

Retaliation against Tumor Cells Showing Aberrant HLA Expression Using Lymphokine Activated Killer-derived T Cells¹

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ABSTRACT

Lymphokine activated killer (LAK) cells represent mixtures of natural killer (NK) and non-MHC-restricted CTLs that have the capacity to lyse a variety of tumor cells and MHC class I-negative target cells. Although it is clear that NK cells are negatively regulated by interactions with MHC class Ia or class Ib molecules, the regulation of LAK-derived T cells has not been clarified to date. In the studies presented here, we demonstrate that IFN- γ treatment of tumor cells can induce their resistance to LAK-derived T cells in a manner similar to that seen for NK cells. The IFN- γ -mediated suppression of LAK activity correlates with increased MHC class I expression by the tumor cells, and the inhibition of LAK-mediated cytotoxicity can be reversed in the presence of class I-specific antibody. Furthermore, the expression of MHC class Ia or class Ib molecules in class I-negative cell lines can reduce their susceptibility to LAK-mediated cytotoxicity. This principle of negative regulation by MHC class I molecules applies to LAK-derived T cells generated from peripheral blood lymphocytes of renal cell carcinoma patients and healthy, control donors. Although LAK-derived T cells can be inhibited in their lytic activity through interactions with MHC class Ia and class Ib molecules, they do not express the known inhibitory receptors specific for these ligands that are found on NK cells. Apparently, LAK-derived T cells are negatively regulated by as yet undefined inhibitory receptors.

INTRODUCTION

LAK³ cells represent a composite of CD3[−] NK cells and CD3⁺ T cells that are derived from PBMCs through *in vitro* culture in the presence of high-dose IL-2 (1–4). A characteristic of LAK cells is their capacity to lyse a variety of tumor cells in a non-MHC-restricted fashion. In addition, LAK cells can kill class I-negative target cells, such as Daudi and K562, which serve as general standards for identification of non-MHC-restricted cytotoxic effector cells (1–5). Separation of LAK populations into CD3[−] and CD3⁺ fractions revealed that both NK cells and T cells could lyse class I-negative target cells and a variety of allogeneic tumor cells (2, 6). A very potent fraction of A-LAK cells was found to be composed predominantly of activated NK cells (7–9).

A number of clinical trials studied the capacity of unseparated LAK cells or A-LAK cells to mediate antitumor activity *in vivo* following the adoptive transfer of large numbers of cells into patients with advanced disease (1, 10–18). Although dramatic regression of some tumor lesions was observed, this occurred rarely, was short-lived, and was associated with high toxicity. Induction of LAK cells was observed in most patients receiving systemic high-dose IL-2 therapy

irrespective of clinical response; thus, if LAK cells contributed to tumor remission, their efficacy was most likely determined at the level of tumor cell recognition (19–21). The failure to identify the basis of LAK-mediated tumor regression, thereby preventing the identification of patients who might benefit from this therapy despite its associated toxicity, limited further clinical development.

The emerging understanding of the negative regulation of lymphocyte function through interactions of inhibitory receptors with MHC class I molecules provides new insight into mechanisms that could influence LAK function. Although activating receptors such as natural cytotoxicity receptors are responsible for the induction of NK-mediated lysis (22–24), the cytolytic capacity of NK cells is ultimately determined by their inhibitory receptor expression. Extensive analyses of different sources of NK cells have shown that essentially all human NK cells bear inhibitory receptors that interact with class I molecules and inhibit cytotoxicity, thereby protecting normal somatic cells from NK-mediated attack (reviewed in Refs. 25–27). Two forms of inhibitory receptors are expressed by NK cells. The KIRs belong to the immunoglobulin superfamily and interact with classical MHC class Ia (HLA-A, -B, -C) molecules. Inhibitory receptors of the C-type lectin superfamily are composed of CD94/NKG2A heterodimers that bind nonclassical MHC class Ib (HLA-E) molecules (28, 29). Through expression of class Ia and Ib molecules, nucleated cells bear two sets of ligands that can independently prevent attack by NK cells bearing either of these inhibitory receptor types.

In the studies presented here, we demonstrate that LAK-T cells obtained from RCC patients and normal, control donors can efficiently lyse class I-negative target cells and allogeneic tumor cell lines that display no or very low levels of corresponding inhibitory class I allotypes. These tumor cells became resistant to lysis after stimulation with IFN- γ . This resistance was associated with up-regulation of class I expression, and cytotoxicity could be restored when class I was masked by specific antibody. Similarly, class I-negative target cells were partially protected from lysis by LAK-T cells following their genetic modification to express HLA class Ia or class Ib proteins. Our results demonstrate that particular class I allotypes inhibit the activity of LAK-T cells in a manner like that known for activated NK cells, suggesting that these effector cells might be used in a clinical setting to combat tumor cells showing low or aberrant expression of these specific HLA molecules.

MATERIALS AND METHODS

Effector Cells and Target Cells. PBMCs obtained from RCC patients undergoing tumor nephrectomy or from healthy donors were used for the generation of LAK cells by culture in RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM pyruvate, 100 units/ml penicillin/streptomycin (complete medium), 15% heat-inactivated, pooled human serum, 1% phytohemagglutinin (PHA; Difco Laboratories, Detroit, MI), and 1000 units/ml recombinant IL-2 (Proleukin; Cetus Corp., Emeryville, CA). These cultures were maintained 3–4 weeks to obtain expanded populations of activated CD4⁺ and CD8⁺ T cells. LAK-derived CD4⁺ and CD8⁺ T cells were enriched by depleting all other subpopulations using magnetic bead separation (Dyna, Oslo, Norway), according to the manufacturer's instructions. Human NK cells were activated as described previously (30), and purified NK cells were obtained by depleting T cells using CD4- and CD3-coated immunomagnetic beads (Dyna). The line of

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³ The abbreviations used are: LAK, lymphokine-activated killer; A-LAK, adherent LAK; NK, natural killer; PBMC, peripheral blood mononuclear cell; IL, interleukin; KIR, killer cell inhibitory receptor; RCC, renal cell carcinoma; RCR, relative cytotoxic response; PE, phycoerythrin; NKC, normal kidney cell; mab, monoclonal antibody; TCR, T-cell receptor.

enriched NK cells used in these studies, designated as B.3NK cells (30), was maintained in complete medium supplemented with recombinant IL-2 (300 units/ml).

