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The Nonclassical Major Histocompatibility Complex Molecule Qa-2 Protects Tumor Cells from NK Cell- and Lymphokine-Activated Killer Cell-Mediated Cytolysis

Eugene Y. Chiang, Maile Henson, and Iwona Stroynowski

The cytotoxic activity of NK cells is regulated by class I MHC proteins. Although much has been learned about NK recognition of class I autologous targets, the mechanisms of NK self-tolerance are poorly understood. To examine the role of a nonpolymorphic, ubiquitously expressed class Ib Ag, Q9, we expressed it on class I-deficient and NK-sensitive B78H1 melanoma. Presence of this Qa-2 family member on tumor cells partially protected targets from lysis by bulk lymphokine-activated killer (LAK) cells. H-2Kb-expressing B78H1 targets also reduced LAK cell activity, while H-2Db offered no protection. Importantly, blocking with F(ab')2 specific for Q9 or removal of this GPI-attached molecule by phospholipase C cleavage restored killing to the level of vector-transfected cells. Experiments with LAK cells derived from H-2Kb SCID and B6 mice established that NK1.1+ TCRγδ NK and NK1.1+ TCRβ+ LAK cells were the prevalent cytolytic populations inhibitable by Q9. Treatment of mice with poly(I:C) also resulted in generation of Q9-regulated splenic cytotoxicity. LAK cells from different mouse strains responded to Q9, suggesting that the protective effect of this molecule is not detectably influenced by Ly49 polymorphisms or the presence/absence of Q9 in NK-harboring hosts. We propose that Q9 expressed on melanoma cells serves as a ligand for yet unidentified NK inhibitory receptor(s) expressed on NK1.1+ NK/T cells. The Journal of Immunology, 2002, 168: 2200–2211.

Recent studies of the class Ia/class Ib family of MHC Ags suggest that these molecules carry out diverse functions in the adaptive and innate immune systems. In addition to presenting pathogen- and tumor-derived ligands to thymically educated T cells, they participate in regulation of NK cell cytotoxicity and in little understood interactions with γδ T cells, intestinal intraepithelial lymphocytes, macrophages, and others. In the mouse system, three categories of class I molecules were reported to interact with NK1.1+ cells: class Ia MHC H-2K and H-2D (1–3), class Ib MHC Qa-1 Ag (4–6), and non-MHC class Ib protein CD1d (7). Each of these molecules plays a different role in NK cell biology and employs a different set of NK receptors to inhibit or modulate their cytotoxicity and/or modulate cytokine production. In H-2b B6 mice, ~25% of NK cells are devoid of the known H-2b class I-specific inhibitory receptors, suggesting that their immunological tolerance toward self is regulated by a set of novel, yet undiscovered NK/target interactions (8).

We have addressed a possibility that the Q region-encoded nonpolymorphic class Ib MHC molecule Q9b (9) is involved in NK cell regulation. The Q9 Ag is a member of the Qa-2 family (10). Its primary amino acid sequence is as similar to class Ia as the alleles of H-2K and H-2D are to each other (9). The three-dimensional crystallographic structure of Q9 is highly homologous to class Ia structures as well as to the class Ib protein HLA-E (11). Furthermore, Q9 binds a wide repertoire of TAP-dependent self and nonself nonanumeric peptides (12), suggesting that Q9 can alert the immune system to the presence of intracellular pathogen infections or malignant transformations. Despite its Ag-presenting properties and the ability to serve as an allogeneic CTL target (13), Q9 is not known to function as a restricting element for pathogen-specific CTL (14, 15), a feature that has been attributed to the inability of Q9 to promote efficient positive selection of thymic T cells (16). The Q9 Ags have a wide tissue distribution and are expressed in immunologically privileged sites/organs/cells: anterior chamber of the eye (17), hair follicles (18), embryo and placenta (19), oocytes and blastocysts (20), and sperm in testis (21). We report in this study that Q9 expressed on melanocyte-derived tumor cells inhibits cytotoxic activity of lymphokine-activated killer (LAK)3 cells. Accordingly, we propose that this molecule plays a role in innate immunity and/or in protecting syngeneic cells from NK-mediated killing, particularly in immunologically privileged tissues, where expression of class Ia and Qa-1 complexed with class Ia leader peptides is very low.

Materials and Methods

Mice

C57BL/6J (abbreviated B6, H2b, Qa-2+), B6.K1 (H2b, Qa-2-), B6 SCID (SCID, H2b, Qa-2+), BALB/cJ (H2d, Qa-2+), 129J (H2n, Qa-2-), C3H/HeJ (C3H, H2b, Qa-2+), β2-microglobulin (β2m) knockout (β2m−/−, H2b), TAP−/− (H2d), and K+β+D− (H2n) mice were either bred and maintained in the Department of Microbiology animal colony at University of Texas Southwestern Medical Center (Dallas, TX) or purchased from The Jackson Laboratory (Bar Harbor, ME). Adult mice (older than 8 wk) were used for all experiments.

Cell lines and cell culture

B78H1 (22) and GM-CSF-transduced B78H1 (23) melanoma cells were generously provided by Drs. H. I. Levitsky (Johns Hopkins School of Medicine, Baltimore, MD) and S. Ostrand-Rosenberg (University of Maryland Cancer Center, Department of Microbiology and Immunology, University of Texas Southwestern Medical Center, Dallas, TX 75390).

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3 Abbreviations used in this paper: LAK, lymphokine-activated killer; β2m, β2-microglobulin; PLC, phospholipase C; bio-, biotinylated.
Baltimore County, Baltimore, MD). Cells were cultured in 50% DMEM/50% RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate (Life Technologies, Grand Island, NY), 0.1 mM nonessential amino acids (Life Technologies), and 10 U/ml penicillin/streptomycin (Life Technologies). NK-sensitive YAC-1 lymphoma cells and NK-resistant P815 mastoyctoma cells were provided by Dr. M. Bennet (University of Texas Southwestern, Dallas, TX) and were cultured in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME (Sigma-Aldrich), 0.1 mM nonessential amino acids, 50X diluted essential amino acids stock (Life Technologies), 1 mM sodium pyruvate, 10 μM HEPE (Life Technologies), and penicillin/streptomycin). All cell lines were grown at 37°C and 5% CO₂. For IFN

**Antibodies**

The following anti-class I MHC Abs were used in this study: anti-α2-μAb 1-1-2 (BD PharMingen, San Diego, CA), anti-IFN-γ mAb Y3 (24), anti-α2-μKb mAb 20-8-4 (25), anti-β2DM mAb 22-18-4 (25), and anti-βm mAb S19.8 (26) with FITC-goat anti-mouse IgG (Cappell, Durham, NC) used as a secondary Ab; biotinylated (bio-) anti-α2-μ mAb M46, bio-γ3, and bio-S19.8 followed by PE- conjugated streptavidin (Biodicine Source International, Camarillo, CA). The following mAbs purchased from BD PharMingen were also used: Fc block anti-mouse CD16/CD32 clone 2.4G2 mAb, PE-conjugated-anti-NK1.1 PK136 mAb, alpholyococcy- and penicillin/streptomycin. All cell lines were grown at 37°C and 5% CO₂. For IFNγ induction, cells were treated with 20 U/ml recombinant mouse IFNγ (Sigma-Aldrich) for 3 days with 20 U/ml recombinant mouse IFNγ (Sigma-Aldrich) for 3 days.

