Isabel Correa[△], **Laura Corral and David H. Raulet**

Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley

Multiple natural killer cell-activating signals are inhibited by major histocompatibility complex class I expression in target cells*

Several lines of evidence indicate that major histocompatibility complex class I molecules expressed by target cells can prevent natural killer cell (NK) lysis, possibly by engaging inhibitory receptors expressed by NK cells. On the other hand it is likely that NK cells must be activated to lysis by the recognition of unidentified NK target structures on target cells. To investigate the relationship between positive activation of NK cells by NK target structures *versus* inhibition by target cell class I molecules, we have examined various NK/target cell interactions for which the expression of inhibitory class I molecules by the target cells is known. The results suggest that specific properties of the target cell other than the absence of class I expression are necessary to activate NK-mediated lysis. Furthermore, different effector cell populations, *i.e.* freshly isolated *versus* interleukin-2 activated NK cells, differ in their capacity to kill class I-deficient lymphoblast target cells. In general, class I-deficient target cells that are resistant to direct lysis by a given NK population can be lysed by the NK cells when the reaction is mediated by antibody-dependent cellular cytotoxicity (ADCC). Most significantly, all types of NK-mediated lysis of lymphoblasts, of tumor cells and of almost any target by ADCC can be inhibited by appropriate class I gene expression in the target cell. These results suggest a model in which lysis by NK cells must be triggered by any one of a set of distinct target cell ligands, but that all of these signals can be overruled by class I-mediated inhibition.

1 Introduction

A variety of evidence indicates that MHC class I molecules play a role in regulating natural killer cell activity. NK cells from normal mice mediate rejection of syngeneic class I-deficient tumor cells [1, 2] and bone marrow cells [3] *in vivo*, as well as lysis of class I-deficient cells *in vitro* [1, 4–7]. Class I expression renders these cells resistant to lysis by syngeneic NK cells.

In some cases, the inhibitory effects of MHC class I molecules have a clear allelic specificity. An example is the phenomenon of hybrid resistance, in which NK cells in H-2 heterozygous animals reject parental bone marrow transplants [8, 9]. In at least some cases, this phenomenon apparently results from the failure of the donor cells to express allelic class I MHC molecules that can inhibit the rejection process [10]. A second example involves human NK clones that lyse allogeneic PHA blasts, apparently due to the failure of the allogeneic cells to express specific allelic HLA-encoded molecules [11–13]. Finally, another recently described example involves the subset of murine NK cells that express the Ly49 marker, which represents about 15 % of freshly isolated NK cells or of IL-2-activated NK cells (adherent lymphokine-activated killer, or A-LAK, cells) [14]. Ly49$^+$ A-LAK cells lysed H-2^b tumor cells but not H-2^d or H-2^k tumor cells. Lysis of H-2^b tumor cells was prevented by transfection of D^d into the cells. It was suggested that Ly49 is an inhibitory receptor that interacts with the D^d molecule, and with an H-2^k class I molecule, but not with the H-2^b class I molecules. It is likely that other related receptors with different MHC specificities also exist, given the existence of several Ly49-related genes [15] and the reports of molecules distinct from Ly49 that may also guide MHC-specific recognition by NK cells [16–18].

The finding that NK cells lyse syngeneic non-transformed lymphoblast target cells from class I-deficient mice indicated that recognition by NK cells does not require the presentation of foreign antigens or tumor antigens [6, 7]. Therefore, it might be thought that class I deficiency by a cell is a sufficient specific condition for it to be lysed by NK cells. However, NK cells are apparently unable to reject class I-deficient non-hematopoietic tissues, for example skin grafts [19]. Furthermore, NK cells fail to lyse class I-deficient fibroblasts *in vitro* [19]. These observations raise the possibility that NK-susceptible hematopoietic cells express cell surface molecules necessary for activation of the NK cell lytic mechanism.

Indeed, it is clear from various studies that other cell surface interactions between NK cells and target cells are generally necessary for the cells to adhere together, and to activate the lytic machinery. Several defined adhesion molecules and other less well defined molecules have been

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Correspondence: David H. Raulet, Department of Molecular and Cell Biology, 489 LSA, University of California, Berkeley 94720 (Fax: 510 642-1443)

Abbreviations: ADCC: Antibody-dependent cellular cytotoxicity **A-LAK:** Adherent lymphokine activated killer cells **β2m:** β2-Microglobulin

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implicated in the NK-target cell interaction (reviewed in [20]). Some of these receptor-ligand interactions may represent specific triggers to activate NK lysis of distinct cell types such as tumor cells or lymphoblasts. The best defined NK triggering molecule is CD16, the FcγR through which NK cells mediate ADCC [21, 22]. If NK cells generally require a specific positive signal to activate lysis, the role of class I recognition might be to overrule this signal, thus preventing lysis. For example, it was reported that both class I^- and class I^+ tumor cells activate a calcium flux in human NK cells, though lysis of the latter target cells was strongly inhibited [23].

We describe experiments herein to address whether class I deficiency in general, or the failure to express the specific inhibitory MHC alleles in the case of $Ly49^+$ NK cells, is a sufficient condition to render lymphoblast target cells or tumor cells sensitive to NK cell-mediated lysis. The results suggest that NK cells and A-LAK cells differ in their spectrum of target antigens, but that in essentially all cases, including ADCC of antibody-coated target cells, lysis can be inhibited by target cell expression of appropriate class I molecules.

