Blastocyst MHC, a Putative Murine Homologue of HLA-G, Protects TAP-Deficient Tumor Cells from Natural Killer Cell-Mediated Rejection In Vivo[1]

Atsushi Tajima,*† Toshitaka Tanaka,*† Tomohiko Ebata,* Kazuyoshi Takeda,* Akemi Kawasaki,* Janice M. Kelly,‡ Phillip K. Darcy,‡ Russell E. Vance,§ David H. Raulet,§ Katsuyuki Kinoshita,† Ko Okumura,* Mark J. Smyth,‡ and Hideo Yagita[2]*

Blastocyst MHC is a recently identified mouse MHC class Ib gene, which is selectively expressed in blastocyst and placenta, and may be the mouse homolog of HLA-G gene the products of which have been implicated in protection of fetal trophoblasts from maternal NK cells and evasion of some tumor cells from NK cell attack. In this study, we identified two blastocyst MHC gene transcripts encoding a full-length α-chain (bc1) and an alternatively spliced form lacking the α2 domain (bc2), which may be homologous to HLA-G1 and HLA-G2, respectively. Both placenta and a teratocarcinoma cell line predominantly expressed the bc2 transcript. When these cDNAs were expressed in TAP-deficient RMA-S or TAP-sufficient RMA cells, only bc1 protein was expressed on the surface of RMA cells, but both bc1 and bc2 proteins were retained in the cytoplasm of RMA-S cells. Significantly, the RMA-S cells expressing either bc1 or bc2 were protected from lysis by NK cells in vitro. This protection was at least partly mediated by up-regulation of Qa-1^b expression on the surface of RMA-S cells, which engaged the CD94/NKG2A inhibitory receptor on NK cells. More importantly, the bc1- or bc2-expressing RMA-S cells were significantly protected from NK cell-mediated rejection in vivo. These results suggested a role for blastocyst MHC in protecting TAP-deficient trophoblasts and tumor cells from NK cell attack in vivo. ***The Journal of Immunology,*** **2003, 171: 1715–1721.**

During mammalian pregnancy, semiallogeneic fetal cells invade the maternal uterine wall but survive without rejection by the maternal immune system (1). Human fetal cytotrophoblasts at the maternal-fetal interface lack classical MHC class Ia molecules due to TAP deficiency (2, 3). Although this prevents recognition by maternal alloreactive T cells, the lack of class Ia molecules leaves these cells susceptible to maternal NK cells, which are specialized to kill cells that have lost MHC class Ia molecules after virus infection or malignant transformation. However, the trophoblasts express a nonclassical MHC class Ib molecule HLA-G, which has been implicated in the protection of trophoblasts from maternal uterine NK cells (4, 5). In support of this, transfection of HLA-G into MHC class Ia-deficient target cells conferred protection from lysis by peripheral blood and decidual NK cells in vitro (6, 7). Moreover, it has been suggested that aberrant expression of HLA-G in tumor cells, which frequently have TAP deficiency (8), may be an escape mechanism from NK cell-mediated immunosurveillance (9, 10).

Recent studies have revealed two types of NK cell surface receptors for MHC class I molecules, which belong to the Ig superfamily or the C-type lectin family and have an immunoreceptor tyrosine-based inhibitory motif in their cytoplasmic domain responsible for inactivation of NK cell effector functions (11). Among them, two Ig-like receptors ILT2 (LIR-1) and p49 (KIR2DL4) directly bind to HLA-G molecules expressed on the surface of target cells and inhibit NK cell-mediated lysis (12–14). In addition, it has been shown that the HLA-G leader fragment can efficiently stabilize and up-regulate the expression of HLA-E, which is another class Ib molecule and a major ligand of the lectin-like CD94/NKG2A receptor (15–17). It has been proposed that the CD94/NKG2A complex acts as the predominant inhibitory receptor involved in the recognition of HLA-G by peripheral blood and decidual NK cells (18).

Despite this remarkable progress in our knowledge about the recognition of HLA-G by NK cells, the physiological functions of HLA-G remain elusive (19), primarily due to the lack of an appropriate animal model. However, it has recently been shown that a mouse MHC class Ib molecule, Qa-1^b, acts as the mouse homolog of HLA-E and is recognized by the mouse CD94/NKG2A receptor (20, 21). Moreover, Sipes et al. (22) have described the cloning of a new mouse MHC class Ib gene, termed blastocyst MHC, that resembles HLA-G in its structure and selective mRNA expression in blastocyst and placenta, although its expression at the protein level and the function are still unknown. In the present study, we investigated the expression and function of blastocyst MHC in TAP-deficient RMA-S cells, which lack classical class Ia molecules and are highly susceptible to NK cells in vitro and in vivo, by introducing two isoforms of blastocyst MHC, which may be homologous to HLA-G1 and HLA-G2. We found that the expression of blastocyst MHC protects TAP-deficient RMA-S cells from NK cell-mediated lysis in vitro by at least partly engaging the CD94/NKG2A inhibitory receptor through up-regulation of Qa-1^b expression. This mouse model enabled us to demonstrate that the aberrant expression of blastocyst MHC in TAP-deficient tumor

*Departments of Immunology and †Obstetrics and Gynecology, Juntendo University School of Medicine, Tokyo, Japan; ‡Cancer Immunology, Peter MacCallum Cancer Institute, East Melbourne, Victoria, Australia; and §Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, CA 94720

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[2] Address correspondence and reprint requests to Dr. Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail address: hyagita@med.juntendo.ac.jp

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cells confers protection from NK cell-mediated rejection in vivo, suggesting a similar role for HLA-G in human tumors.