Specificity of cytotoxicity was assessed using EBV-transformed lymphoblastoid cell lines derived from the L721 cell line. The L721.112 cell line represents a hemizygous variant of L721 that expresses the A1, Cw7, B8 haplotype (kindly provided by T. Spies, Fred Hutchinson Cancer Research Center, Seattle, WA). The L721.221 cell line does not express any MHC class I molecules (31). This line was transfected to express the class I alleles, *B*3501*, *B*3702*, *Cw*0602*, or *Cw*0702*, as described (30, 32). An HLA-E transfectant of K562 was generated by transfecting hybrid DNA, cloned into the pcDNA3 vector, encoding exon 1 of HLA-A2 and exons 2–7 of HLA-E. This construct allows HLA-E surface expression through stabilization of the HLA-E heavy chain with an HLA-A2-derived peptide. Daudi cells transfected with the gene encoding β 2-microglobulin (33) were kindly provided by P. Parham (Stanford University, Palo Alto, CA).

The RCC-26 tumor line was established from a primary stage I (T_1, G_2, N_0, M_0) tumor of patient 26 (HLA alleles: *A*0201*, *A*3303*, *B*4101*, *B*5101*, *Cw*1502*, and *Cw*1701*), and the NKC-26 line was established from normal kidney parenchyma obtained at the time of tumor nephrectomy (34). Both cell lines were cultured in 10% FCS containing complete medium without antibiotics. RCC-26 cells were transduced with the human IFN- γ cDNA, using the retroviral system described previously (35). Two IFN- γ -expressing lines (designated as endo γ 1 and endo γ 2) were generated by independent retroviral transduction (36). A control line (RCC-26VC) was made using the same vector without IFN- γ cDNA. Exogenous IFN- γ stimulation of tumor cells was performed for 96 h in medium containing 1000 units/ml of recombinant IFN- γ (Roche, Basel, Switzerland).

Cell-mediated Cytotoxicity Assay. Cell-mediated lysis was quantitated using standard 4-h ^{51}Cr -release assays (37). Spontaneous release was determined by incubating target cells alone; total release was determined by directly counting labeled cells. The percentage of cytotoxicity was calculated as follows: % specific lysis = (experimental cpm – spontaneous cpm / total cpm – spontaneous cpm) $\times$ 100. Duplicate measurements of four step titrations of effector cells were used for all experiments. To combine data from independent experiments, % RCRs were calculated using specific lysis of untransfected L721.221 or K562 cells in each experiment as reference values of 50%. The percentage of lysis of other target cells was normalized to the reference value and expressed as % RCR (37).

To mask MHC class I molecules, the class I-specific mab, W6/32, was added to target cells 30 min prior to addition of effector cells. mab W6/32 was used as ascites diluted 1:100, and purified immunoglobulin (UPC10; Sigma Chemical Co., Deisenhofen, Germany) was used as the isotype control.

Immunophenotyping of Effector Cells and Target Cells. Effector cells were characterized using a panel of lymphocyte-specific mabs: FITC- or PE-labeled mabs specific for CD3 (UCHT1), CD4 (13B8.2), CD8 (B9.11), and CD56 (NKH-1) were purchased from Beckmann/Coulter (Westbrook, ME). Inhibitory receptor expression was analyzed with FITC- or PE-labeled mabs specific for p58.1 (KIR2DL1, EB6), p58.2 (KIR2DL3, GL183), and CD94 (HP-3B1), all purchased from Beckmann/Coulter and NKb1 (KIR3DL1, DX9) from PharMingen (San Diego, CA). Cells were incubated for 30 min on ice, washed twice, fixed with PBS-1% paraformaldehyde, and analyzed using flow cytometry (FACS-Calibur; Becton/Dickinson, San Jose, CA).

Tumor cells were tested for surface expression of MHC class I molecules by flow cytometry using culture supernatants of the W6/32 hybridoma (American Type Culture Collection, Rockville, MD). The HLA-C-specific mab L31 was kindly provided by P. Giacomini (Regina Elena Cancer Institute, Rome, Italy; Ref. 38). mab UPC10 and MOPC21 (Sigma Chemical Co.) were used as negative controls. Cells were incubated with mabs for 90 min on ice, washed twice with PBS, and incubated with PE-conjugated goat-antimouse immunoglobulin [F(ab) $_2$, 115–116-146; Dianova, Hamburg, Germany] for 30 min and analyzed using flow cytometry.