2 Materials and methods

2.1 Mice

C57BL/6J (B6, H-2^b), B10.BR (H-2^k) and B10.D2 (H-2^d) mice were purchased from the Jackson Laboratories. Class I-deficient H-2^b $\beta2m^-$ mice [24], backcrossed five-times to B6, were bred in the animal facility at the University of California, Berkeley. H-2^k $\beta2m^-$ mice were derived by backcrossing the B6-$\beta2m^-$ mice to B10.BR mice and interbreeding the offspring [25]. B6 nude mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

2.2 Antibodies

Anti-Ly49 (A1, [26]), anti-CD8 (AD4-15, [27]), anti-CD4 (RL-172.4) and anti-HSA (J11d, [28]) mAb have been described. The mouse anti-D^d hybridoma 34-2-12s [29], the mouse IgG2a anti-D^b hybridoma 28-14-8s [30], and the anti-NK1.1 hybridoma PK136 [31] were obtained from the ATCC, and the antibodies were purified by passage over a Protein A Sepharose column. Rat IgG2b anti-mouse Thy 1.2 mAb (30H12), FITC-anti-LFA-1 (2D7), FITC-anti-ICAM-1 (3E2) and FITC-anti-ICAM-2 (MIC2-4(3C4)) were purchased from Pharmingen (San Diego, CA). Rabbit anti-mouse IgG and goat anti-mouse Ig antibodies were purchased from Cappel Laboratories (Durham, NC). FITC-conjugated goat anti-hamster Ig was purchased from Caltag (South San Francisco, CA). Anti-TcRαβ H57-597 [32] and anti-TcRγδ GL3 [33] antibodies were purified on Protein A Sepharose columns.

2.3 Preparation of freshly isolated NK cells

NK cells were enriched by nylon wool passage of splenocytes of B6 normal or nude mice, as indicated, that had been pretreated 18 h earlier with 150 μg polyinosinic:polycytidylic acid (poly I:C). As determined by flow cytometry, approximately 10% or 60% of these cells were $NK1.1^+TcR^-$ cells in the case of normal or nude donors, respectively.

2.4 Preparation of A-LAK cells

A-LAK cells were prepared as described [14], with modifications. Nylon wool-passed B6 splenocytes were cultured in medium containing 200 ng/ml of recombinant IL-2 generously provided by Cetus Inc. (Emeryville, CA). After 3 days of culture, adherent cells were harvested and depleted of T cells [14]. The surviving cells were returned to culture with fresh IL-2, and after 3 additional days some of the cells were used to prepare $Ly49^+$ and $Ly49^-$ A-LAK cells. The cells were incubated with 20 μg/ml A1 (anti-Ly49) mAb and washed extensively before panning on petri dishes that had been coated with 2.5 μg/ml goat anti-mouse IgG. The non-adherent population was removed and negatively panned twice more to deplete residual $Ly49^+$ cells. The adherent and non-adherent cells, as well as unseparated cells, were left in culture overnight, then harvested with PBS/EDTA (0.02%), washed, and returned to culture for an additional 2 days in IL-2-containing medium. The $Ly49^+$ and $Ly49^-$ A-LAK populations were each greater than 95% positive for NK1.1 and CD16 expression, and expressed these molecules at similar cell surface densities.

2.5 Tumor cells

C1498 and YAC-1 tumor cells were obtained from the ATCC. EL-4(R) designates a subline of the B6-derived T lymphoma cell line EL-4, maintained in our laboratory for several years, whereas EL-4(P) designates a subline of EL-4 that we obtained from Dr. Terry Potter (National Jewish Hospital, Denver, CO). EL-4(P) cells transfected with D^d (EL-4(P)-D^d) were also obtained from Terry Potter, as were the R8.15, and R8.15-D^d cell lines [34]. Dr. Michael Bevan provided the mutant RMA/S and wild-type RMA tumor cell lines [1] which are derived from the H-2^b RBL-5 tumor cell line.

2.6 Transfection of RMA cells with D^d

RMA cells were transfected by electroporation with a plasmid (pD^d1) containing the D^d gene driven by its own promoter [35]. The plasmid pMC1NeoPoly A (Stratagene Inc, La Jolla, CA), containing the bacterial neo^R gene, was co-transfected. Transfectants were selected by culture in medium containing G418, and D^d positive transfectants were sorted and cloned. A clone that stained brightly with 34-2-12s (anti-D^d) was employed for the experiments.

2.7 Target cells

The experiments in Figs. 1 and 2 employed Con A blasts that were prepared by culturing spleen cells in medium containing 2 μg/ml Con A for 2 days. The Con A blasts employed for the experiment in Fig. 3 were prepared by

depleting spleen cells of $CD8^+$ cells and B cells by treatment with anti-CD8 and anti-HSA mAb and complement, followed by incubation in medium containing 2 μg/ml Con A for 1–3 days. Con A blasts were depleted of Con A by treatment with α-methyl mannoside and washed extensively before the cytotoxicity assay. For comparisons of T cells activated for different lengths of time, cultures were set up on 3 different days for assay on the same day. Unactivated T cell targets were prepared by nylon wool passage of splenocytes. Tumor cells and T cell targets were labeled with ^{51}Cr for 1.5–2.5 h at 37 °C, purified over Ficoll/Isopaque, and washed extensively before the assay.