Materials and Methods

Mice

C57BL/6 (B6) mice, 8–12 wk of age, were purchased from Clear Japan (Tokyo, Japan) or The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). B6 RAG-$2^{-/-}$ mice were kindly donated by Drs. K. Kobayashi and M. Ito in the Central Institute for Experimental Animals (Kawasaki, Japan). Timed pregnant B6 mice were killed by cervical dislocation, and the whole uterus including placenta and embryos (day 3.5 of gestation) and the placenta (day 7.5 and 13.5 of gestation) were dissected for RT-PCR.

Cell lines

A murine teratocarcinoma cell line, F9, was kindly provided by Dr. N. Minato (Kyoto University, Kyoto, Japan) and cultured in DMEM containing 10% FCS, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, 2.3 mg/ml HEPES, and 2.0 mg/ml $NaHCO_3$. A B6 mouse-derived T lymphoma cell line, RMA, and its TAP-deficient mutant, RMA-S, were obtained from American Type Culture Collection (ATCC; Manassas, VA) and cultured in RPMI 1640 containing 10% FCS, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, 2.3 mg/ml HEPES, and 2.0 mg/ml $NaHCO_3$. Chinese hamster ovary (CHO)[3] cells stably expressing Qa-1^b (Qa-1^b/CHO) were prepared as described previously (23).

RT-PCR and generation of transfectants

Total RNA was isolated from F9 cells and placentas by using RNA STAT-60 (TEL-TEST, Friendswood, TX). Single-stranded cDNA was synthesized with oligodeoxythymidylate primer and Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) from 5 μg of RNA and then used for PCR. The PCR was performed at 94°C for 5 min, 60°C for 1 min, and 72°C for 2 min for 30 cycles. According to the published blastocyst MHC sequence (22), 5′-GTACTCGAGATGGCGCAGC GAACGCTA-3′ corresponding to the first six codons and tagged with an *Xho*I site was used as the 5′ primer, and 5′-TCCGGATCCGGATGCA GATGATCCCTT-3′ corresponding to the last six codons except stop codon and tagged with a *Bam*HI site was used as the 3′-primer. β-Actin was used as an internal control as described previously (24). The PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

The PCR products were subcloned into the *Xho*I and *Bam*HI sites of the mammalian expression vector pcDNA3.1(−)/Myc-His (Invitrogen, Carlsbad, CA), and sequenced by a fluoresceinated dye terminator cycle sequencing method on an automated sequencer (Applied Biosystems, Foster City, CA). RMA-S cells were transfected with the pcDNA3.1(−)/Myc-His vector containing the blastocyst MHC cDNA by electroporation (300 V, 800 μF) with a Gene Pulser (Bio-Rad, Hercules, CA) and selected with 1 mg/ml G418 (Sigma-Aldrich, St. Louis, MO). The transfectants were then cloned by limiting dilution. Alternatively, the C-terminal Myc-His-tagged cDNA was subcloned into the MSCV-IRES-green-fluorescent protein (GFP) retroviral vector (kindly provided by Dr. A. Nienhuis, St. Jude Children's Research Hospital, Memphis, TN; Ref. 25) and infected into RMA-S and RMA cells as described previously (26). Stable GFP^+ cells were isolated by FACS and used without further cloning.

Immunofluorescence, flow cytometry, and confocal microscopy

For detection of C-terminal Myc-His-tagged blastocyst MHC proteins, cells were treated with Cytofix/Cytoperm solution (BD PharMingen, San Diego, CA) and stained with anti-His mAb (3D5; Invitrogen) followed by PE-conjugated goat anti-mouse IgG Ab (Caltag, Burlingame, CA). The stained cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA), and data were processed by CellQuest program (BD Biosciences). To determine subcellular localization, Cytofix/Cytoperm-treated cells were stained with anti-His mAb followed by Alexa594-conjugated goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR) and analyzed on an MRC-1024 confocal microscope (Bio-Rad, Hercules, CA). To analyze cell surface expression of Qa-1^b, H-$2K^b$, and H-$2D^b$, cells were stained with biotinylated anti-Qa-1^b mAb (6A8.6F10.1A6; BD PharMingen), anti-H-$2K^b$ mAb (AF6-88.5; BD PharMingen), and anti-H-$2D^b$ mAb (KH95; BD PharMingen), respectively, followed by PE-conjugated streptavidin (BD PharMingen). In some experiments, cells were preincubated with 20 μM peptides at 26°C for 24 h before the staining. The stained cells were then analyzed on a FACSCalibur as above. Dead cells were excluded by propidium iodide staining.

Western blotting

Cells were lysed with a lysis buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.3), 250 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF at a concentration of 1×10^7 cells/ml. Total lysates were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA). After blocking with Block Ace (Yukijirushi, Sapporo, Japan), the membrane was probed with anti-Myc mAb (9E10; ATCC). Ab binding was detected using HRP-conjugated rabbit anti-mouse IgG Ab (Jackson ImmunoResearch, West Grove, PA) and the chemiluminescence system (ECL Western blotting detection reagents; Amersham Pharmacia Biotech, Little Chalfont, U.K.).