RESULTS

LAK Cells from RCC Patients and Healthy Donors Recognize MHC Class I-negative Target Cells and Allogeneic Tumor Lines. LAK cells generated from PBMCs of patients with RCC and healthy donors were tested for cytotoxic activity directed against MHC class

I-negative target cells and various tumor cell lines. Fig. 1 shows representative results of several independent experiments in which the HLA class I-negative target cells, L721.221, K562 and Daudi, were efficiently lysed by four different LAK populations. No substantial differences in levels of cytotoxic activity were observed between LAK-26 and LAK-53, derived from two RCC patients (Fig. 1A), and LAK-CP176 and LAK-CP41, derived from two healthy control donors (Fig. 1B). The patient-derived LAK-26 cells lysed the autologous RCC tumor line (RCC-26) and various allogeneic RCC lines (SKRC) as well as the MEL-25 melanoma line. Obviously, the induction of cytotoxicity was independent of the individual HLA background of the tumor cells (Fig. 1C). The control donor-derived LAK-CP176 line lysed these allogeneic tumor cell lines with a similar specificity (Fig. 1D). These tumor lines were shown to be class I positive through binding of the class I-specific mab, W6/32 (data not shown). These results demonstrated that all LAK populations were able to recognize target cells in a non-MHC-restricted manner, and that lysis was not specific for any single tumor entity.

LAK Populations Are Composed Primarily of CD4 $^{+}$ and CD8 $^{+}$ T Cells. Phenotype analyses of lymphocyte subsets were made of the expanded LAK cultures derived from the four different donors used in the experiments shown above. Table 1 shows the distribution of CD3 $^{+}$ CD56 $^{+}$ NK cells and CD3 $^{+}$ T cells. Differences between LAK cells derived from RCC patients and healthy donors were not evident; CD3 $^{+}$ T cells were the major cell type, representing >95% of the cells in all samples. The LAK-CP176 culture was dominated by CD8 $^{+}$ T cells, whereas the other LAK samples contained approximately equal numbers of CD4 $^{+}$ and CD8 $^{+}$ T cells. CD3 $^{+}$ CD56 $^{+}$ NK cells were present in only very low numbers, ranging from 0 to 6% of total cells. These results revealed that the culture conditions used for

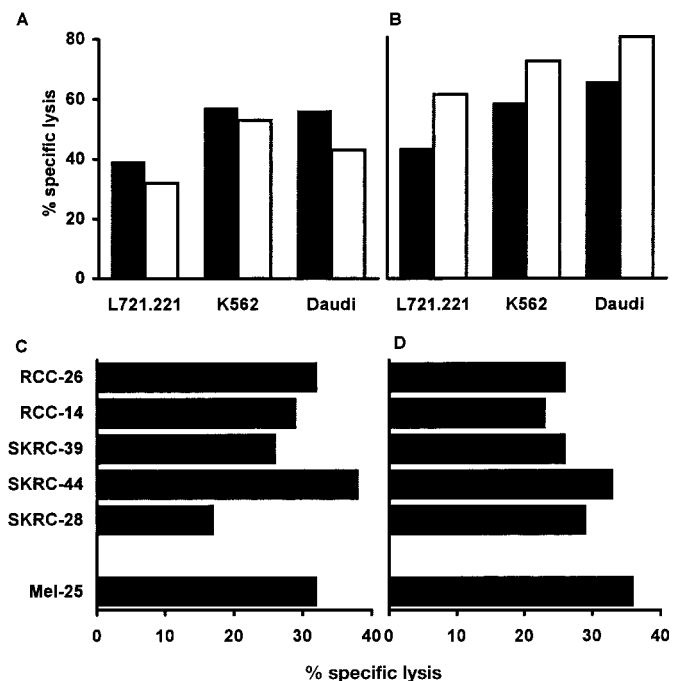


Fig. 1. Cytotoxic activities of LAK cells derived from RCC patients and healthy donors against MHC class I-negative and malignant target cells. LAK cells generated from PBMCs of two RCC patients (A), LAK-26 (■) and LAK-53 (□), and two healthy donors (B), CP-176 (■) and CP-41 (□), were analyzed with three MHC class I-negative target cells, L721.221, K562, and Daudi. Results represent the percentage of specific lysis of one representative experiment of seven independent analyses. LAK-26, LAK-53, LAK-CP176, and LAK-CP41 cells were tested at an E:T of 20:1. C, specific cytotoxic activity of LAK-26 cells against various RCC lines (RCC and SKRC) and one melanoma line (MEL-25) at an E:T of 20:1. D, specific cytotoxic activity of LAK-CP176 cells against the same RCC and MEL lines at an E:T of 20:1.

generation and maintenance of these LAK cells led primarily to expansion of T cells rather than adherent NK cells. These phenotypes indicated that the major cytolytic component was attributable to activated T cells. In fact, depletion of the small remaining fraction of NK cells from the LAK populations did not alter their cytotoxic potential or specificity (data not shown).

IFN- γ -mediated Inhibition of LAK-T Cells and Activated NK Cells Is Associated with Enhanced MHC Class I Expression. It has been shown previously that IFN- γ treatment of tumor cells can lead to resistance to purified NK cells and to LAK cells composed predominantly of activated NK cells (39, 40). To determine whether IFN- γ could also influence tumor cell sensitivity to enriched LAK-T cells, RCC-26 cells were analyzed either after exogenous stimulation with IFN- γ or after transduction with human IFN- γ cDNA (36). Susceptibility of IFN- γ -modulated RCC-26 cells to lysis was assessed using autologous LAK-26 T cells (Fig. 2, A and B). Activated NK cells (B.3NK cells), which were shown previously to be negatively regulated through p58.2 receptors of the KIR family that bind HLA-C molecules of the Cw1, w3, w7 subgroup (30), were included for comparison (Fig. 2, C and D). Unmodified RCC-26 cells and the control line carrying empty vector (RCC-26VC) were lysed by both effector cell types; however, the activated NK cells showed a consistently stronger lytic capacity. Both tumor lines displayed substantial resistance to lysis by the LAK-T cells and NK cells after stimulation with exogenous IFN- γ (Fig. 2, A and C). A similar degree of resistance was observed with one IFN- γ transductant (endoy1), whereas the

Table 1 Phenotype analysis of LAK populations

	CD3 ⁺ CD56 ⁺	CD3 ⁺ CD56 ⁺	CD3 ⁺ CD4 ⁺	CD3 ⁺ CD8 ⁺
LAK-26	1 $\pm$ 0 ^a	9 $\pm$ 2	52 $\pm$ 5	50 $\pm$ 0
LAK-53 ^b	5	6	63	33
LAK-CP176	3 $\pm$ 2	17 $\pm$ 7	19 $\pm$ 6	78 $\pm$ 7
LAK-CP41	6 $\pm$ 2	14 $\pm$ 1	46 $\pm$ 1	46 $\pm$ 1

^a The data represent mean percentages (with SDs) of positive cells derived from four or more experiments.