2.8 Cytolytic assay

NK cells or A-LAK cells were incubated for 3.5–4 h with ^{51}Cr-labeled target cells in duplicate or triplicate as previously described [6]. For the ADCC reaction, target cells, after ^{51}Cr labeling, were incubated with 10 μg/ml rat IgG2b anti-Thy-1 mAb or mouse IgG2a anti-mouse D^b as indicated for 30 min on ice, and then washed extensively before the assay.

2.9 Immunofluorescent staining

For analysis of NK cells and A-LAK cells, populations were stained in the first step with anti-NK1.1-biotin and fluorescinated antibodies specific for adhesion molecules, and in the second step with streptavidin-phycoerythrin. Gated $NK1.1^+$ cells were analyzed for fluorescence intensity. For the analysis of activated and unactivated T cells, cells were stained in the first step with anti-TcRβ-biotin and fluorescinated antibodies specific for adhesion molecules, and in the second step with streptavidin-phycoerythrin. Gated $TcR\beta^+$ cells were analyzed for fluorescence intensity. For analysis of tumor cells, the cells were stained directly with fluorescinated antibodies specific for adhesion molecules. A Coulter Epics Elite flow cytometer (Hialeah, FL) or a FACScan analyzer (Becton Dickinson, Mountain View CA) were employed. Dead cells were excluded on the basis of forward and side light scatter.

3 Results

3.1 Adherent LAK cells fail to lyse class I-deficient lymphoblast targets

A-LAK cells were compared with freshly isolated NK cells for their capacity to lyse Con A blast target cells from $\beta2m^+$ and $\beta2m^-$ B6 mice (Fig. 1). Con A blasts from $\beta2m^-$-deficient mice were lysed very poorly by A-LAK cells in this experiment and four others. A-LAK cells efficiently lysed the YAC-1 tumor target cell. Freshly prepared NK cells from poly I:C treated mice, tested in parallel, lysed $\beta2m^-$ Con A blasts but not $\beta2m^+$ Con A blasts, as previously reported [6, 7] (Fig. 1A).

To determine whether Con A blast targets were intrinsically resistant to lysis by A-LAK cells, we tested whether A-LAK cells mediate ADCC of T cell targets. The A-LAK cells efficiently lysed anti-Thy 1-coated $\beta2m^-$ Con A blasts (Fig. 1C). These experiments indicate that Con A blasts can be lysed by A-LAK cells, as long as a suitable positive stimulus is provided (ADCC).

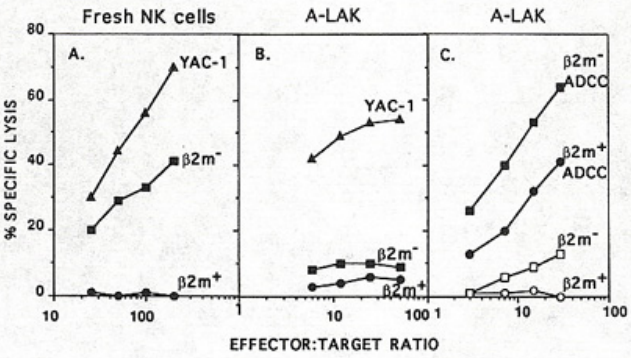

Figure 1. Class I-deficient Con A blasts are sensitive to lysis by freshly isolated NK cells, but resistant to lysis by A-LAK cells. (A) Activity of freshly isolated NK cells prepared from normal B6 mice. (B) Activity of A-LAK cells from B6 mice. (C) Activity of A-LAK cells in ADCC reaction mediated by anti-Thy-1 mAb. Con A blasts were prepared from $\beta2m^-$ and $\beta2m^+$ $H\text{-}2^b$ mice. The data in panels A and B were generated in the same experiment, while panel C depicts a separate experiment.

Although the B6 A-LAK cells lysed antibody-coated $\beta2m^+$ $H\text{-}2^b$ target cells, the lysis was less efficient than in the case of $\beta2m^-$ $H\text{-}2^b$ target cells (Fig. 1C). β2m expression resulted in depressed ADCC by unseparated A-LAK cells, resulting in a two- to fivefold reduction in the efficiency of lysis in different experiments. Therefore, syngeneic class I expression inhibits ADCC mediated by the A-LAK cell population, but the inhibition is not nearly as profound as the inhibition observed in the case of direct lysis of Con A blast targets by freshly isolated NK cells (compare to Fig. 1A; [6, 7]).

3.2 Role of class I MHC expression in preventing ADCC

To test the inhibitory function of different MHC molecules in the case of ADCC, A-LAK cells were separated into $Ly49^+$ and $Ly49^-$ fractions, and tested for ADCC of various Con A blast target cells. Previous studies demonstrated that $H\text{-}2^d$ and $H\text{-}2^k$ expression by tumor target cells inhibits lysis mediated by $Ly49^+$ A-LAK cells, whereas $H\text{-}2^b$ tumor cells were lysed efficiently [14]. When tested on $\beta2m^+$ Con A blast target cells, $Ly49^+$ A-LAK cells mediated ADCC of $H\text{-}2^b$ but not $H\text{-}2^k$ or $H\text{-}2^d$ target cells (Fig. 2A). Furthermore, Con A blasts from $\beta2m^-$ mice of either the $H\text{-}2^b$ or $H\text{-}2^k$ haplotypes were lysed efficiently (Fig. 2B). The strongly depressed killing of $\beta2m^+$ $H\text{-}2^k$ cells compared to $\beta2m^-$ $H\text{-}2^k$ cells indicates that class I molecules of $H\text{-}2^k$ (and presumably $H\text{-}2^d$) haplotypes inhibit killing. Similar results were obtained in four other experiments.