**Flow cytometry**

For target cell staining, 1 × 10⁶ cells were washed once in staining buffer (PBS with 1% FCS and 0.1% sodium azide) and pelleted in a 1-ml poly- styrene conical tube. A saturating amount of primary Ab was added to the cell pellet in a volume of 100 μl, vortexed, and incubated on ice for 15 min. Excess unconjugated Ab was removed by washing the suspension once with staining buffer. A dilution of FITC-labeled secondary Ab was added in a final volume of 100 μl and incubated on ice for 15 min. The samples were washed twice, resuspended in 300 μl of staining buffer, and filtered through 35-μm nylon mesh. A total of 1 × 10⁶ cells were collected on a FACScan flow cytometer (BD Biosciences, Palo Alto, CA). For effector cell staining, 1 × 10⁶ cells were washed once in staining buffer then pelleted in 96-well U-bottom polystyrene plates. FcR were blocked by incubating cells with 1 μg of anti-CD16/CD32 mAb in 20 μl of staining buffer for 15 min on ice. Effector cells were either analyzed for NK.1.1 expression using single-color flow cytometry by staining with PE-conjugated anti-NK.1.1 mAb or a detailed analysis was performed using multicolor flow cytometry. For multi-color analysis, a saturating amount of experimental Ab(s) was added to samples in a volume of 100 μl and samples were incubated on ice for an additional 30 min. The stained cells were washed twice, resuspended in 300 μl of staining buffer, and filtered through 35-μm nylon mesh. A total of 1 × 10⁶ cells were collected on a FACScanLibr flow cytometer (BD Biosciences). Fluorescence compensation was performed when the samples were analyzed using multicolor flow cytometric analysis. Gates were set using forward and side scatter parameters to exclude dead cells. All data was analyzed using CellQuest version 3.1f software (BD Biosciences).

**RNA isolation, cDNA synthesis, and RT-PCR**

Total RNA was isolated using the RNA STAT-60 method (Tel-Test, Friendswood, TX) as previously described (17). First-strand cDNA was synthesized from 5 μg of RNA using the Life Technologies SuperScript II synthesis kit according to the manufacturer’s instructions. PCR was performed using the HotStartTag DNA polymerase system (Qiagen, Valencia, CA). A total of 0.5–2 μl of cDNA was added to a total PCR mixture of 25 μl containing 2.5 μl of PCR buffer containing Tris-Cl, KCl, (NH₄)₂SO₄, and MgCl₂, 5 μl of Q-Solution, 0.5 μl of 10 mM dNTPs, 1 μl of 20 μM upstream primer, 1 μl of 20 μM downstream primer, 0.625 U of HotStartTag DNA polymerase, and diethylpyrocarbonate-treated sterile H₂O. The primer pair TAP2F1 (5’-GATCAACGTTG GATACAGAG) and TAP2R (5’-CCGAGTTCAGAATGACCCAG) was designed from published TAP2 sequence (29) and detects a 523-bp TAP2 product; ACT1 (5’-ACCTGACACTACCTCATGAA) and ACT23 (5’-ACTTGGGTGTGACAGTGAGG) detects a 570-bp β-actin product (30). Primers were initially designed based on the annotations of the gene. The PCR product was isolated in a GeneAmp PCR System 7900 thermal cycler (PerkinElmer, Foster City, CA) and heated to 94°C for 15 min followed by 35 cycles at the settings of 94°C for 1 min for denaturation, 55°C for 1 min for annealing, and 72°C for 1 min for extension, followed by a final incubation at 72°C for 7 min. The PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

**Generation of LAK cells**

LAK cells were generated using a protocol adapted from previously published methods (2, 30, 31). Briefly, B6 spleen cells were aseptically harvested and cultured in complete DMEM medium supplemented with 500 U/ml murine rIL-2 (provided by Dr. M. Bennett). Cultures were maintained in 24-well plates in a total volume of 2 ml in a humidified incubator at 37°C 10% CO₂ and were harvested on day 5 or 6, unless indicated otherwise. LAK cells to be sorted were taken after 5 days of culture and were washed and resuspended in sterile sorting buffer (PBS with 1% BSA) at a concentration of 20 × 10⁶ cells/ml. A total of 4 × 10⁶ cells in a volume of 50 μl of sorting buffer were aliquoted into wells of a 96-well U-bottom plate. Cells were added to stained with 20 μM PE-conjugated anti-NK.1.1 mAb in a total volume of 100 μl. Live cells of lymphocyte size by forward and side scatter were gated and then sorted by the FACStar (BD Biosciences). Collected cells were resuspended in complete DMEM supplemented with 200 μg/ml hygromycin B (Intronotes). Cells were cultured by limiting dilution and TAP2 expression was confirmed by RT-PCR. Q9TAP.11, Q9TAP.17, KβTAP.1-2.9, KβTAP.1-2.25, DβTAP.1-3.4, and DβTAP.1-3.5 were generated by single cell sort of bulk transfections. All clones were monitored by flow cy-
**Generation of poly(I:C)-activated killer cells**

Poly(I:C) activation was performed essentially as described by Chang et al. (8). Mice were injected i.p. with 200 μg of poly(I:C) (Sigma-Aldrich). After 18–20 h, spleen cells were harvested and cultured on tissue culture-treated plates for 1 h at 37°C. The nonadherent cells were used as effector cells.

**Generation of Con A lymphoblasts**

Con A-activated T cell blasts were generated by culturing 30 × 10^6 freshly isolated spleen cells in 6 ml of complete DMEM supplemented with 3 μg/ml Con A type IV (Sigma-Aldrich) for 60–72 h. Cells were spin down and resuspended in HBSS. Viable cells were isolated by density gradient centrifugation by collecting the cells at the interface of HBSS and the lymphocytic-specific gradient isomylph (Gallard-Schlesinger, Carle Place, NY). Excess isomylph was removed by washing cells with 10 ml of HBSS.