^b Only two experiments could be performed because of limited growth.

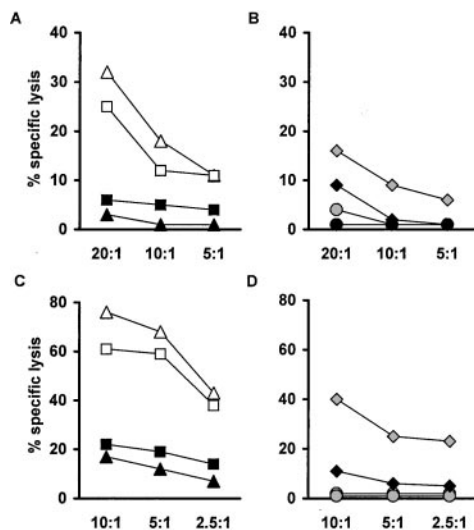


Fig. 2. Inhibition of lysis by endogenous or exogenous IFN- γ modulation of RCC-26 cells. Specific cytotoxic activity of LAK-26 T cells (A and B) and B.3NK cells (C and D) against unmodified RCC-26 cells (Δ), vector control cells RCC-26VC ($\square$) without IFN- γ (open symbols), or after 96 h of stimulation with 1000 units/ml IFN- γ (black symbols). Unmodified RCC-26 and RCC-26VC cells were directly compared for their sensitivity to lysis by LAK-26 (A) and B.3NK (C) cells. Cytotoxic activity of LAK-26 (B) and B.3NK (D) cells against two endogenous IFN- γ -expressing RCC-26 lines, endoy1 ($\circ$) and endoy2 ($\diamond$), without (gray symbols) or after 96 h of stimulation with exogenous IFN- γ (black symbols). Data are shown as the percentage of specific lysis for one of four independent experiments.

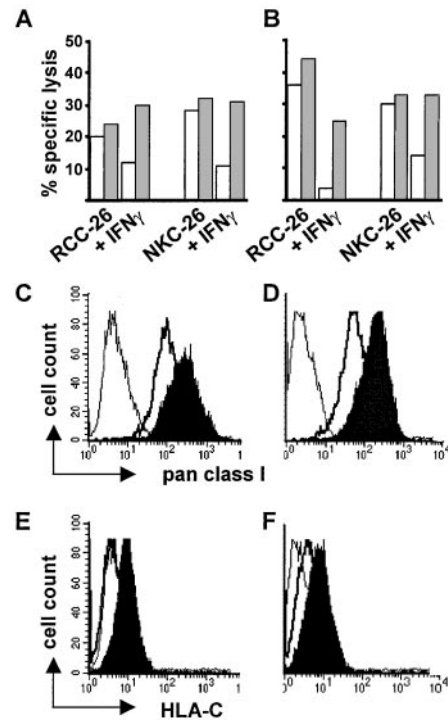


Fig. 3. LAK and B.3NK activities are not tumor specific, and inhibition can be reversed by blocking with HLA class I mab. LAK-26 (A) and B.3NK (B) cells were tested in parallel against tumor (RCC-26) cells and normal kidney cells (NKC-26), both preincubated with control mab (MOPC21), without IFN- γ ($\square$), or after 96 h of IFN- γ stimulation ($\blacksquare$). Cells preincubated for 30 min with class I-specific mab (W6/32) in both graphs. RCC-26 (C and E) and NKC-26 (D and F) were analyzed for HLA class I surface expression using W6/32 for general class I staining (C and D) and the HLA-C-specific mab, L31 (E and F). Unstimulated RCC-26 or NKC-26 cells are shown in bold open graphs, IFN- γ -stimulated cells are displayed as filled graphs, and control staining with mab MOPC21 is shown with open thin graphs.

second transductant (endoy2) showed only partial resistance to both effector populations (Fig. 2, B and D). However, further stimulation with exogenous IFN- γ led to an increased resistance to lysis by both LAK-26 T cells and B.3NK cells (Fig. 2, B and D). In parallel studies, the LAK-53, LAK-CP41, and LAK-CP176 T cells showed cytotoxic patterns similar to those of LAK-26 T cells and B.3NK cells, demonstrating a general correlation between IFN- γ -induced effects and resistance to LAK-T cells (data not shown).