These results suggest that MHC molecules of $H\text{-}2^k$ and $H\text{-}2^d$ haplotypes strongly inhibit ADCC by $Ly49^+$ A-LAK cells, whereas $H\text{-}2^b$ MHC molecules inhibit lysis much less. However, $H\text{-}2^b$ molecules may inhibit $Ly49^+$ A-LAK cells to a limited extent, based on the more efficient lysis of

$\beta 2m^-$ $H\text{-}2^b$ target cells compared to $\beta 2m^+$ $H\text{-}2^b$ target cells (compare Fig. 2A and B, see Sect. 4). Although this effect was modest, it was reproducible in each of four additional experiments.

$Ly49^-$ A-LAK cells mediated ADCC of all three $\beta 2m^+$ targets to a roughly similar extent, although $H\text{-}2^b$ target cells may be slightly more sensitive (Fig. 2E). However, $\beta 2m^-$ target cells were lysed more efficiently than $\beta 2m^+$ target cells by two- to eightfold in different experiments (Fig. 2E versus F and data not shown), indicating that class I molecules also inhibit lysis by $Ly49^-$ A-LAK cells, although modestly.

3.3 Specificity of fresh NK cells for activated T cells

The lysis of $\beta 2m^-$ lymphoblast target cells by freshly isolated NK cells suggested that lymphoblasts express target structures for NK cells. In order to determine the effect of cellular activation on the susceptibility of lymphoid cells to NK-mediated lysis, we compared the capacity of freshly isolated NK cells to lyse splenic $CD4^+$ T cells at various stages of activation: either as freshly isolated cells (mostly unactivated cells) or after 1, 2 or 3 days of activation with Con A. As shown in Fig. 3A, freshly isolated $CD4^+$ T cells (day 0) from $\beta 2m^-$ mice were not lysed by freshly isolated NK cells. $\beta 2m^-$ $CD4^+$ T cells activated for 1 day with Con A were lysed efficiently by NK cells. Activation for longer times resulted in a reduction in susceptibility, which was marked in the case of target cells stimulated for 3 days with Con A. The day 3 $CD4^+$ Con A blasts were highly activated and healthy cells as assessed by phase contrast microscopy. The failure of day 0 T cells to be lysed was observed in each of five additional experiments, and the diminished lysis of day 3 Con A blasts was observed in each of two additional experiments.

To determine whether the resistant T cell targets were inherently resistant to the NK cell's lytic machinery, the panel of target cells were tested in parallel for susceptibility to ADCC mediated by the same freshly isolated NK cells (Fig. 3B). $\beta 2m^-$ blasts activated with Con A for 1, 2 or 3 days were approximately equal in their sensitivity to ADCC mediated by freshly isolated NK cells. Unactivated lym-

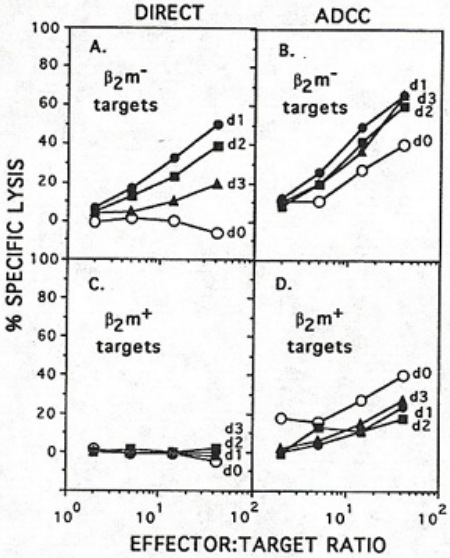

Figure 3. Varying susceptibility of T cells to NK cells dependent on activation state. $CD4^+$ T cells activated for 1–3 days with Con A, or unactivated T cells, were employed as target cells for freshly isolated NK cells prepared from B6 nude mice. The target cells were derived from $\beta 2m^+$ or $\beta 2m^-$ mice of the $H\text{-}2^b$ haplotype as indicated. Direct lysis (panels A and C) and ADCC-mediated by anti-Thy 1 mAb (panels B and D) are plotted separately.