**Cytotoxicity assays**

⁵¹Cr release assays were performed according to the protocol of Dr. M. Bennett’s laboratory (2), with few modifications. Briefly, effector cells at various E:T ratios in a volume of 100 μl were added to the wells of a U-bottom 96-well plate. Target cells were labeled by incubating 2 × 10⁶ cells in a volume of 200 μl with 150–200 μCi of Na⁵¹CrO₄ (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at 37°C and 5% CO₂. A total of 2000 ⁵¹Cr-labeled target cells in 100 μl of medium were added to each well, and the plates were incubated at 37°C and 10% CO₂ for 4 h. For anti-Qa-2 blocking experiments, target cells were preincubated with either 100 μg/ml F(ab')₂, 20-8-4 mAb or 100 μg/ml control IgG F(ab')₂ (Caltag Laboratories) for 15 min on ice, and Ab was present throughout the killing assay. For NK cell receptor blocking, effector cells were preincubated with 2.5–5 μg/well Ly-49R-specific F(ab')₂ for 15–30 min at 37°C before addition of target cells, and Ab was present throughout the killing assay. For removal of GPI-attached molecules from the surface of melanoma targets, cells were treated with 2 U of phospholipase C (PLC; Sigma-Aldrich) and 40 μg of brefeldin A (Sigma-Aldrich) during the 1-h ⁵¹Cr labeling incubation. A total of 0.4 μg of brefeldin A was then added to each well during the 4-h incubation with effector cells. After incubation for 4 h, 100 μl of the supernatant was removed from each well and transferred to Skatron macrowell tubes (Skatron, Sterling, VA). Radioactivity was counted in a Micromedic Gamma Counter (ICN Biomedicals, Costa Mesa, CA). Data are expressed as the percentage of specific release, calculated as follows: ([experimental release — spontaneous release]/maximum release — spontaneous release) × 100. Maximum release was determined by incubating target cells with 100 μl of 1% SDS. All experiments were performed in triplicate.

**Results**

**Melanoma targets expressing Q9, H-2Kb, and H-2Dd Ags**

Although the Q9 and the classical H-2K and H-2D Ags are remarkably similar in their structural composition, they differ in specific details of Ag presentation pathways. For example, at low temperatures the bulk of the class Ia H chains reach the cell surface of TAP2-negative RMA-S cells, where they can be selectively stabilized by exogenously added class Ia-specific peptides (32). In contrast, <5–10% of Q9 H chains come out to the surface of TAP-negative cells (Ref. 33 and data not shown). Because the vast majority of peptide-receptive Q9 chains remain intracellular, it is not possible to efficiently stabilize Q9 on the surface of transfected RMA-S or other TAP-negative cells. This property precludes the use of the standard stabilization approach (34–36) for studies of Q9 in NK target recognition.

To overcome this difficulty, we sought to identify an NK-sensitive cell line that will support expression of Q9 in the absence of other class I Ags. Based on a number of different criteria, the B78H1 derivative (22) of B16 melanoma was selected as a recipient for Q9 transfections. Its most important properties can be summarized as follows (E. Y. Chiang and I. Stroyanowski, manuscript in preparation): 1) B78H1 is deficient in H-2Kb and H-2Dd transcription (23) and stains negative for surface β₂m; 2) this phenotype is not altered by incubation with IFN-γ; 3) β₂m is constitutively transcribed in B78H1, suggesting that β₂m expression is intact and available for interactions with transfected class I H chains; 4) B78H1 is TAP2 negative, but this deficiency is reversed upon IFN-γ stimulation.

We generated a panel of B78H1 transfectants expressing Q9 or empty vector and characterized their phenotype with anti-Q9 and anti-β₂m Abs (Fig. 1). The analysis of these transfectants showed that B78H1 expressing Q9 cDNA driven by a CMV promoter
(Q9.A7 clone) expressed low but detectable cell surface levels of Q9-specific 1-1-2 and 20-8-4 epitopes, as well as B2-m-specific S19.8 epitope (Fig. 1A, Q9.A7 panel). None of these epitopes was detected on empty vector-transfected B78H1 (Fig. 1A, vector panel). Supertransfection of the Q9.A7 clone with a plasmid expressing TAP2 cDNA (Q9TAP.C1 clone) resulted in a detectable enhancement of cell surface Q9/B2-m epitopes (Fig. 1A, Q9TAP.C1 panel). Independently generated Q9TAP.11 and Q9TAP.17 clones (Fig. 1C) recapitulated this phenotype but presented a more uniform display of Q9 proteins. The up-regulation of Q9 in TAP-positive cells is consistent with the predicted enhancement of the transport of peptide-filled Q9 Ags to the cell surface in TAP-positive cells compared with TAP-negative cells (33). Treatment of B78H1 transfectants with IFN-γ (Fig. 1A, right panels) also resulted in increases of surface Q9/B2-m complexes, because this cytokine induced TAP2 transcription in transfected cells (Fig. 1B) and may have had additional stimulatory effects on the Q9 Ag presentation pathway. IFN-γ stimulation did not induce B2-m or class I expression on vector-transfected B78H1 (Fig. 1A) despite detectable up-regulation of TAP2 revealed by RT-PCR (Fig. 1B). The latter result formally demonstrates that the parental B78H1 tumor remains class I negative in IFN-γ-treated cells and that this phenotype is independent of TAP2 expression status.

Because B78H1 cells were not previously characterized as targets for NK/LAK cell killing or for their ability to inhibit cytotoxic effects by class Ia Ags, we constructed two additional sets of reference targets: TAP2–H-2Kb (KbTAP.1-2.9 and KbTAP.1-2.25) and TAP2–H-2Db6 (DbTAP.1-3.4 and DbTAP.1-3.5). The class Ia-positive clones were confirmed to constitutively express TAP2 by RT-PCR (data not shown) and to display class Ia surface epitopes characteristic of H-2Kb or H-2Db6 (Fig. 1C). Comparisons of flow cytometry profiles of Q9 on Q9/TAP+ clones with Kb on KbTAP+ clones using Q9/Kb cross-reactive 20-8-4 mAb indicate that the two Ags are expressed at comparable levels.

