and they have been considered “nonspecific” effector cells. Yet, NK cells do exhibit specificity in the recognition of Hh antigens and it has been difficult to understand how they accomplish this. NK cells have Fc receptors for Ig and could potentially reject BMC grafts using “natural antibodies” via antibody-dependent cellular cytotoxicity (21). However, mice with severe combined immune deficiency (SCID), which are devoid of Igs, also specifically reject incompatible BMC, using NK cells (22). Thus, recognition may be a property of the NK cell itself. The data presented in this report, that reactivity against  $Hh-1^d$  antigens resides in a distinct subpopulation of NK cells, strongly support this

notion. Elimination of the 5E6<sup>+</sup> subset of NK cells abrogated the ability of inbred mice to resist an *Hh-1<sup>d</sup>* incompatible BMC graft, but their ability to resist an *Hh-1<sup>b</sup>* incompatible BMC graft was unaffected.

The observation that both 5E6<sup>+</sup> and 5E6<sup>-</sup> NK cells were capable of lysing YAC-1 tumor cells in vitro and in vivo, yet differ in their ability to reject *Hh-1<sup>d</sup>* and *Hh-1<sup>b</sup>* BMC, suggests that NK cells may use different mechanisms to recognize Hh antigens on BMC and "target structures" on tumor cells.

In addition to reactivity to allogeneic stem cells, NK cells are known to interact with autologous stem cells both in vitro and in vivo (23). Thomson et al. (24) reported that NK cells are capable of eliminating syngeneic stem cells in vivo after certain viral infections. Therefore it is particularly interesting that *Hh-1<sup>d</sup>* mice (BALB/c and B10.A(3R)), like *Hh-1<sup>b</sup>* and *Hh-1<sup>k</sup>* mice, possess functional 5E6<sup>+</sup> cells that can lyse YAC-1 cells (Figs. 2 and 5). Since 5E6<sup>+</sup> cells (*Hh-1<sup>d</sup>* reactive) exist in *Hh-1<sup>d</sup>* mice, some mechanism apparently prevents potentially self-reactive 5E6<sup>+</sup> cells from eliminating their own stem cells. This notion is supported by the differential effect of SW5E6 antibodies on in vivo NK activity in mice whose BMC do or do not express *Hh-1<sup>d</sup>*. When injected into mice, SW5E6 antibodies had a minimal effect on the clearance of YAC-1 cells in *Hh-1<sup>d</sup>*-type mice, but the effect in animals with other *Hh-1* types was much more pronounced (Fig. 5). This difference was more dramatic after lethal irradiation (Fig. 6, A and B). Thus it seems that in *Hh-1<sup>d</sup>* mice, the function of 5E6<sup>+</sup> NK cells is somehow downregulated, by mechanisms that remain to be explored. It will be interesting to investigate the role of 5E6<sup>+</sup> NK cells in the regulation of hemopoiesis in *Hh-1<sup>d</sup>* mice.

What would be the advantage of having distinct subsets of NK cells for *Hh-1* antigens? Distinct subsets of NK cells with different specificities for *Hh-1* antigens would allow the reactivity to an individual *Hh-1* antigen to be easily altered without affecting other necessary NK cell functions. There is no evidence yet that the 5E6 molecule itself is involved in the recognition of the *Hh-1<sup>d</sup>* antigen. Although the 5E6<sup>+</sup> subset appears to contain all *Hh-1<sup>d</sup>* reactive NK cells, it cannot be concluded that all 5E6<sup>+</sup> cells express receptors for *Hh-1<sup>d</sup>*. However, if in fact all 5E6<sup>+</sup> NK cells do express receptors for *Hh-1<sup>d</sup>*, it seems surprising that such a large percentage of NK cells would be devoted to recognition of *Hh-1<sup>d</sup>* but not to any of the other four *Hh-1* antigens. It is conceivable that receptors for *Hh-1* antigens on NK cells are not clonally distributed, i.e., NK cells may express more than one anti-*Hh-1* receptor. Allelic exclusion of Ig or TCR genes is necessary for the expression and regulation of B and T cell receptors that must recognize a potentially infinite number of foreign antigens, but NK cells involved in the rejection of BMC grafts only need to recognize five or less *Hh-1* antigens. Therefore, the mechanisms for the expression and regulation of NK cell receptors need not be similar to those used by other lymphocytes. The advantage of having NK cells able to recognize more than one *Hh-1* antigen would be that a larger amount of the NK cell pool could be responsive to each *Hh-1* specificity. Although we have so far only detected subsets of NK cells specific for the *Hh-1<sup>d</sup>* antigen, it will be interesting to investigate whether or not other functions attributed to NK cells can be placed into distinct subsets.

### Summary

NK cells demonstrate many immune functions both in vitro and in vivo, including the lysis of tumor or virus-infected cells and the rejection of bone marrow allografts.

However it remains unclear whether or not all NK cells can mediate these various functions or if NK cells exist in functionally distinct subsets. We have developed a new NK-specific mAb, SW5E6, which binds to ~50% of murine NK cells. The 5E6 antigen identifies a distinct and stable subset of NK cells and is expressed on about one-half of fresh or rIL-2-activated murine NK cells. Both 5E6<sup>+</sup> and 5E6<sup>-</sup> NK cells are capable of lysing YAC-1 tumor cells in vitro and in vivo. By treating animals with SW5E6, we demonstrate that the 5E6<sup>+</sup> subset is necessary for the rejection of *H-2<sup>d</sup>/Hh-1<sup>d</sup>* but not *H-2<sup>b</sup>/Hh-1<sup>b</sup>* bone marrow cells. Thus NK cells exist as functionally separable subsets in vivo.

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