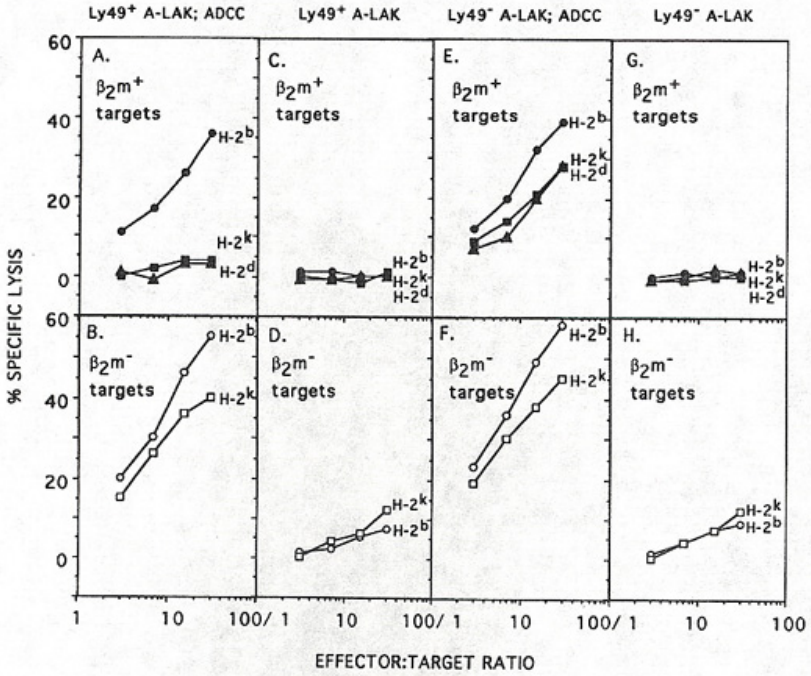

Figure 2. Specific inhibition by $H\text{-}2^d$ and $H\text{-}2^k$ but not $H\text{-}2^b$ class I molecules of ADCC mediated by $Ly49^+$ A-LAK. $Ly49^+$ and $Ly49^-$ A-LAK were used as effectors against Con A blast target cells, from $\beta 2m^+$ or $\beta 2m^-$ mice of the $H\text{-}2^b$, $H\text{-}2^d$ or $H\text{-}2^k$ haplotypes. Panels A, C, E, and G depict results with $\beta 2m^+$ target cells, while panels B, D, F, and H depict results with $\beta 2m^-$ target cells. Panels A, B, E, and F depict ADCC reactions accomplished by coating the target cells with anti-Thy 1 mAb, while the remaining panels depict direct lysis.

phocytes were less sensitive, but only marginally so, by a factor of two to eight, in this and several other experiments. These data suggest that T cell targets at different states of activation do not differ dramatically in their sensitivity to NK lytic machinery activated by ADCC.

As in our previous studies, only $\beta 2m^-$ blasts, and not $\beta 2m^+$ blasts, were lysed by NK cells in the absence of facilitating antibody, indicating efficient inhibition of this lysis by target cell class I MHC expression (Fig. 3A, C). In the case of ADCC, lysis of activated T cell targets was consistently lower when the targets were $\beta 2m^+$ than when they were $\beta 2m^-$ (Fig. 3B, D). In eight experiments, the efficiency of lysis of day 1 Con A blasts was reduced between two- and eightfold as result of $\beta 2m$ expression. Hence, class I expression inhibits ADCC of Con A blasts by freshly isolated NK cells, but the inhibition is less marked than observed in the case of direct lysis of Con A blasts (*e.g.* Fig. 1A). Interestingly, ADCC of unactivated T cell targets was essentially unaffected by class I expression (Fig. 3B, D). In each of four experiments, ADCC of $\beta 2m^+$ $CD4^+$ T cells was equal to that of $\beta 2m^-$ $CD4^+$ T cells, or only slightly reduced (less than twofold).

3.4 Specificity of tumor cell lysis by NK cells

A previous study suggested that $Ly49^+$ A-LAK cells lyse $H\text{-}2^b$ tumor cells but not $H\text{-}2^d$ or $H\text{-}2^k$ tumor cells [14]. Consistent with the earlier results, we found that three $H\text{-}2^b$ tumor cells lines, C1498, R8.15, and an EL-4 subline from Terry Potter (EL-4(P)), were lysed efficiently by unseparated A-LAK cells or $Ly49^+$ A-LAK cells (Figs. 4, 5).

However, two other $H\text{-}2^b$ tumor cell lines, RMA and an EL-4 subline maintained for several years in our laboratory (EL-4(R)), were resistant to lysis by $Ly49^+$ A-LAK cells or unseparated A-LAK cells (Fig. 4A, B). The different susceptibility of the EL-4 sublines suggests that cloned cell lines can undergo variations influencing their susceptibility to lysis by A-LAK cells. A variant of the RMA cell line with a mutation in the peptide transporter molecule, RMA/S [36, 37], was also lysed poorly by $Ly49^+$ A-LAK cells, though better than non-mutant RMA cells (Fig. 4A, B). $Ly49^+$ A-LAK cells efficiently lysed the resistant EL-4, RMA and RMA/S cells by ADCC (*i.e.* when the target cells were coated with anti-Thy-1 mAb), demonstrating that these tumor cells were not inherently resistant to lysis (Fig. 4C). These results demonstrate that $H\text{-}2^b$ lymphoid tumor cells vary in their capacity to activate lysis by $Ly49^+$ A-LAK cells.