IFN- γ stimulation of a cell line derived from normal kidney parenchyma of patient 26, NKC-26 (34), revealed that resistance to LAK-T (Fig. 3A) and B.3NK (Fig. 3B) cytotoxicity also occurred in normal epithelial cells after IFN- γ stimulation. These results demonstrated that neither the effector cells nor the IFN- γ -induced resistance to lysis of target cells was tumor specific. Parallel studies analyzing the levels of general class I expression directly on the target cells used in Fig. 3, A and B, showed increases in total MHC class I expression in both RCC-26 (Fig. 3C) and NKC-26 cells (Fig. 3D). To resolve the composite pattern of the pan-class I staining into individual allelic components, both cell lines were separately analyzed for the expression of HLA-A2 molecules using several HLA-A2-specific mabs, HLA-B51 using a Bw4-specific mab and HLA-B41 using a Bw6-specific mab (data not shown). HLA-A and HLA-B represented the majority of class I molecules, whereas the level of HLA-C molecules was very low (Fig. 3, E and F). Nevertheless, IFN- γ stimulation substantially increased the level of HLA-C molecules, which serve as the ligands for the p58.2 inhibitory receptors that govern the activity of B.3NK cells but had only weak effects on HLA-A and HLA-B expression (Fig. 3, E and F, and data not shown). The weak staining pattern observed with mab L31 may be explained by its preferential

Table 2 Reversal of MHC class I-mediated inhibition by α MHC class I mabs

Effector cells	E:T ^a	mab ^b	Target cells					
			RCC-26	+IFN- γ	RCC-26VC	+IFN- γ	endo γ 1	endo γ 2
LAK-26	20:1	Isotype	20	15	25	19	8	7
		α class I	24	30	32	31	15	16
LAK-CP41	20:1	Isotype	9	4	NT ^c	NT	4	4
		α class I	26	15	NT	NT	20	21
B.3 NK	20:1	Isotype	59	15	33	15	8	13
		α class I	69	55	46	46	41	42

^a E:T cell ratio.^b mab concentrations: 10 μ g/ml of isotype control; 1:100 diluted ascites of the class I-specific mab W6/32.^c NT, not tested.

reactivity with β_2 -microglobulin-free heavy chains of HLA-C molecules (38). Although RCC-26 and NKC-26 cells showed bright expression of total class I, the levels of individual allotypes may determine the threshold between cytotoxicity and inhibition. In studies not shown, it was found that allogeneic LAK-T cells could lyse the NKC-26 line and that IFN- γ stimulation led to partial protection, demonstrating that resistance was not limited to autologous LAK-T cells.

Masking of MHC Class I Restores Killing by LAK-T Cells.

Because IFN- γ regulates the expression of many different genes, studies using monoclonal antibody to mask class I expression on target cells were used to demonstrate that these molecules directly contributed to the down-modulation of LAK-T cell and NK cell activity. Preincubation of IFN- γ -stimulated RCC-26 or NKC-26 cells with W6/32 mab substantially increased lysis by both effector populations (Fig. 3, A and B). Thus, resistance of both normal epithelial cells and tumor cells was mediated by class I molecules, and masking them with antibody increased target cell susceptibility. The class I-dependent inhibition of LAK-T cell cytotoxicity was confirmed by extended blocking studies (Table 2). Cytotoxic activities of LAK-T cells (LAK-26, LAK-CP41) and control B.3NK cells against various target cells were analyzed in the presence of W6/32 or isotype control mab. Preincubation of unstimulated RCC-26 and RCC-26VC cells with W6/32 mab led to small increases in lysis by the different effector cells as compared with isotype controls. Addition of W6/32 mab led to increased lysis of the IFN- γ -stimulated RCC-26 and RCC-26VC cells. Moreover, lysis of both IFN- γ -expressing transductants was substantially increased in the presence of W6/32 mab. These results demonstrated that class I molecules, at least in part, contributed directly to the IFN- γ -mediated inhibition of LAK-T cells and activated NK cells. The improved cytotoxicity consistently achieved through masking of class I was also observed with a second RCC line and a corresponding IFN- γ transductant, revealing that this was not an effect limited to this single tumor line (data not shown).

HLA Class Ia and Class Ib Molecules Can Directly Inhibit LAK-T Cell Cytotoxicity. Because LAK-T cells efficiently lysed class I-negative target cells, we investigated whether expression of class I molecules in such cells could directly inhibit LAK-T cell activity. The class I-negative cell line L721.221 was derived from a class I-positive lymphoblastoid cell line (L721) by irradiation-induced mutagenesis and is HLA class I negative (31). L721.112 represents a hemizygous cell line generated in this series that still expresses one of the two parental haplotypes (HLA-A1, B8, Cw7). L721.221 cells were very sensitive to LAK-26 T cells, whereas the expression of class I molecules in L721.112 cells provided partial protection (Fig. 4A). The Daudi cell line does not produce β_2 -microglobulin and thus does not express MHC class I at the cell surface. Transfection with the β_2 -microglobulin gene reconstituted class I expression (33), which was confirmed by staining with W6/32 mab (data not shown). These transfectant cells also showed substantial resistance to lysis compared

with untransfected cells (Fig. 4B). The genetic HLA class I background and the expression levels of individual allotypes are likely to influence the degree of inhibition mediated by these transfectant cell lines. In addition, K562 cells that are also class I negative acquired substantial resistance to LAK-26 T cells after transfection with the class Ib gene encoding HLA-E molecules (Fig. 4C). L721.221 cells genetically modified to express HLA-B35, HLA-Cw6, or HLA-Cw7 molecules were also analyzed as target cells for LAK-26 T cells. Whereas inhibition was not induced by HLA-B35 molecules, partial resistance was induced by HLA-Cw6 and Cw7 expression (Fig. 4D). These results confirmed that class I molecules alone could directly inhibit LAK-26 T-cell cytotoxicity.