Q9 expression inhibits cytotoxic activity of LAK cell populations in vitro

Many different methods are currently used to generate LAK cells with cytotoxic activity. We have initially chosen a classical approach used by Bennett et al. (2) as well as others (30, 31). B6 LAK cells prepared according to the protocols, whereby splenic cells were cultured with IL-2 for 5–6 days, typically contained a very large proportion (30–65%) of NK1.1+ TCRγδ LAK cells and a smaller fraction (<10%) of classical NK1.1+ TCRαβ NK cells (Fig. 2A). Four-color flow cytometry analysis of these LAK cells revealed that the NK1.1+ TCRαβ LAK cell population was composed primarily of CD8+CD4− cells (Fig. 2B) and expressed predominantly CD8αβ heterodimers (Fig. 2C). NK1.1+ TCRγδ T cells in LAK cell cultures were predominantly CD8− or CD4+ T cells (Fig. 2B); NK1.1+ TCRαβ NK cells were CD4 or CD8 negative (data not shown). The LAK cell phenotype observed in our experiments is consistent with previously published descriptions (30, 31).

We used such IL-2-generated LAK cells in an extensive series of in vitro killing experiments designed to test the ability of Q9 to modulate their cytotoxicity. A representative experiment in Fig. 3A demonstrates that B6-derived LAK cells efficiently killed class I-negative, vector-transfected B78H1 tumor targets. Significantly, the killing of B78H1 was partially reduced when target cells expressed Q9 Ag. To test reproducibility and clonal variability of this observation, the killing assays were repeated more than 60 times using three independently isolated Q9 transfectants (Q9TAP.C1, Q9TAP.11, and Q9TAP.17) and TAP2-negative parental as well as TAP2-positive transfected vector controls (Fig. 3 and data not shown). In each case we observed partial inhibition (~30% reduction in percentage of specific lysis) of LAK cell cytotoxicity in the presence of Q9. Data for E:T ratios of 100:1 and 30:1 are summarized in Fig. 3B. Similar differential killing between B78H1 and its Q9-transfected derivative was observed in experiments using LAK cells cultured in the presence of IL-2 for 3 days (data not shown).

As internal controls we monitored cytotoxicity of LAK cells against NK-sensitive YAC-1 targets and NK-resistant P815 targets. Killing of class I-negative B78H1 and the YAC-1 control targets was always more pronounced than killing of any other cell line/transfectant used in our studies. However, the relative hierarchy of killing of YAC vs vector-transfected B78H1 tumor varied in different experiments, suggesting that individual LAK cell preparations differed in their active cytotoxic specificity against these two distinct tumors. The susceptibility of P815 control tumor was less than that of the Q9-transfected B78H1 tumor in all experiments.
Ab against Q9 restores LAK cell-mediated lysis of Q9-positive targets

The differential cytotoxic activity of bulk LAK cells against B78H1 tumor and Q9 transfectants suggested that cell surface display of Q9 Ag partially inhibited the lysis of the Q9-positive targets. To confirm this interpretation, we tested whether Abs blocking the access to Q9 during the killing assay would interfere with the inhibition. F(ab')2 of 20-8-4 mAb, which interacts with α2 domains of Q9 (37), were used to diminish the possibility of Ab-dependent cellular cytotoxicity. At the concentration of 100 μg/ml, the anti-Q9 blocking F(ab')2 fully restored killing of the Q9TAP.C1 targets but had no effect on killing of the vector-transfected tumors (Fig. 4A). As an additional control, we tested F(ab')2 of an isotype-matched control Ab. At the same 100 μg/ml concentration, the control F(ab')2 neither blocked nor enhanced the killing of vector-transfected B78H1 or Q9TAP.C1. Titration of the blocking anti-Q9 F(ab')2 demonstrated that full restoration of killing is observed at concentrations greater than 10 μg/ml (Fig. 4B).

FIGURE 3. Q9 partially inhibits the cytotoxic activity of LAK cells against B78H1 targets. Day 5–6 IL-2-cultured LAK cells were used in 4-h 51Cr release assays against tumor targets. A, Lysis (as percentage of specific release) of vector-transfected and Q9TAP2-transfected (Q9TAP.C1) targets as a function of E:T ratio. Inset, Sensitivity of vector-transfected B78H1 (■) to LAK-mediated killing relative to YAC-1 (○) and P815 (△) control targets. Results are shown as mean ± SE. One representative experiment of over 25 performed is shown. B, Q9 inhibition of LAK cytotoxic activity is statistically significant. The ability of IL-2-generated LAK cells to lyse three independently generated Q9-expressing targets was examined. Lysis of Q9TAP2 transfectants was compared with that of vector-transfected B78H1 at two E:T ratios: 100:1 and 30:1. Percentage of inhibition of lysis was calculated as [1 – (Q9TAP % specific lysis/vector % specific lysis)] × 100. Percentage of inhibition of lysis for Q9TAP.C1 was 31.2 ± 2.2% at 100:1 and 30.8 ± 3.4% at 30:1; percentage of inhibition of lysis for Q9TAP.11 was 26 ± 2% at 100:1 and 28 ± 3% at 30:1; percentage of inhibition of lysis for Q9TAP.17 was 29.9 ± 3.2% at 100:1 and 34.7 ± 5.9% at 30:1. Inhibition was significant in all cases by Student’s t test (*, p < 0.0005). Data are expressed as mean ± SE.

FIGURE 4. Antibody against Q9 completely restores LAK cell cytotoxic activity against Q9-expressing B78H1 targets. A, Vector-transfected B78H1 or Q9TAP.C1 targets were preincubated with either no blocking Ab, 100 μg/ml anti-Q9 blocking 20-8-4 F(ab')2, Ab, or 100 μg/ml control F(ab')2 Ab, then incubated with day 6 LAK cells at an E:T ratio of 100:1 for 4 h at 37°C. This experiment was performed six times, with the results shown above. B, Target cells were preincubated with increasing concentrations of anti-Q9 blocking 20-8-4 F(ab')2 Ab, then incubated with day 6 LAK cells at an E:T ratio of 100:1 for 4 h at 37°C. In data not shown, preincubation with control F(ab')2 Ab had no effect on susceptibility of targets to effector cell cytolyis. Killing in the presence of control F(ab')2 Ab was similar to killing without Ab at all concentrations. The blocking Ab titration experiment was performed twice with similar results. All Ab blocking treatments were conducted in triplicate, with data shown as mean ± SE.

Similar data were observed with two other Q9-expressing targets (data not shown). These results indicate that cell surface expression of Q9 is likely to be responsible for inhibiting LAK cell cytotoxicity in the in vitro assays.