Cell surface biotinylation and immunoprecipitation

Cells (1×10^7/ml in PBS) were surface biotinylated with 100 μg/ml sulfo-*N*-hydroxysuccinimide-biotin (Sigma-Aldrich) at room temperature for 40 min and then lysed with the lysis buffer. The lysates were immunoprecipitated with anti-Myc mAb and protein G-conjugated beads (Amersham Pharmacia). The imuunoprecipitates were subjected to 10% SDS-PAGE and transferred onto a PDVF membrane. The biotinylated proteins were detected using an HRP-containing ABC kit (Vector Laboratories, Burlingame, CA) and ECL.

Cytotoxicity assays

Splenic mononuclear cells were prepared from untreated, anti-asialo-GM1 Ab-treated (20 μg i.p. on days −2 and −1), and/or polyinosinic-polycytidylic acid (poly(IC))-treated (100 μg i.p. 18 h before) B6 mice as previously described (27). NK cells (>90% $NK1.1^+$) were prepared from splenic mononuclear cells of B6 RAG-$2^{-/-}$ mice by depleting adherent macrophages as previously described (27) and used as effector cells immediately or after activation with 250 U/ml human IL-2 (Shionogi, Osaka, Japan) for 2 days. In some experiments, IL-2-activated NK cells were separated into $NKG2A^-$ and $NKG2A^+$ populations by FACS after staining with FITC-conjugated anti-NKG2A/C/E mAb (20d5; BD PharMingen) and used as effector cells after culturing with IL-2 for an additional 3 days. A standard ^{51}Cr release assay was performed as previously described (27). In some experiments, a cytotoxicity assay was performed in the presence of Fab prepared from control rat IgG (Sigma-Aldrich), anti-mouse NKG2A/C/E mAb (20d5) (28), or anti-Qa-1^b mAb (BD PharMingen) by using the Fab preparation kit (Pierce, Rockford, IL) at 10 μg/ml. The peptide-mediated protection assay using Qa-1^b/CHO cells was performed as previously described (23). An N-terminal octamer (MAQRTLFL) of the blastocyst MHC signal peptide (bcSP) was newly synthesized. The canonical Qa-1^b-binding peptide, Qdm (Qa-1 determinant modifier; AMAPRTLLL) (29), was used as a positive control, and an H-$2K^b$-binding OVA peptide (SIINFEKL) (30) was used as a negative control.

In vivo tumor assays

Groups of five B6 mice were inoculated s.c. with increasing numbers of mock or blastocyst MHC-transfected RMA-S cells in 0.2 ml of PBS. Some groups of mice were depleted of NK cells by i.p. administration of anti-asialo-GM1 Ab (20 μg; Wako Pure Chemical, Osaka, Japan) on days −1, 0, 7, and 14. All mice were observed every 2 days for tumor growth using a caliper square measuring along the perpendicular axes of tumors, and the tumor size was determined as the product of two diameters. In another set of experiments, groups of 10 untreated or anti-asialo-GM1 Ab-treated B6 mice were inoculated i.p. with various numbers of mock or blastocyst MHC-transfected RMA-S cells in 0.2 ml of PBS, and survival of recipient mice was monitored for 60 days.

Statistical analysis

Statistical analysis was performed by the two-sample *t* test in the cytotoxicity assay or by the unpaired Mann-Whitney *U* test in the s.c. tumor growth and survival assays. Values of $p < 0.05$ were considered significant.

[3] Abbreviations used in this paper: CHO, Chinese hamster ovary; PVDF, polyvinylidene difluoride; bcSP, blastocyst MHC signal peptide; GFP, green-fluorescent protein; bc1, blastocyst MHC isoform 1; bc2, blastocyst MHC isoform 2; poly(IC), polyinosinic-polycytidylic acid.

Results

Identification of an alternatively spliced isoform of blastocyst MHC

To isolate the cDNA for blastocyst MHC, we first performed RT-PCR using total RNA prepared from the placentas of timed pregnant B6 mice or a teratocarcinoma cell line F9 of 129 mouse origin with a 5′-primer corresponding to the first six codons and a 3′-primer corresponding to the last six codons (except stop codon) of the reported blastocyst MHC gene sequence (22). As shown in Fig. 1*A*, we unexpectedly found a shorter transcript of 774 bp, which was predominant over the expected band of 1050 bp corresponding to the reported blastocyst MHC gene transcript both in the placentas and F9 cells. The transcripts were then subcloned into the pcDNA3 vector, and the cDNAs were sequenced. The nucleotide sequence of the 1050-bp cDNA was completely identical with the reported blastocyst MHC gene sequence isolated from 129 genomic library (22), which encodes the full length blastocyst MHC molecule composed of a leader, three Ig-like extracellular (α1, α2, α3) domains, a transmembrane domain, and a cytoplasmic domain (Fig. 1, *B* and *C*). In contrast, the 774-bp cDNA was devoid of the 276-bp sequence corresponding to exon 3 of the reported blastocyst MHC genomic sequence encoding the α2 domain (Fig. 1*B*), suggesting that this cDNA encodes an alternatively spliced isoform of blastocyst MHC lacking the α2 domain (Fig. 1*C*). Several alternatively spliced isoforms have been identified for human HLA-G, including the full length HLA-G1 and the HLA-G2 lacking an α2 domain (19). Thus, we tentatively designated the full length blastocyst MHC encoded by the 1050-bp cDNA as bc1, and the α2-lacking blastocyst MHC encoded by the 774-bp cDNA as bc2, which might be homologous to HLA-G1 and HLA-G2, respectively.