Insight into the fine specificity of MHC inhibition was also ob-

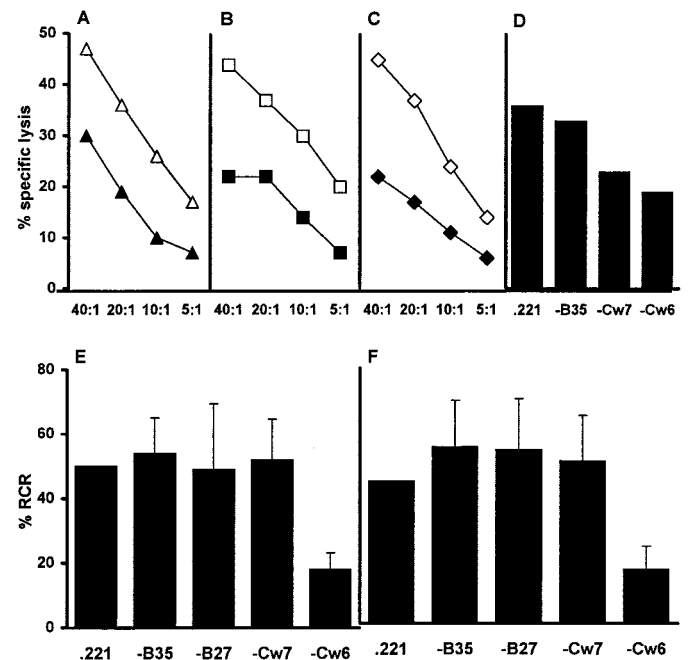


Fig. 4. Inhibition of LAK cytotoxicity by HLA class Ia and Ib molecules transfected into class I-negative cells. A-D, cytotoxicity of LAK-26 cells was tested using different class I-negative target cells after modification to express class I molecules. A, the class I-negative L721.221 (Δ) line compared with the class I-positive HLA-hemizygous variant L721.112 line expressing the HLA-A1, Cw7, B8 haplotype ($\blacktriangle$). B, class I-negative Daudi cells ($\square$) in comparison with class I-positive Daudi/ β_2 -microglobulin transfectant potentially expressing A*0102, A*6601, B*5801, B*5802, Cw*0302, and Cw*0602 ($\blacksquare$; Ref. 33). C, class I-negative K562 cells ($\diamond$) and the corresponding HLA-E-expressing transfectant ($\blacklozenge$). D, L721.221 cells compared with transfectants expressing single HLA alleles (B*3501, Cw*0702, Cw*0602) showing comparable levels of MHC expression (data not shown). Data represent the percentage of specific lysis of one of three independent experiments at an E:T of 20:1. LAK-CP41 (E) and LAK-CP176 (F) lysis of L721.221 cells and transfectants expressing HLA-B*3501, B*2705, Cw*0702, and Cw*0602 at an E:T of 20:1. Data for A-D represent the percentage of specific lysis, and E-F represent mean values of the % RCR using 50% lysis of L721.221 as a reference from three or more independent experiments (37); bars, SD. The values of the percentage of specific lysis of L721.221 ranged from 35% to 58%.

tained for other LAK-T cells (LAK-CP41 and LAK-CP176); lysis of L721.221 cells could be inhibited by >50% by HLA-Cw6 (Fig. 4, E and F). In contrast, HLA-B35, -B27 and -Cw7 molecules did not provide protection.

LAK-derived CD4⁺ and CD8⁺ T Cells Are Also Negatively Regulated by MHC Molecules. A mixed LAK-T cell population was enriched for CD4⁺ and CD8⁺ lymphocytes, respectively, by depletion of other cell types to confirm directly that both T-cell fractions were cytolytic and to determine whether both lymphocyte subsets were down-regulated by IFN- γ -stimulated tumor cells (Fig. 5, A and B). The highly purified CD4⁺ and CD8⁺ T cells were able to lyse RCC-26, NKC-26, and K562 target cells with a specificity pattern similar to that of the unseparated LAK-T population shown in Figs. 1 and 2 (Fig. 5, C and D). Furthermore, IFN- γ modulation (exogenous stimulation or endogenous expression) of RCC-26 and NKC-26 cells inhibited both the purified CD4⁺ and CD8⁺ LAK-T cells.

LAK-T Cells Do Not Express Known Inhibitory Receptors. The demonstration that LAK-T cells were negatively regulated by interaction with class I molecules suggested that they might express inhibitory receptors similar to those carried by NK cells. Therefore, surface phenotyping was made using mabs specific for several known inhibitory receptors that interact with HLA-B, -C, or -E molecules. As summarized in Table 3, inhibitory receptors belonging to the KIR immunoglobulin superfamily (p58.1, p58.2, or NKB1) were not present on the T cells. Small percentages of both CD3⁺ and CD3⁻ cells (3–7%) expressed CD94 molecules. It has been found that CD94 associates with the NKG2A coreceptor molecule in NK cells to create an HLA-E-specific heterodimeric inhibitory receptor. Because K562 cells expressing HLA-E substantially inhibited LAK-26 T-cell-mediated cytotoxicity (Fig. 4C), we expected to find T cells bearing such receptors in this population. However, <1% of LAK-26 T cells bound NKG2A antibody (data not shown). Likewise, because LAK-26, LAK-CP41, and LAK-CP176 were partially inhibited by HLA-Cw6 molecules (Fig. 4, D–F), some T cells expressing p58.1 receptors were expected but were also not found in these populations. Thus, the known inhibitory receptors governing NK cell inhibition by HLA-C

Table 3 Analysis of inhibitory receptor expression on LAK populations

	NK cell markers							
	p58.1 ^a		p58.2 ^a		NKB1 ^a		CD94 ^a	
	CD3 ⁺	CD3 ⁻	CD3 ⁺	CD3 ⁻	CD3 ⁺	CD3 ⁻	CD3 ⁺	CD3 ⁻
LAK-26	0 $\pm$ 0 ^b	0 $\pm$ 0	1 $\pm$ 1	1 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	7 $\pm$ 7	7 $\pm$ 8
LAK-CP176	0 $\pm$ 0	0 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	6 $\pm$ 2	3 $\pm$ 2
LAK-CP41	0 $\pm$ 0	0 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 1	0 $\pm$ 0	0 $\pm$ 0	3 $\pm$ 1	5 $\pm$ 2
B.3NK	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	95 $\pm$ 4	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	85 $\pm$ 15 ^c

^a Mixed NK populations served as positive controls for these mabs (data not included).