PLC treatment renders Q9-expressing targets sensitive to LAK cell-mediated cytolysis

Q9 Ags are attached to the cell membrane via GPI lipid anchors that are sensitive to cleavage by PLC (38). To further demonstrate that inhibition of killing is regulated by Q9 expressed on target cells, B78H1 target cells were treated with PLC before incubation with LAK cell effectors to remove Q9 from the cell surface. In addition, brefeldin A was added to block egress of newly synthesized Q9 Ags to the cell surface after PLC cleavage. This combined treatment resulted in complete elimination of Q9 from the surface of Q9-positive B78H1 transfectants throughout the culture conditions simulating the cytotoxicity assay (Fig. 5A). The PLC-treated and untreated Q9 transfectants exhibited comparable levels of killing by LAK cells (Fig. 5B). In contrast, survival of vector-transfected tumor cells was enhanced by ~30–45% compared with conditions that did not include PLC and brefeldin A (Fig. 5C). This pattern is most consistent with an explanation that PLC/brefeldin A reduced LAK cytotoxicity toward both targets, but removal of Q9 from Q9 transfectants compensated reproducibly for this effect by enhancing Q9 but not vector target sensitivity. The differential effect of PLC/brefeldin A on killing of targets lends further support for the protective effect of Q9 expression in LAK cell-mediated killing.
The PLC/brefeldin A-induced down-regulation of LAK cell cytotoxicity may have resulted from impairment of their activation pathway at the level of LAK cells or at the level of target cells. In the latter case, we considered a possibility that one of the NK-activating molecules expressed on B78H1 is GPI-linked and is cleaved off the cell surface by PLC. Staining of B78H1 for RAE-1, a GPI-linked class I-like protein that is a ligand for NKG2D-activated NK cells, was negative, but low levels of RAE-1 mRNA were detected by RT-PCR (data not shown). It is not known whether this basal level of RAE-1 is sufficient to account for nearly 30% of LAK cell cytotoxic activity in these assays or whether other activating molecules are also involved in the observed effects.

Regulatory effects of class Ia Ags displayed on B78H1 targets

Our results suggest that Q9 has a modest, 30% down-regulatory effect on bulk LAK cell killing. It is important to emphasize that the degree by which Q9 inhibits LAK cells in this system is, by itself, insufficient to draw conclusions about the strength of the signal induced by LAK cell/Q9 interactions or the clonal distribution/density of the putative Q9-reactive LAK cell inhibitory receptors (see Discussion). To provide an independent reference for the B78H1 killing/inhibitory effects, we tested whether H-2K\(^{\alpha}\), a known NK regulatory element that can inhibit selected NK populations (2, 34, 36), has a detectable inhibitory effect on killing of B78H1 tumor cells. Using B78H1 cells cotransfected with K\(^{\beta}\) and TAP2 (K\(^{\beta}\)TAP.1-2.9 and K\(^{\beta}\)TAP.1-2.25 in Fig. 1C) as targets, we showed that the inhibitory effects of K\(^{\beta}\) were very similar to those of Q9 (Fig. 6A, ~35% inhibition at 100:1 E:T ratio) and quantitatively comparable to inhibitory effects of peptide-loaded K\(^{\beta}\) expressed on RMA-S or blasts (2, 34, 39). In contrast, B78H1 targets expressing TAP2 and H-2D\(^{b}\) (D\(^{b}\)TAP.1-3.4 and D\(^{b}\)TAP.1-3.5) were not protected from killing by LAK cells (Fig. 6B). This result is consistent with previous findings in which D\(^{b}\)-positive hematopoietic cells were used as targets (2, 39).

Negative regulation of LAK cells by Q9 may require a threshold of Q9 expression on target cells

To determine whether inhibition of LAK cells by Q9 molecules is dependent on a threshold level of surface expression on target cells, we compared the killing of TAP-negative Q9\(^{\text{low}}\) and TAP2-positive, IFN-\(\gamma\)-treated, Q9\(^{\text{high}}\)/Q9.A7 transfectants (characterized in Fig. 1A). The results in Fig. 7 demonstrate that the constitutively expressed, heat-stable Q9 on Q9.A7 did not reduce LAK cell cytotoxicity to a statistically significant degree. In contrast, TAP2-enhanced levels of Q9 present on IFN-\(\gamma\)-treated Q9.A7 cells were sufficient to down-regulate cytotoxicity by ~30–50%. These results suggest that the regulatory effects of Q9 expression on B78H1 targets are dependent on a threshold level of surface expression on target cells.
are consistent with the density-dependent effect of Q9. However, we cannot exclude the possibility that the nature of the peptides/ligands bound to Q9 under the TAP2-negative vs TAP2-positive (and IFN-γ-induced) conditions contributes to these observations. For instance, specific sets of peptide/Q9 complexes that can differentially influence LAK cell activity may be favored.

**Q9 inhibits activity of LAK cells generated from Q9-positive and -negative mouse strains of different H2 haplotypes**

Because Q9 is structurally invariant and its polymorphism affects predominantly quantitative expression, we asked whether the putative Q9-reactive LAK cell inhibitory receptors are functionally present in different strains. To address this point, we examined the killing of Q9 and control tumor targets by LAK cells isolated from Qa-2-negative B6.K1 (H2b, Qa-2null) and C3H (H2k, Qa-2null) and Qa-2-positive 129 (H2a, Qa-2inhb) and BALB/c (H2d, Qa-2med) inbred mice. The results in Fig. 8 demonstrate that LAK cells from these different mouse strains were sensitive to down-regulation of LAK cell activity by Q9. The quantitative variations discerned between the different strains were not statistically significant and we do not know, at present, whether they represent true differences. The results are most consistent with the notion that the Q9 inhibitory effect on LAK cells is achieved via a conserved receptor and that presence of this putative receptor in mice is not detectably functionally significant.

Our experiments with IL-2-cultured LAK cells did not identify the active populations of cytotoxic cells that can be inhibited by Q9. Based on previous studies (30), we reasoned that the two most likely candidate effectors are classical NK1.1+ TCR− NK cells, which represent a minor fraction (<10%) of our LAK cells, and NK1.1+ TCR+ LAK cells, which represent a major fraction of IL-2-derived LAK cells (30–65%). To directly address whether Q9 inhibits activity of NK cells, we used effectors from SCID mice, which do not produce mature T cells or B cells and lack CTL as well as IL-2-induced NK1.1+ TCR− LAK cells. The flow analysis in Fig. 9A confirms this point and identifies NK cells as the major population of SCID LAK cells. The results in Fig. 9B indicate that the NK1.1+ cells from SCID killed B78H1 and that this killing was reduced in the presence of Q9. Furthermore, inhibition of NK cell-mediated cytotoxicity against Q9-expressing targets. SCID LAK cells were stained for TCR and NK1.1. Percentages of cells for each mouse strain.