3.5 Role of MHC in inhibiting tumor cell lysis

Lysis of susceptible tumor cell lines R8.15 and EL-4(P) by $Ly49^+$ A-LAK cells was inhibited if the cells were transfected with the D^d gene (Fig. 5A, C), as had been previously shown for the C1498 cell line [14]. D^d expression made little or no difference in the lysis of these target cells by $Ly49^-$ A-LAK cells (Fig. 5B, D). These data corroborate the role of D^d in inhibiting tumor cell-specific lysis. In addition, when the reaction is mediated by ADCC, lysis by A-LAK cells of non-susceptible tumor cells is also inhibited by D^d expression. As shown in Fig. 4D, 8 times more $Ly49^+$ A-LAK cells were required to lyse D^d-transfected RMA cells, compared to untransfected RMA cells. Similar results were obtained in two additional experiments.

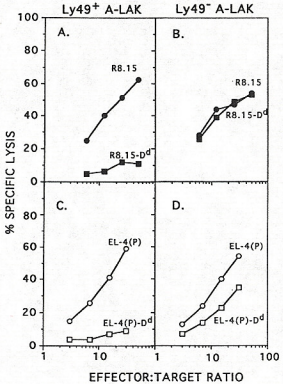

Figure 5. Direct lysis by $Ly49^+$ A-LAK of two $H\text{-}2^b$ expressing tumor cell lines is inhibited by transfection of the D^d gene. $Ly49^+$ (A, C) and $Ly49^-$ (B, D) A-LAK were tested for direct lysis of the indicated tumor cells.

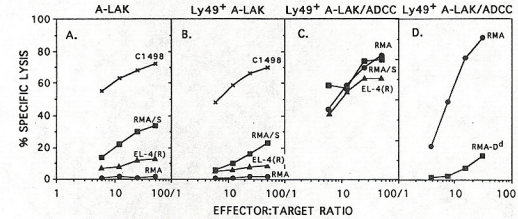

Figure 4. Tumor cell lines were tested for susceptibility to lysis by unseparated A-LAK (A), $Ly49^+$ A-LAK (B), or $Ly49^+$ A-LAK in an ADCC reaction (C, D). In panel D, ADCC by $Ly49^+$ A-LAK of RMA cells and of RMA cells stably transfected with D^d are compared. ADCC reactions employed anti-Thy 1 mAb in all cases except the EL-4(R) target, for which anti-D^b mAb was employed.

3.6 Relationship between expression of adhesion molecules and susceptibility to NK-mediated lysis

The foregoing studies have identified several instances where specific insensitivity to NK- or A-LAK-mediated lysis is apparently unrelated to MHC expression, including (a) the lysis of $\beta 2m^-$ Con A blasts by freshly isolated NK cells but not A-LAK cells; (b) the lysis by freshly isolated NK cells of day 1 Con A blasts but not unactivated T cells or day 3 Con A blasts; (c) the insensitivity of H-2^b tumor cells RMA and EL4(R) to lysis by Ly49$^+$ A-LAK cells. It is possible that in one or more of these instances the differential expression of adhesion molecules is responsible. Therefore, in each case we have determined whether sensitivity to lysis correlates with the cell surface expression levels of the adhesion molecule LFA-1, or its ligands ICAM-1 and ICAM-2, by flow cytometric analysis of cells following direct staining with fluoresceinated antibodies.

Freshly isolated NK cells and A-LAK cells expressed roughly similar levels of LFA-1 (Table 1). ICAM-1 levels were higher on freshly isolated NK cells than on A-LAK cells in two experiments, but not in another. ICAM-2 levels were higher on NK cells in all three experiments, but only by 1.5- to threefold. Hence the differential capacity of these cells to directly lyse Con A blasts is unlikely to be due to differences in expression of these adhesion molecules.

Table 1. Adhesion molecule expression by A-LAK cells *versus* NK cells[a]

Effectors	Reagent			
	Control[b]	LFA-1	ICAM-1	ICAM-2
Expt. 1				
A-LAK	0.7	32.8	3.3	1.6
NK	0.7	26.4	8.1	4.7
Expt. 2				
A-LAK	0.5	27.1	4.5	1.9
NK	0.4	39.2	15.6	5.9
Expt. 3				
A-LAK	0.7	26.9	3.6	1.6
NK	0.5	19.3	3.1	2.4

a) Values represent mean fluorescence intensity. Cells were from B6 mice.
b) Control staining was with the irrelevant 34-2-12s anti-D^d mAb.

In the case of activated *versus* unactivated T cell targets, ICAM-1 and ICAM-2 levels were highest on day 1 Con A blasts, followed in order by day 2 Con A blasts, day 3 Con A blasts and finally unactivated T cells (Table 2). Sensitivity of the cells to lysis followed the same order. LFA-1 levels were lowest on the unactivated T cells, the least sensitive target cells; T cells activated for 1–3 days with Con A expressed two- to threefold higher levels of LFA-1. Therefore, the sensitivity of the different T cell populations to direct lysis by NK cells correlated reasonably well with ICAM-1 and ICAM-2 levels, and less well with LFA-1 levels.

In the case of the tumor target cells, the levels of the adhesion molecules did not correlate well with sensitivity to lysis by A-LAK cells (Table 3). For example, the RMA cell line had relatively high levels of LFA-1 and ICAM-2, but was insensitive to lysis by A-LAK. Also, the EL-4(P) subline expressed the lowest levels of ICAM-1 and ICAM-2 of the tumor cells tested, but was a reasonably good target for A-LAK cells. Although these cells had higher levels of LFA-1 than the other tumor target cells, antibodies against LFA-1, which blocked lysis of C1498 cells, failed to block lysis of EL-4(P) (data not shown).