Expression of bc1 and bc2 proteins in RMA-S and RMA cells

Expression of blastocyst MHC at the protein level has not been determined because of the lack of a specific Ab reagent. To overcome this problem, bc1 and bc2 cDNAs were tagged with anti-Myc and anti-His epitope sequences at the C terminus by subcloning into the pcDNA3/Myc-His vector. A T lymphoma cell line, RMA-S, of B6 mouse origin was used for transfection, because these cells do not express functional MHC class Ia molecules on their surface due to TAP deficiency and thus are susceptible to NK cell-mediated cytolysis in vitro and in vivo (31, 32). The bc1-Myc-His and bc2-Myc-His cDNAs were expressed in RMA-S and TAP-sufficient RMA cells by using MSCV-IRES-GFP retroviral vector and the stable transfectants were identified by the expression of GFP. Immunofluorescent staining of permeabilized cells with anti-His mAb and flow cytometry revealed substantial levels of bc1-Myc-His and bc2-Myc-His protein expression in both RMA-S and RMA transfectants (Fig. 2*A*). Confocal imaging analysis of these cells showed that only bc1-Myc-His, but not bc2-Myc-His, protein was apparently localized at the plasma membrane of RMA cells and that both bc1-Myc-His and bc2-Myc-His proteins were mostly retained in the cytoplasm of RMA-S cells (Fig. 2*B*). Western blotting analysis of the whole cell lysates with anti-Myc mAb detected the 47-kDa bc1-Myc-His protein and the 38-kDa bc2-Myc-His protein expected from their primary amino acid sequences in both RMA-S and RMA transfectants and an additional 51-kDa protein in bc1-Myc-His-transfected RMA cells, which appeared to represent a glycosylated form (Fig. 2*C*). Cell surface biotinylation and immunoprecipitation with anti-Myc mAb detected only the 51-kDa protein in bc1-Myc-His-transfected RMA cells (Fig. 2*D*) These results indicated that only bc1, but not bc2, could be expressed on the cell surface in TAP-sufficient RMA cells, and that both bc1 and bc2 were retained in the cytoplasm in TAP-deficient RMA-S cells.

Protection of bc1- or bc2-transfected RMA-S cells from NK cell cytotoxicity

We examined the susceptibility of mock, bc1-, or bc2-transfected RMA-S cells to NK cell cytotoxicity in a standard 4-h ^{51}Cr release assay using freshly isolated splenocytes from untreated or poly(IC)-treated B6 mice as the effector cells. As shown in Fig. 3*A*, the bc1- or bc2-transfected RMA-S cells, but not mock transfected cells, were significantly protected from the lysis by freshly isolated B6 splenocytes. Even when poly(IC)-activated splenocytes were used as the effector, the bc1- or bc2-transfected cells were significantly protected from the lysis as compared with mock transfected cells (Fig. 3*B*). In both experiments, the cytotoxic activities were completely abolished by NK cell depletion by the anti-asialo-GM1

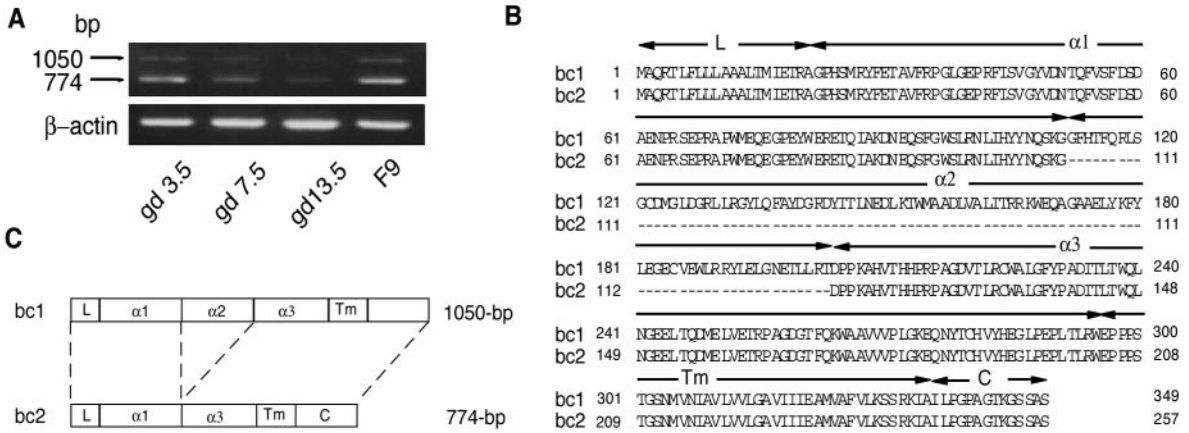

FIGURE 1. Expression of full length and a splice variant blastocyst MHC mRNA in placenta and F9 cells. *A*, RT-PCR analysis for blastocyst MHC mRNA. Total RNA was extracted from the placenta of B6 mice at day 3.5, 7.5, or 13.5 of gestation (gd) and a teratocarcinoma cell line, F9. RT-PCR analysis was performed with primers specific for full length blastocyst MHC or β-actin. Two bands (1050 bp and 774 bp) were obtained for blastocyst MHC, and a band (540 bp) was obtained for β-actin. *B*, Amino acid sequences encoded by the two RT-PCR products for blastocyst MHC. The 1050-bp band encoded a full length blastocyst MHC (bc1) and the 774-bp band encoded a splice variant devoid of 92 aa encoding the α2 domain (bc2). *C*, Structures of bc1 and bc2. L, leader; α1–α3, extracellular domains; Tm, transmembrane domain; C, cytoplasmic domain.