**FIGURE 8.** Q9 inhibits activity of LAK cells derived from both Q9+ and Q9− mouse strains of different haplotypes. Day 5–6 LAK cells were generated from Qa-2-negative B6.K1 (H2b) and C3H (H2k) and Qa-2-positive 129 (H2a) and BALB/c (H2d) mice in the same manner as B6 LAK cells. All effectors were tested against vector-transfected B78H1 and at least one Q9-expressing transfectant. Results are shown as mean ± SE. Experiments were performed at least three times with effector cells from each mouse strain.

**FIGURE 9.** Q9 inhibits “classical” NK cells. Effector cells were generated by culturing splenocytes from SCID mice in the presence of IL-2 for 5–6 days. A, Multicolor flow cytometry analysis of effector populations. SCID LAK cells were stained for TCR and NK1.1. Percentages of cells for each quadrant are shown below the dot plot. In data not shown, cells were gated on the TCR−NK1.1− (lower right) quadrant and analyzed for CD8α vs CD4 and CD8α vs CD8β2 expression. These cells were ~95% negative for these surface markers. Profile shown is representative of three separate stainings. B, SCID LAK cells were used against vector-transfected and Q9-expressing Q9TAP.C1 B78H1 targets. Inset, Sensitivity of vector-transfected B78H1 (■) to LAK-mediated killing relative to YAC-1 (○) and P815 (△) control targets. Results are shown as mean ± SE. One representative experiment of five is shown. C, Ab against Q9 completely restores NK cell-mediated cytotoxicity against Q9-expressing targets. SCID LAK cells were tested against target cells preincubated with F(ab′)2. Ab as described in Fig. 4. Percentage of specific lysis was determined at an E:T ratio of 100:1, and data are shown as mean ± SE.
killing was reversed in the presence of anti-Q9 F(ab')2 (Fig. 9C).
This demonstrates that NK cell-mediated cytotoxicity against
B78H1 can be inhibited by Q9 Ag.

To determine whether the NK1.1+ TCR+ LAK cell fraction in
our LAK cell preparations had lytic capacity, we examined cyto-
xic effects of the sorted NK1.1+ TCR+ population in parallel
with the bulk unsorted LAK cells generated in the same experi-
ment (Fig. 10A). The results in Fig. 10B demonstrated that
NK1.1+ TCR+ LAK cells, similarly to bulk LAK cells, were
effective in killing B78H1 and were inhibited by Q9 expression on
target cells. Approximately 20–30% of the sorted NK1.1+ TCR+
LAK cells stained positive for 5E6 mAb specific for Ly49CI re-
ceptors that recognize K+ (Fig. 10C). Sorted NK1.1+ TCR+ cells
did not display cytotoxicity against B78H1 that was detectable
above P815 control levels (data not shown). Accordingly, we con-
clude that NK1.1+ TCR+ cells are the most numerous lytic ef-
fec tors in IL-2-induced LAK cell cultures that can be inhibited by Q9.

In vivo activated cytotoxic effectors are inhibited by Q9

Up to this point, all our experiments used IL-2-activated LAK cells
as effectors. Because this procedure generated a biased com-
position of NK1.1+ TCR+ LAK cells over classical NK cells, we
prepared activated NK cells by in vivo injection of poly(I:C).
Comparison of TCR/NK1.1 expression on splenocytes from
poly(I:C)-injected mice (Fig. 11A) with that from un.injected mice
(Fig. 11B) showed that the experimental and the control animals
had similar proportions of NK1.1+ TCR+ and NK1.1+ TCR+ cells.
Both the poly(I:C)-activated as well as the nonactivated spleno-
cytes killed YAC-1 targets, albeit with lower efficiency than LAK
cells. In contrast, vector-transfected B78H1 targets were detect-
ably killed only by poly(I:C)-activated cells. As observed with
LAK cells, the poly(I:C)-induced killing was reduced by expres-
sion of Q9 on target cells (Fig. 11A). These results suggest that Q9
offers protection from poly(I:C)-induced cytotoxicity against
B78H1. The differential killing of B78H1 and YAC-1 tumors by
poly(I:C) activated vs nonactivated splenocytes indicates that the
B78H1-specific effectors may depend on activation through a dif-
ferent pathway than YAC-1 killers. Alternatively, the threshold for
killing of YAC-1 by constitutively activated NK cells is lower than
the threshold for killing of B78H1. Furthermore, absence of de-
tectable B78H1 killing by normal splenocytes provides formal ev-
idence that poly(I:C) nonresponsive splenic populations do not
contribute to the cytotoxicity against B78H1 in our in vitro assays.

Qa-2 expression on lymphoblasts is not sufficient for protection
from LAK cell-mediated cytolysis

This study demonstrates that Q9 expressed on the surface of neural
crest-derived melanoma cells inhibits NK and LAK cells. To test
whether Q9 expressed on nontransformed/other tissue type cells
has the same protective effect, LAK cells were tested in vitro
cytotoxicity assays against Con A splenic blast targets derived
from different B6-background mouse strains. The panel included
Q9-positive strains B6 and K/17 half-B/17 (deficient for class Ia,
expressing class Ib; Ref. 14) and Q9-negative strains B6.K1 (lack-
ing Qa-2 genes; Ref. 44), and Kb deficient for class IB, and
TAP-/- deficient for class Ib and many class Ib, including Qa-2; Refs.
39 and 45–48). Flow cytometry studies in Fig. 12A establish that T cell blast surface
levels of Kb, Qa-2, and Kb m are reduced at least 10-fold in Kb m/-
or TAP-/- mice compared with B6, while T cell blasts of
Kb m/- or TAP-/- mice react ~30% of their surface Kb m. The great
majority of class Ib molecules associated with Kb m in Kb m/- or
TAP-/- are likely to be Qa-2, as the Qa-2 expression levels on T cells
approach H-2K (49). Nevertheless, we find that Kb m/- TAP-/- blasts are

FIGURE 11. Q9 inhibits the ability of poly(I:C)-activated NK cells to
lyse B78H1. Spleen cells harvested from mice injected i.p. with poly(I:C)
(A) or freshly isolated splenocytes from untreated mice (B) were used as
effectors against 51Cr-labeled vector and Q9TAP.11 B78H1 transfectants.
Cells were stained with mAb specific for TCR and NK1.1. Numbers in-
dicate percentages of cells in each corresponding quadrant. Data points in
killing assays are mean ± SE. Similar results were obtained in four sepa-
rate experiments.
self MHC class I proteins. In the mouse system, two types of structurally divergent, lectin-like inhibitory receptors have been characterized: Ly49 (51) and CD94/NKG2 (4–6). Both types of receptors are present on NK cells, as well as on subsets of T cells, and are distributed clonally in an overlapping fashion such that different NKT cells express different combinations of class I specific inhibitory receptors. While Ly49 receptors bind directly to different alleles of self and nonself class I MHC molecules, the CD94/NKG2 heterodimers sense class I indirectly by binding to the class Ib molecule, Qa-1, associated with selected class I-derived leader peptides.