Table 2. Expression of adhesion molecules by Con A blasts and T cells[a]

Target cells	Reagent			
	Control[b]	LFA-1	ICAM-1	ICAM-2
Day 0	0.4	10.7	1.1	4.4
Day 1	1.2	30.9	18.8	9.3
Day 2	1.3	25.7	12.5	3.9
Day 3	1.3	28.1	10.3	2.3

a) Values represent mean fluorescence intensity. Cells were CD4$^+$ cells from B6 mice.
b) Control staining was with the irrelevant 34-2-12s anti-D^d mAb.

Table 3. Expression of adhesion molecules by tumor target cells[a]

Tumor cell	Reagent			
	Control[b]	LFA-1	ICAM-1	ICAM-2
C1498	4	52	40	68
R8.15	4	32	34	34
EL-4(P)	4	83	6	22
EL-4(R)	4	62	17	59
RMA	3	66	27	59
RMA/S	2	43	17	55

a) Values represent mean fluorescence intensity.
b) Control staining was with the irrelevant goat anti-hamster IgG.

4 Discussion

4.1 Factors other than MHC expression determine target cell sensitivity to NK cells

The results of these studies suggest that while the failure of target cells to express inhibitory class I molecules is generally a necessary condition for optimal lysis by NK cells, other factors are also critical to activate lysis. For example, although it was recently reported that all of several H-2^b tumor cell lines tested were lysed by Ly49$^+$ A-LAK cells [14], our results indicate that several other H-2^b tumor cells are lysed poorly or not at all by Ly49$^+$ A-LAK cells, unless the reaction was mediated by ADCC. Furthermore, H-2^b Con A blasts were not lysed by Ly49$^+$ A-LAK cells.

It was previously suggested that the failure of Ly49$^+$ A-LAK cells to lyse H-2^b Con A blasts might be due to the expression of specific peptide/H-2^b complexes by Con A blasts, absent on the susceptible target cells, that can engage the Ly49 receptor and cause inhibition of lysis [14]. The resistant H-2^b tumor cells might be explained by a

similar argument. However, several results argue against this hypothesis in the case of both Con A blast target cells and the resistant tumor cells. First, not only H-2^b blasts, but also $\beta2m^-$ (class I-deficient) blasts, were resistant to lysis by $Ly49^+$ A-LAK cells. Second, H-2^b blasts were susceptible to ADCC by $Ly49^+$ A-LAK cells, while H-2^d blasts were not, indicating that the H-2^b on T-blasts is not strongly inhibitory to lysis by $Ly49^+$ A-LAK cells, in contrast to H-2^d or H-2^k molecules. In the case of the resistant H-2^b tumor cell line RMA, we found that the peptide-transporter-deficient RMA/S mutant was also lysed relatively poorly by $Ly49^+$ A-LAK cells. This suggests that transported H-2^b-associated peptides are unlikely to fully account for the resistance of RMA cells to lysis, although we have not ruled out the possibility that a relevant peptide is transported by an alternative pathway in RMA/S cells. The resistant H-2^b tumor cells could be lysed by ADCC, arguing that these cells are not resistant to A-LAK cell lytic machinery. Finally, RMA cells transfected with the D^d gene were resistant to ADCC by $Ly49^+$ A-LAK, suggesting that the untransfected cells do not express an inhibitory MHC/peptide complex.

These results suggest that A-LAK cells distinguish other specific features of the target cells in addition to the MHC molecules expressed by these cells. The resistance of some tumor cells to lysis by A-LAK cells could be explained if some tumor cells but not others express a specific target structure(s) recognized by activating NK cell receptors. Activating signals can also be provided through the CD16 Fc receptor, via the ADCC reaction, resulting in the lysis of all tested target cell types.

Similar arguments may account for the failure of A-LAK cells to lyse $\beta2m^-$ Con A blasts at any state of activation, and the selective lysis of day 1 Con A blasts by freshly isolated NK cells. A-LAK cells may fail to express a receptor for an NK target structure(s) expressed by activated T cells. The putative NK-activating ligands may be expressed selectively by recently activated T cells, accounting for the lower or absent lysis of unactivated T cells and day 3 Con A blasts by freshly isolated NK cells.

If, as suggested above, a specific NK target structure is expressed by activated T cells, it may reflect one mechanism whereby NK cells can detect and destroy newly transformed cells. Because A-LAK cells fail to lyse T blasts but lyse various tumor target cells, it is possible that the susceptible stable transformed cell lines express distinct target structures or that the same structure is recognized with the aid of the increased levels of adhesion molecules found on the tumor cell lines as compared to T blasts. However, given the complexity of NK specificities reported, for some but not all tumor cells, for virus-infected cells, for bone marrow stem cells, and for activated lymphocytes, it is reasonable to suggest that NK cells can be activated to lysis through any of several distinct activating receptors, each with a distinct specificity. The only well characterized example is the FcγR CD16.