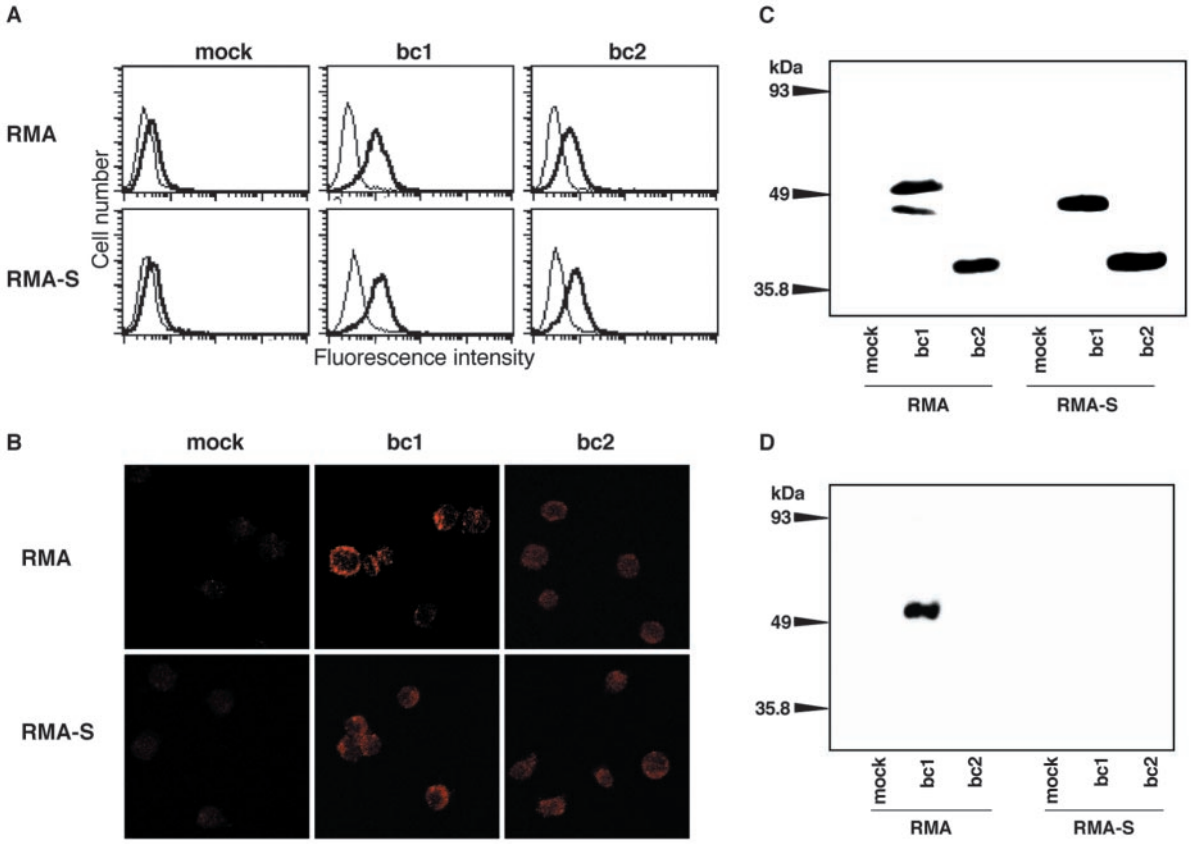

FIGURE 2. Expression of bc1 and bc2 proteins in cDNA transfectants. *A*, Flow cytometric analysis. Mock, bc1-, or bc2-transfected RMA and RMA-S cells were fixed/permeabilized and then stained with anti-His mAb followed by PE-conjugated goat anti-mouse IgG Ab. The bold histograms represent staining with anti-His mAb, and the thin histograms represent background staining with control mouse IgG. *B*, Confocal imaging analysis. Mock, bc1-, or bc2-transfected RMA and RMA-S cells were adhered onto glass slides, fixed/permeabilized, stained with anti-His mAb followed by Alexa594-conjugated goat anti-mouse IgG Ab, and then imaged by confocal microscopy. *C*, Western blotting analysis. Mock, bc1-, or bc2-transfected RMA and RMA-S cell lysates were subjected to SDS-PAGE and then blotted onto a PVDF membrane. The membrane was probed with anti-Myc mAb followed by HRP-conjugated rabbit anti-mouse IgG and then subjected to chemiluminescent detection. *D*, Cell surface biotinylation and immunoprecipitation. Mock, bc1-, or bc2-transfected RMA and RMA-S cells were cell surface biotinylated, and the lysates were immunoprecipitated with anti-Myc mAb. The immunoprecipitates were subjected to SDS-PAGE and then blotted onto a PVDF membrane. The membrane was probed with an HRP-containing ABC kit and then subjected to chemiluminescent detection.