^b The data represent mean percentages (with SDs) of positive cells derived from four or more experiments.

^c These CD94 molecules are associated with an activating NKG2C molecule (data not shown).

and HLA-E ligands were not expressed to any appreciable degree by LAK-T cells, although the specific class I ligands characteristic for these receptors were apparently delivering inhibitory signals to these T cells.

DISCUSSION

The regulation of LAK activity has been reported to be influenced by many distinct molecules (41), but the major factors impacting on LAK function remain undefined to date. In this study, we demonstrate that LAK-T cells generated from PBMCs of RCC patients and healthy donors are negatively regulated through interactions with MHC class Ia and Ib molecules in a manner similar to that seen with activated NK cells. It is clear that leukemic cell lines such as K562 and Daudi, which are used as indicator cells for NK and LAK activity, respectively, are excellent target cells because they lack HLA class I expression. Indeed, all LAK-T cell samples showed strong lysis of these class I-negative target cells; however, they became partially resistant after genetic modification to express either class Ia or class Ib molecules. Class I-positive tumor cells could also be lysed by mixed LAK-T cell populations, enriched CD4⁺ and CD8⁺ LAK-T cells, and activated NK cells. Nevertheless, the role of HLA molecules as key regulators of all these effector cells was clearly demonstrated through their inhibition by tumor cells showing up-regulated class I expression after IFN- γ stimulation. Moreover, direct inhibition by HLA molecules was confirmed by masking their expression using class I-specific mabs that partially restored target cell lysis. The failure to completely restore all cytotoxicity may be related to the failure of the W6/32 antibody to optimally bind to all class Ia and class Ib allotypes, particularly under the culture conditions (*i.e.*, 37°C) required to detect cytotoxicity.

In general, both the levels and the class I allotypes, rather than the mere presence or absence of HLA, determined resistance or susceptibility of tumor cells to lysis by these various non-MHC-restricted effector cells. This supports the contention that LAK-T cells behave as molecular densitometers detecting specific class I allotype expression. Quantitative assessment of class I expression through binding of the pan class I mab, W6/32, was not predictive of the degree to which tumor cells were resistant to LAK-T cells. This is most likely attributable to the fact that the tumor lines may vary in their expression of individual HLA allotypes. The levels of different allotypes could not be analyzed because of the lack of appropriate allele-specific mabs, so that the relative contribution of individual class Ia and class Ib molecules to inhibition could not be defined. Class I transfectant cell lines provided some insight into the role of different HLA allotypes conferring resistance. The ability of L721.112 and Daudi/ β_2 -microglobulin cells to partially inhibit LAK-26 cells suggested that inhibition was not limited to any unique HLA molecule because these two lines were derived from unrelated donors with different HLA allo-

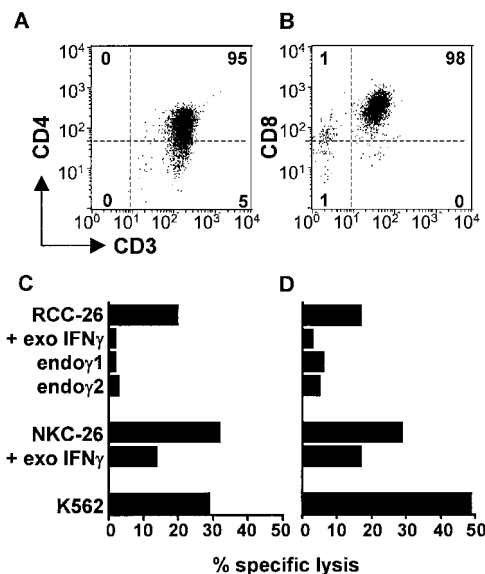


Fig. 5. Negative regulation of cytotoxicity of CD4⁺ and CD8⁺ LAK-T cells. A and B, phenotype analysis of enriched CD4⁺ (A) and CD8⁺ (B) LAK-26-derived T cells. CD3/CD4 and CD3/CD8 staining is shown. The cytotoxic pattern of CD4⁺ LAK-26 cells (C) was compared with that of enriched CD8⁺ LAK-26 T cells (D). Data represent the percentage of specific lysis at an E:T of 20:1 for (C) and (D).

types. Furthermore, the partial resistance of transfectants expressing HLA-C and HLA-E molecules revealed that both class Ia and class Ib molecules contributed to inhibition. Therefore, it appears most likely that various class I allotypes each inhibit some cells in mixed LAK-T populations. Interestingly, HLA-C molecules could substantially inhibit activity of several LAK-T cell populations. Because HLA-C expression is generally lower than that of HLA-A or HLA-B, HLA-C molecules may play a particularly important role in regulating these non-MHC-restricted T cells. IFN- γ stimulation of RCC-26 and NKC-26 led to enhanced expression of the adhesion molecules, intercellular adhesion molecule-1 (CD54) and lymphocyte function-associated antigen-3 (CD58), along with class I (data not shown). Because these adhesion molecules have been associated with activation of LAK cells (42, 43), it was considered that IFN- γ stimulation might increase tumor cell susceptibility to LAK-T- and NK-mediated cytotoxicity. However, IFN- γ stimulation caused almost complete resistance, demonstrating the dominance of class I-mediated inhibition in LAK-T cells. A similar finding was reported previously for NK-mediated lysis of IFN- γ -stimulated melanoma cells (44). These findings now provide a plausible explanation for older studies demonstrating an IFN- γ -induced resistance of breast carcinoma lines to lymphokine-activated NK cells (40, 41).