It is difficult at present to dissect the relative importance of the expression of putative NK target structures *versus* the expression of adhesion molecules in the success or failure of the NK-target cell combinations analyzed here. The expression of adhesion molecules, particularly ICAM-1 and ICAM-2, are correlated with the sensitivity of T blasts to lysis by freshly isolated NK cells. However, the sensitivity of tumor cell targets to lysis did not correlate well with the expression of these adhesion molecules. Comparing NK and A-LAK effector cells, differential expression of these adhesion molecules was not consistently observed, and therefore seems unlikely to account for the superior activity of freshly isolated NK cells in the lysis of Con A blasts. It is possible, of course, that the differential expression of other adhesion molecules contributes to the observed pattern of lysis. It appears likely that the differential expression of both general adhesion molecules and NK-specific triggering molecules contribute to the reactivity of NK cells for a given target cell.

4.2 The role of MHC class I molecules

The previous discussion emphasizes the possibility that various different activating signals may activate lysis by NK cells and/or A-LAK cells. The question arises whether class I MHC expression can inhibit lysis induced by any or all of these signals. In fact, in each system studied, there was strong evidence for inhibition by class I MHC molecules.

Our results demonstrate strong inhibition of tumor cell lysis by class I molecules, strong class I-mediated inhibition of the lysis of Con A blasts by freshly isolated NK cells, strong H-2^d and H-2^k-mediated inhibition of ADCC by $Ly49^+$ A-LAK cells of Con A blast target cells, and D^d-mediated inhibition of ADCC of non-susceptible tumor target cells by $Ly49^+$ A-LAK. Inhibition of direct lysis of tumor cells and of Con A blasts by class I molecules were also reported in earlier studies [1–7]. The results suggest a model in which two specific conditions must be met for target cell susceptibility to NK cells: expression of a target structure recognized by NK cells (including antibody-mediated recognition), and failure to express inhibitory MHC molecules that can be recognized by these NK cells. This model predicts that the inhibitory signal mediated by Ly49 and related molecules interferes with the activation or effector pathways initiated by CD16 and other activating receptors on NK cells. The requirement for two conditions for lysis may represent a fail-safe mechanism operative in NK cells, to minimize the possibility that normal autologous cells will be attacked.

As in previously published studies [14], we found that $Ly49^+$ A-LAK cells were inhibited more strongly by H-2^d or H-2^k MHC molecules than by H-2^b MHC molecules. However, we find that lysis of $\beta2m$-deficient target cells was modestly but reproducibly higher than lysis of $\beta2m^+$ H-2^b cells, suggesting that H-2^b class I molecules are somewhat inhibitory for $Ly49^+$ effector cells. It is possible therefore, that Ly49 interacts weakly with H-2^b class I molecules, resulting in modest inhibition. An alternative explanation is that a significant fraction of $Ly49^+$ A-LAK cells express, in addition to Ly49, other inhibitory receptors with specificity for H-2^b class I molecules. Interaction of these other receptors with H-2^b class I molecules might inhibit these A-LAK cells in a dominant fashion. It is presently not established that a given NK cell can express more than one member of the inhibitory receptor family. However, we have found that a fraction of A-LAK cells express both

Ly49 and another molecule, 5E6 [16, 38], that defines a distinct MHC specificity in NK cells. Presently it is not clear whether 5E6 is an inhibitory receptor. Two inhibitory molecules on human NK cells have also been shown to be co-expressed on some human NK clones [17, 18].

Freshly isolated NK cells are strongly inhibited in their direct lysis of Con A blasts by syngeneic class I molecules [6, 7, 39], suggesting that most effector cells in the fresh NK effector population express receptors that can be inhibited by autologous class I molecules. However, ADCC of lymphoblasts mediated by freshly isolated NK cells or unfractionated A-LAK cells was only modestly (two- to eightfold) inhibited by syngeneic MHC expression. In contrast, ADCC mediated by $Ly49^+$ A-LAK cells was profoundly inhibited by expression of $H\text{-}2^d$ or $H\text{-}2^k$ molecules. At least two mechanisms can be proposed to account for the weak inhibition by syngeneic class I molecules of ADCC-mediated by NK cells and A-LAK cells. First, the antibody-mediated interaction, but not the target structures expressed by Con A blasts, may activate a subpopulation of effector cells that is refractory to inhibition by syngeneic class I molecules. Alternatively, the efficiency of inhibition by class I molecules may be a complex property, determined by an interplay between the strength of the activating signal and the strength of the inhibitory signal. ADCC may impart a more potent activating signal to NK cells than that imparted by Con A blasts, and may therefore require a stronger inhibitory signal. Some of the freshly isolated NK cells in $H\text{-}2^b$ mice may be sufficiently reactive with $H\text{-}2^b$ molecules for inhibition of direct lysis but not for inhibition of ADCC.

In the present study there was only a single instance where class I expression had essentially no effect on lysis: ADCC of unactivated T cell targets mediated by fresh NK cells. This may be accounted for by the observation that the level of class I expression by these cells was somewhat lower than the levels observed on inhibitory T cell blasts (data not shown). Alternatively, perhaps the class I molecules on unactivated cells are unable to interact with corresponding inhibitory receptors on NK cells. For example they may lack a peptide or peptides necessary for interactions with inhibitory receptors. It is notable that unactivated T cells are refractory to lysis by NK cells in the absence of facilitating antibody, possibly due to their failure to express NK target structures and/or sufficient levels of necessary adhesion molecules (see above). Presumably this accounts for the failure of these cells to be destroyed by autologous NK cells *in vivo*, despite the failure of their MHC molecules to inhibit NK cells *in vitro*.

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