Ab pretreatment. Similar results were obtained when IL-2-activated NK cells from B6 RAG-$2^{-/-}$ mice were used as effector cells (see below). These results indicated that the expression of either bc1 or bc2 in RMA-S cells conferred significant protection from NK cell cytotoxicity in vitro.

Contribution of Qa-1 and CD94/NKG2A

We next explored the mechanism by which bc1 and bc2 conferred protection from NK cell-mediated lysis. Because the murine homologs of ILT-2 or KIR2DL4 have not been identified and the bc1 and bc2 proteins were not apparently expressed on the surface of RMA-S cells (Fig. 2), we examined the possible contribution of CD94/NKG2A and Qa-1^b. As represented in Fig. 4*A*, the bc1- or bc2-transfected RMA-S cells exhibited the increased expression of Qa-1^b on their surface as compared with the mock transfected cells, whereas the levels of H-$2K^b$ or H-$2D^b$ were not significantly changed. Addition of Fab of either anti-mouse NKG2 mAb or anti-Qa-1^b mAb significantly reversed the resistance of bc1- or bc2-transfected RMA-S cells to IL-2-activated NK cell cytotoxicity (Fig. 4*B*). Moreover, when IL-2-activated NK cells were fractionated into NKG2A^- and NKG2A^+ populations, the protection by bc1 and bc2 was observed only against the NKG2A^+ population (Fig. 4*C*). These results suggested that the protection conferred by bc1 and bc2 was at least partly mediated by the CD94/NKG2A-inhibitory receptor on NK cells that recognized the up-regulated Qa-1^b expression on bc1- or bc2-transfected RMA-S cells.

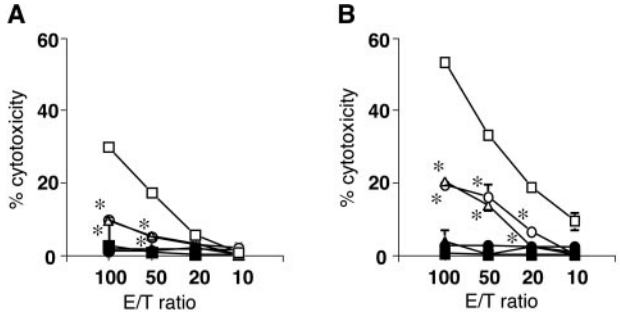

FIGURE 3. Protection of bc1- or bc2-transfected RMA-S cells from NK cell cytotoxicity. Cytotoxic activity of whole (□, △, and ○) or NK cell-depleted (■, ▲, and ●) splenocytes from untreated (*A*) or poly(IC)-treated (*B*) B6 mice was tested against mock (□, ■), bc1 (○, ●), or bc2 (△, ▲)-transfected RMA-S cells by 4-h ^{51}Cr release assay at the indicated E:T ratios. Data are shown as the mean ± SD of triplicate wells. Similar results were obtained in three independent experiments. *, $p < 0.05$ compared with the mock transfectant.