In B6 mice, only Ly49C/I of the known Ly49 inhibitory receptors has a clearly defined specificity for self (H2b) class I Ags (8). In addition, the CD94/NKG2A recognizes self H-2D\textsuperscript{b} leader peptide in the context of Qa-1. Importantly, staining of NK cells with Abs and/or tetramers against these two classes of receptors (5, 6, 52) and single cell RT-PCR analyses (53) revealed that approximately one-fourth of NK cells in B6 mice do not express either of these known receptors to self (8). Furthermore, extensive polymorphisms of MHC class Ia ligands and their independently inherited Ly49 receptors suggest that NK cells of individuals who inherit selected sets of Ly49 receptors/MHC alleles may fail to recognize their own class Ia Ags. Similarly, because CD94/NKG2A binds only a subset of the known class Ia leader peptides, the inhibition mediated through this receptor may be inactive in some haplotypes. This may also occur during transcriptional shutdown of class Ia genes in certain virally infected or malignantly transformed cells or in immunologically privileged sites where expression of class Ia is very low. The potential self-reactivity of NK cells in syngeneic systems raises a question of whether additional inhibitory receptors/class Ia ligands contribute to the maintenance of tolerance to self cells.

We report in this work that the nonpolymorphic Q9 Ag can inhibit cytolytic activity of NK cells and LAK cells, and we propose that this phenomenon may be due to the interaction of Q9 with conserved inhibitory receptor(s) expressed on NK and other activated cells. The Q9 Ag is one of the best-characterized murine class Iib molecules, yet its physiological function(s) remains unknown. This MHC molecule shares many similarities with class Ia proteins: it binds \( \beta_m \) and a wide repertoire of peptides delivered by TAP-dependent pathway (33); its tissue distribution includes hemopoietic and nonhemopoietic cell types; and the pattern of expression, while quantitatively different, overlaps with H-2K, H-2D, and H-2L (21). It also has some unique properties: it is attached to membrane via GPI (38) and, upon cross-linking with Abs, activates T cells on which it resides (54, 55). Like many other class Iib molecules, such as HLA-G (56), Q9 are also synthesized as soluble molecules (27, 38). The diverse soluble forms of Q9 are found in murine blood and their levels are quantitatively induced by poly(I:C) with kinetics coinciding with induction of NK activation (57). Although Q9 is present on thymic epithelial cells (14, 15), it fails to select substantial populations of CD8\textsuperscript{T} cells (14, 15), thus suggesting that it is unlikely to be an efficient restriction element for pathogen-restricted T cells (14, 15). Despite this possible deficiency in T cell immunity, Q9 binds endogenous as well as viral/bacterial peptides (12), raising a possibility that it may function independently of class Ia in recognition of self vs nonself. Consistent with this assumption is the observation that the transcription of Q9 is modulated independently of class Ia in many tumors (21).

To test whether Q9 interacts with NK cells, we used the B16 melanoma-derived B78H1 tumor and its transfected derivatives. The salient features of the B78H1 melanoma include the total absence of all \( \beta_m \)-dependent class I Ags on the cell surface of the

![Diagram](http://www.jimmunol.org/)

**FIGURE 12.** Killing of B6, \( \beta_m^{-/} \), TAP\textsuperscript{−/−}, and \( K^b^{-/} \) Con A blasts by IL-2-activated LAK cells. A, Flow cytometry histograms of blasts derived from various mouse strains, as indicated for each profile. Blasts were stained for Qa-2 (bio-M46, upper histograms), K\textsuperscript{b} (bio-Y3, middle histograms), and \( \beta_m \) (bio-S19.8, lower histograms). The degree of biotinylation for each Ab is variable and the specific activity of bio-M46 is lower than that of bio-Y3. The light gray shaded area is irrelevant control Ab staining. B, \( ^{51} \text{Cr} \)-labeled Con A-activated T cell blast target cells were incubated with day 5–6 B6 LAK effector cells for 4 h. Results are shown as mean ± SE. One representative experiment of three is shown.
parental tumor and its high susceptibility to LAK cell-mediated killing. In the present study, we demonstrated that display of Q9 on the B78H1 surface led to reduced killing of the targets by splenic IL-2-induced LAK cells and that this inhibitory effect was specifically prevented by anti-Q9 F(ab’)2 Ab fragments. Further evidence supporting the protective role of Q9 was provided by PLC treatment of Q9-expressing targets. Enzymatic removal of Q9 from the cell surface resulted in cytolytic activity that compensated for the reduction in overall capacity of LAK cells to recognize and kill the tumor targets.

We have also examined the nature of the effector cells responsible for killing of the B78H1 targets and showed that different activation protocols such as poly(I:C) injection in vivo or culturing of splenic cells with IL-2 in vitro lead to generation of cytolytic cells inhibitable by Q9. Experiments with LAK cells derived from SCID mice established that NK cells were the active cytolytic population that is inhibited by Q9 in this strain. In B6 mice, the most prevalent lytic effectors generated by IL-2 activation were NK1.1+ TCR+ CD8+ LAK cells. These CD1d-independent splenic NK1.1+ TCR+ CD8+ LAK cells, characterized previously by other laboratories (30, 31), are thought to arise from NK1.1+ CD8+ IL-2Rα precursor T cells upon IL-2 incubation in vitro or viral infection in vivo (31) and are known to express a variety of NK cell receptors (31). We showed in our study that these CD8+ LAK cells are inhibited by Q9 to a degree comparable to that seen with NK cells from SCID mice. Taken together, the results are most easily reconciled with the hypothesis that the nonpolymorphic Q9 protects the syngeneic mice. Taken together, the results are most easily reconciled with the hypothesis that the nonpolymorphic Q9 protects the syngeneic mice. Taken together, the results are most easily reconciled with the hypothesis that the nonpolymorphic Q9 protects the syngeneic mice. Taken together, the results are most easily reconciled with the hypothesis that the nonpolymorphic Q9 protects the syngeneic mice.