KIR and CD94/NKG2A inhibitory receptors are highly characteristic for NK cells, but small numbers of T cells in healthy donors have been found to express KIR or CD94 molecules. Expanded populations of T cells bearing these receptors were also found in several clinical situations (reviewed in Ref. 45). KIR⁺ T cells were present among RCC tumor-infiltrating lymphocytes (46), in PBMCs of HIV-infected individuals (47), and in PBMCs of patients after bone marrow transplantation (48). Ikeda *et al.* (49) initially demonstrated the functional relevance of inhibitory receptor expression by MHC-restricted T cells. They showed that CTLs coexpressing tumor-specific TCRs and p58.2 KIR could not lyse autologous melanoma cells simultaneously expressing the MHC-peptide ligand of the TCRs and the HLA-Cw7 ligand of the KIRs. The dominant-negative regulation via signaling through an inhibitory receptor in these CTLs may have enabled the melanoma cells to escape immune elimination. TCR-mediated lysis occurred, however, with tumor cell variants that lost expression of HLA-Cw7. Because the LAK-T cells studied here were partially inhibited by HLA-C and HLA-E molecules, NK inhibitory receptors with corresponding specificities were primary candidates to explain inhibition of lysis. However, KIR and CD94-specific antibodies failed to bind to any LAK-T cells. Thus, their negative regulation appears to be mediated by as yet undefined inhibitory receptors that interact with class Ia and class Ib ligands. Molecular analyses have revealed that the loci encoding the known KIR and CD94/NKG2 inhibitory receptors are members of complex families in which many loci remain to be characterized with respect to patterns of cellular expression and function (50, 51). Therefore, there are many potential candidates to account for the putative inhibitory receptors used by LAK-T cells.

Rosenberg *et al.* (10, 52) were the first to explore the antitumor potential of LAK cells for immune therapy of cancer patients. After adoptive transfer of LAK cells into patients with advanced disease, some patients showed dramatic responses, but many tumors failed to regress. Numerous animal studies and analyses of human LAK cells *in vitro* pinpointed activated NK cells as the major effector component mediating tumor regression (41), although weaker cytotoxic function was also found in the T-cell fraction (2, 6). However, clinical trials using A-LAK cells, applied either alone or in combination with high-dose IL-2 to retain NK viability, failed to improve clinical efficacy (7–9). Furthermore, clinical benefits were severely limited by concurrent toxicity, particularly when IL-2 was coadministered with the A-LAK cells. This toxicity, combined with the inability to under-

stand why only some tumors regressed, led to the abandonment of LAK cells in favor of therapeutic strategies designed to adoptively transfer tumor-infiltrating lymphocytes (reviewed in Ref. 53) or to induce MHC-restricted T-cell responses (17, 18). These newer strategies show promise, but once again, tumor regression has only been observed in some patients. Interestingly, in several well-studied examples, it was found that tumor variants emerged that showed partial or complete loss of MHC class I expression in patients who had generated strong class I-restricted CTL responses *in vivo* (49, 54–56). Extensive immunohistochemical studies of tumors have revealed a high prevalence of cells showing aberrant expression of HLA molecules, often limited to selected HLA allotypes (57). Although such tumors may still bind pan class I antibodies, such as W6/32, their disturbed MHC allotype expression might allow them to escape elimination by MHC-restricted CTLs (58, 59).

The insight provided by our results demonstrating a class I-mediated inhibition of LAK-T cells reveal that these cytolytic lymphocytes might be effective in eliminating tumor cells that show low or aberrant MHC class I expression. Our results support the contention that tumor cells showing high expression of all class I specificities would not be sensitive to LAK-mediated cytotoxicity because they could most efficiently deliver inhibitory signals, whereas they should be more susceptible to MHC-restricted CTLs. In contrast, tumor cells showing low or aberrant class I expression may evade some MHC-restricted CTLs, but as a consequence, they should be more susceptible to LAK cytotoxicity. In particular, our preliminary observations suggest that tumor cells lacking adequate expression of HLA-C and HLA-E molecules might be particularly sensitive to LAK-T cell cytotoxicity. Thus, development of strategies implementing both MHC-restricted, tumor-specific CD4⁺ and CD8⁺ T cells and non-MHC-restricted LAK-T cells might enable a counterbalanced immune attack of tumor cells that would prevent selection of escape variants attempting to avoid both types of effector T cells. Future studies are required to assess whether LAK-derived CD4⁺ and CD8⁺ T cells persist longer *in vivo* than NK cells in the absence of systemic high dose IL-2, thereby providing a means to reduce treatment toxicity. Furthermore, it needs to be determined whether LAK-T cells have a better capacity than NK cells to home and infiltrate tumors. For example, infiltrates of both MHC-restricted and non-MHC-restricted T cells have been found in RCCs, whereas these tumors contain fewer NK cells (53, 60). Although the LAK-T cells described here were found to lyse normal kidney parenchyma cells *in vitro*, they may not invade healthy tissue in large numbers *in vivo*, thereby limiting collateral damage. Whether LAK-T cells should be administered simultaneously with MHC-restricted CTLs or only applied when tumor variants with aberrant HLA expression are detected is also a central question that would need to be resolved to determine how LAK-T cells might be used most effectively in the immunotherapy of cancer.

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