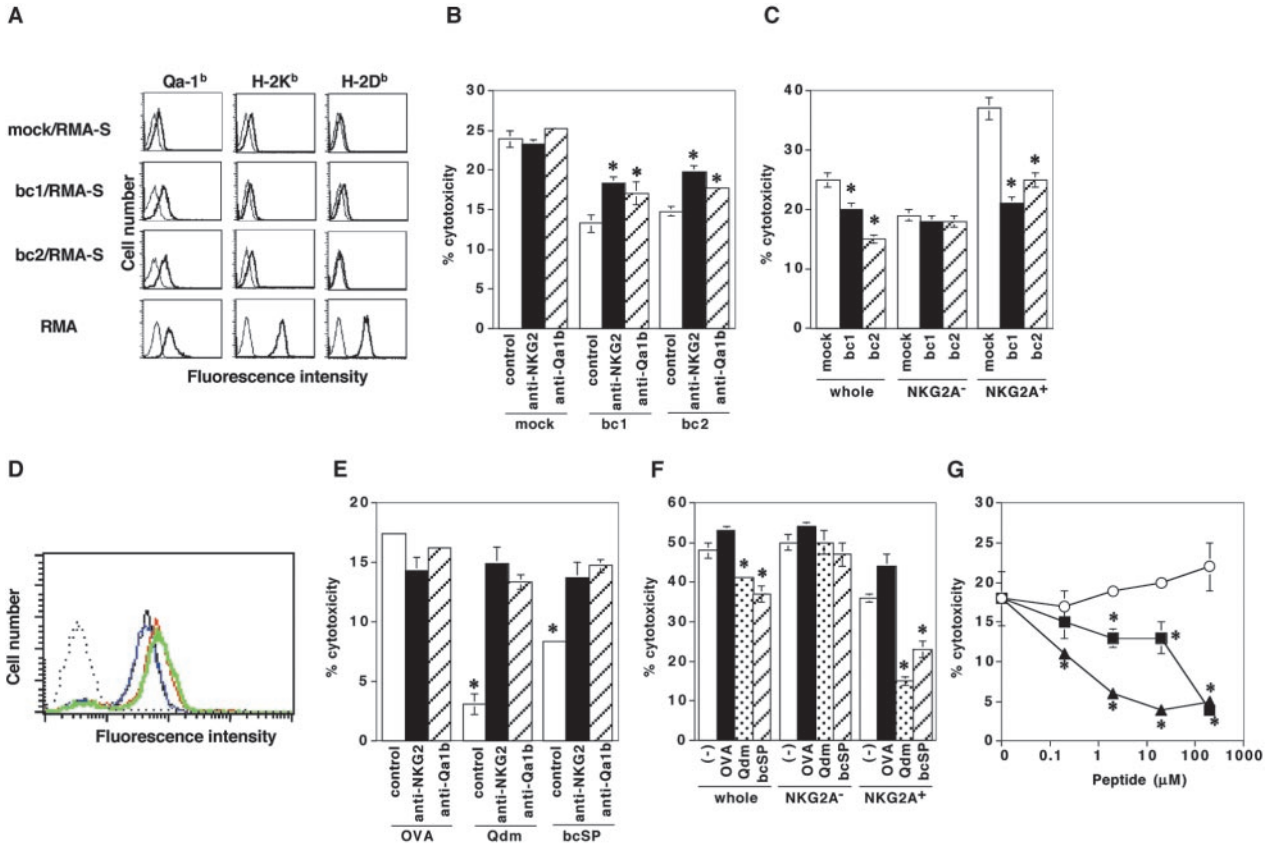

FIGURE 4. Involvement of Qa-1 and CD94/NKG2A in the protection of bc1- or bc2-transfected RMA-S cells from NK cell cytotoxicity. *A*, Increased Qa-1^b expression on bc1 or bc2 transfectants. Mock, bc1-, or bc2-transfected RMA-S cells and RMA cells were stained with biotinylated mAbs against Qa-1^b, H-2K^b, and H-2D^b, followed by PE-conjugated streptavidin (thick histograms). Thin histograms indicate the staining with biotinylated control IgG. *B*, Reversal of the protection by anti-NKG2 and anti-Qa-1^b mAbs. Cytotoxic activity of IL-2-activated NK cells was tested against mock, bc1-, or bc2-transfected RMA-S cells by a 4-h ^{51}Cr release assay at E:T 50 in the presence of 10 μg/ml Fab of anti-NKG2 mAb, anti-Qa-1^b mAb, or control IgG. Data are indicated as the mean $\pm$ SD of triplicate wells. $*$, $p < 0.05$ compared with control IgG. Statistically significant differences were consistently observed in three independent experiments. *C*, Protection of bc1- or bc2-transfected RMA-S cells from NKG2A$^+$, but not NKG2A$^-$, NK cells. Cytotoxic activity of IL-2-activated whole, NKG2A$^-$, or NKG2A$^+$ NK cells was tested against mock, bc1-, or bc2-transfected RMA-S cells by a 4-h ^{51}Cr release assay at E:T 10. Data are indicated as the mean $\pm$ SD of triplicate wells. Similar results were obtained in three independent experiments. $*$, $p < 0.05$ compared with mock transfectant. *D*, Up-regulation of Qa-1^b expression on Qa-1^b-transfected CHO cells by Qdm and bcSP peptides. Qa-1^b-transfected CHO cells were preincubated with 20 μM blastocyst MHC signal peptide (bcSP; green histogram), canonical Qa-1^b-binding peptide (Qdm; red histogram), or K^b-binding OVA peptide (OVA; blue histogram), and then stained with biotinylated anti-Qa-1^b mAb and PE-conjugated streptavidin. The black histogram indicates the staining of untreated cells with anti-Qa-1^b mAb and the dotted histogram indicates the staining with control IgG. Similar results were obtained in two independent experiments. *E*, Protection of Qa-1^b-transfected CHO cells from NK cell cytotoxicity by Qdm and bcSP peptides. Cytotoxic activity of freshly isolated NK cells was tested against Qa-1^b-transfected CHO cells by a 4-h ^{51}Cr release assay at E:T 50 in the presence of 20 μM bcSP, canonical Qa-1^b-binding peptide (Qdm), or K^b-binding OVA peptide (OVA) and 10 μg/ml Fab of anti-NKG2 mAb, anti-Qa-1^b mAb, or control IgG. Data are indicated as the mean $\pm$ SD of triplicate wells. $*$, $p < 0.05$ compared with OVA. Statistically significant differences were consistently observed in three independent experiments. *F*, Protection of Qa-1^b-transfected CHO cells from NKG2A$^+$, but not NKG2A$^-$, NK cells by Qdm and bcSP peptides. Cytotoxic activity of IL-2-activated whole, NKG2A$^-$, or NKG2A$^+$ NK cells was tested against Qa-1^b-transfected CHO cells by a 4-h ^{51}Cr release assay at E:T 4 in the presence or absence of 200 μM bcSP, Qdm, or OVA peptide. Data are indicated as the mean $\pm$ SD of triplicate wells. Similar results were obtained in three independent experiments. $*$, $p < 0.05$ compared with OVA. *G*, Dose response of protection by Qdm and bcSP peptides. Cytotoxic activity of IL-2-activated NKG2A$^+$ NK cells was tested against Qa-1^b-transfected CHO cells by a 4-h ^{51}Cr release assay at E:T 4 in the presence or absence of the indicated doses of bcSP (■), Qdm (▲), or OVA (○) peptide. Data are indicated as the mean $\pm$ SD of triplicate wells. Similar results were obtained in three independent experiments. $*$, $p < 0.05$ compared with OVA.