The nature of this putative receptor is speculative at this time. It is possible that NK cell receptors recognizing Q9 correspond to one or more of the known murine inhibitory receptors from the Ly49, CD94/NKG2, or paired Ig-like receptor PIIR (58)-related families, or perhaps different, as yet unidentified, proteins. To date, 21 Ly49 proteins have been detected based on reactivity patterns of mAbs. Of these, Ly49A, B, C, E, F, G, I, J, O, Q, S, T, and V have been shown or are predicted to be inhibitory receptors (40, 41). However, not all mouse strains express all of these receptors, and of those receptors that are common to several strains, polymorphisms may exist (43). LAK cells from different strains were influenced by Ly49 to a similar degree, suggesting that the protective effect of Q9 is not detectably influenced by Ly49 polymorphism among the strains (B6 vs 129 vs BALB/c vs C3H), and the absence or presence of Q9 in the NK harboring hosts (B6 vs B6.K1 vs C3H). Furthermore, Ab blocking of Ly49C/I and G2, the Ly49 inhibitory receptors that are expressed by the four strains used in this study, did not restore killing, suggesting that Q9 is not a ligand for any of these known inhibitory receptors. The possibility exists that Q9 may interact with numerous different inhibitory receptors, and that blocking of one or two members is insufficient to remove the inhibitory effect. For example, the class Ia MHC molecule H-2Dα is a putative ligand for inhibitory receptors Ly49A, C, and G expressed in B6 mice (40). Experiments using a mixture of 5E6 and 4D11 have been inconclusive (data not shown).

Because H-2Kb expressed on B78H1 TAP-positive targets reduced LAK cell killing in our in vitro assays to approximately the same degree as Q9, one could propose that the Q9-specific inhibitory receptor is expressed on a similar proportion of LAK cells as Kb-reactive Ly49C/I (2), which we detected on 12–25% of NK cells (data not shown) and 20–30% of NK1.1+ TCR+ LAK cells (Fig. 9C) in bulk LAK cell cultures. An important caveat of such a proposition is that the observed results may be influenced by qualitative differences in the putative receptor interactions with their respective class I ligands. The class Ia-mediated NK cell inhibition has been previously reported to depend on the density of Kb ligands on target cells (59). In this study we also presented evidence suggesting that the Q9-mediated NK inhibition may require a minimal threshold of Q9 on the surface of target cells. It is thus conceivable that the Q9-mediated inhibition is dependent on the density of Q9 on the target cells in a way that differs from Kb interactions with Ly49C/I. It is also possible that the negative signals delivered to NK cells by Kb and Q9 are qualitatively and/or quantitatively different in countervailing the activation pathway against B78H1 tumor. Finally, Q9 may reduce the killing of LAK cells by as yet unknown mechanisms that are not paralleled by Ly49C/I/H-2Kb signaling.

The inhibitory effect of Q9 on LAK cell-mediated cytotoxicity is clearly demonstrated when Q9 is expressed on B78H1 tumor cells. However, Q9 expression on T cell blasts does not seem to confer the same protection. B6 blasts expressing their full repertoire of class I molecules are recognized as self by B6 LAK cells and are thus spared from lysis. Q9 is not expressed by B6.K1 blasts, but these too are resistant to LAK cells because other class I molecules such as Kb are present. Targets lacking class I such as β2mβ/ and TAP′/− blasts are readily lysed by LAK cells. Interestingly, blasts expressing Qa-2 in the absence of class Ia molecules (Kbβ/−/Dαβ/−/−/ blasts) are killed to the same degree as β2mβ/−/− blasts. This finding recapitulates observations of Grigoriadou et al. (39), who also recently reported that inhibition of NK cells involved in bone marrow graft rejection requires negative signaling from H-2Kb and H-2Dα, but found no evidence that class Ib molecules contribute significantly to this process. Their conclusions were based on experiments which B6 mice were engrafted with bone marrow from mutant Kbα/−, Dββ/−, or Kbα/− Dββ/− mice or experiments in which IL-15-activated LAK cells or poly(I:C)-activated NK cells were tested for killing of class Iα-deficient Con A blasts. Several differences between B78H1 tumor cells and hemopoietic cells may explain the inability of Qa-2 to protect blast targets from LAK cell-mediated cytolysis. B78H1 does not express β2m-dependent class I Ags, while Kbα/− Dββ/− blasts express other class Ib molecules in addition to Qa-2. It is conceivable that the full complement of class Ib proteins expressed in Kbα/− Dββ/− mice influences negatively as well as positive NK regulatory signals and that the net effect of these interactions is not detectable with blast targets. Alternatively, NK cells activated by blasts may require qualitatively different inhibitory signals than NK cells activated by tumors or nonhemopoietic cells. A precedent for different activation pathways being inhibited differentially by class I has been reported for RMA vs RAE-1-transfected RMA cells (60). It is also possible that peptides presented in the context of Qa-2 on T cell blasts vs melanomas may have an influence on the inhibitory effect, as peptide dependence has been observed with some human and murine NK cell receptor/class I interactions (34, 61). In this respect it is of interest that peptide resides higher up in the shallow groove of Q9 than peptides in other MHC molecules (11). Peptide presented in the context of Q9 also has a very high accessibility to solvent, suggesting that it is more likely to interact with other ligands than peptides buried deeper in MHC pockets (11).

The NK inhibitory effects of Q9 molecules are interesting in the context of the known biological properties of this class Ib Ag. Q9 are expressed as both membrane-bound and soluble proteins in several immunologically privileged sites where expression of class Ia is low and NK cell attack may be detrimental (17–21). These features are reminiscent of the human class Ib molecule HLA-G, the major inhibitory NK ligand recognized by killer cell inhibitory receptor (62), leukocyte Ig-like receptor (63), and CD94/NKG2A (64) receptors in placenta. Q9 also shares similarities with HLA-C, a ubiquitously expressed human class Ia Ag that functions as a
dominant ligand for human killer cell inhibitory receptors (65) and is rather ineffective as a restriction element for T cells (66). The separation of NK cell and T cell functions in HLA-G and HLA-C (67) allows them to avoid “compromises” facing other class Ia molecules confronted with opposing selections by T cells and NK cells.

Recent evidence suggests that many molecules dominating mouse and human NK cell/class I recognition evolved independently in these species but nevertheless show functional homologies (68). Q9 and HLA-G/HLA-C may constitute another example of convergent evolution. It appears that NK-cell-regulating properties of these molecules developed at the expense of their T cell-relevant functions. This specialized adaptation was convincingly demonstrated for HLA-G and HLA-C and allows them to confer selective advantage to cells threatened with destruction by NK cells (67). It remains to be established whether similar mechanisms operate on Q9, when it down-regulates cytotoxicity of NK cells.

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