We next examined whether the signal peptide of bc1 and bc2 can confer the Qa-1^b-mediated protection, because the N-terminal octamer (bcSP: MAQRTLFL, see Fig. 1B) contained the Qa-1^b-binding motif (MAxxTLxL) common to the canonical Qa-1^b-binding peptide (Qdm: AMAPRTLLL) derived from the signal sequence of MHC class Ia molecules (33, 34). Because it was reported that exogenously added Qdm peptide could not up-regulate the low expression of Qa-1^b or confer protection from NK cells on RMA-S cells (35), we used CHO cells expressing Qa-1^b at a high level that were efficiently protected from mouse NK cells when Qdm peptide was exogenously added (23) to test the protective ability of bcSP peptide. As shown in Fig. 4*D*, preincubation of the Qa-1^b-tranfected CHO cells with the bcSP peptide, as well as the Qdm peptide, but not an H-2K^b-binding OVA peptide substantially up-regulated the cell surface expression of Qa-1^b, suggesting the binding and stabilization of Qa-1^b by bcSP. Moreover, as shown in Fig. 4*E*, the bcSP peptide conferred significant protection from freshly isolated NK cells as compared with the OVA peptide, although it was not as potent as the Qdm peptide. The protection conferred by bcSP and Qdm was totally abrogated by Fab of anti-NKG2 mAb or Qa-1^b mAb. Furthermore, when IL-2-activated NK cells were fractionated into NKG2A$^-$ and NKG2A$^+$

populations, only the NKG2A$^+$ population was inhibited by the Qdm and bcSP peptides (Fig. 4*F*). Titration of the peptides showed a comparable inhibitory effect of bcSP and Qdm at 200 μM, but Qdm was more potent than bcSP at lower doses against NKG2A$^+$ IL-2-activated NK cells (Fig. 4*G*).

Protection of bc1- or bc2-transfected RMA-S cells from NK cell-mediated rejection in vivo

We finally examined whether the bc1- or bc2-transfected RMA-S cells could be protected from NK cell-mediated rejection in vivo. When 5×10^3 cells were inoculated s.c. into B6 mice, three of five mice developed progressively growing tumors, whereas none of five mice inoculated with mock/RMA-S cells developed a palpable tumor (Fig. 5*A*). Although all mice developed s.c. tumors when inoculated with 5×10^4 or 5×10^5 cells, the growth of bc1- or bc2-transfected cells was significantly accelerated as compared with the mock transfected cells. When the mice were depleted of NK cells by anti-asialo-GM1 Ab treatment, the difference between bc1/bc2 and mock transfectants was abrogated. When 1×10^3 or 1×10^4 cells were inoculated i.p., survival of the bc1/RMA-S or bc2/RMA-S recipients was significantly shortened as compared with that of the mock/RMA-S recipients (Fig. 5*B*). This difference was abrogated again by NK cell depletion by anti-asialo-GM1 Ab treatment. In both systems, similar results were obtained when either transfected clones or retrovirally transduced bulk cell lines were used (data not shown). These results indicated that the expression of either bc1 or bc2 in RMA-S cells did confer protection from NK cell-mediated rejection in vivo and thus increased the malignancy of the tumor cells.

Discussion

In this study, we identified an alternatively spliced isoform of blastocyst MHC (bc2) that was expressed at a higher level in the placenta and a teratocarcinoma cell line than the previously identified full length isoform (bc1) and demonstrated that both the bc1 and bc2 isoforms protected MHC class Ia-deficient RMA-S cells from NK cells in vitro and in vivo. It has previously been reported that the expression of either HLA-G1 or HLA-G2 protected MHC class Ia-deficient K562 cells from human NK cells in vitro (7). Therefore, the bc1 and bc2 molecules may be the mouse homologs of HLA-G1 and HLA-G2, respectively, not only in their structures and expression but also in their functions.

We first characterized the expression of C-terminally Myc-His-tagged bc1 and bc2 proteins in TAP-sufficient RMA and TAP-deficient RMA-S transfectants. Only bc1 was apparently expressed on cell surface in the RMA transfectant, but not in the RMA-S transfectant. This suggests that the TAP-dependent peptide loading is required for transportation of bc1 from endoplasmic reticulum to cell surface through Golgi apparatus, just like MHC class Ia molecules. This notion is consistent with the prediction from the structural feature of bc1 that has an intact peptide-binding region (22). Similarly, the cell surface expression of HLA-G1 has been reported to be TAP-dependent (36). The physiological role of TAP-dependent Ag presentation by bc1 remains to be addressed in future studies. In contrast to HLA-G1, the cell surface expression of HLA-G2 remains controversial (19). Because bc2 lacks the α2 domain and thus cannot accommodate peptides, bc2 appeared not to be transported to cell surface even in the TAP-sufficient RMA transfectant. Consequently, the TAP-deficient RMA-S transfectants did not express either bc1 or bc2 on cell surface at a detectable level.

Despite no apparent expression of bc1 or bc2 on cell surface, the RMA-S transfectants expressing either bc1 or bc2 were efficiently protected from NK cell-mediated lysis. This protection was at least partly mediated by the Qa-1^b molecules up-regulated on the bc1/bc2 transfectants and the CD94/NKG2A-inhibitory receptor on NK cells, because Fab of anti-NKG2 mAb and anti-Qa-1^b mAb significantly reversed the bc1/bc2-conferred protection. This notion was further substantiated by the fact that the protection by bc1 and bc2 was observed against the NKG2A$^+$ NK cell population, but not against the NKG2A$^-$ population.

The ability of bc1/bc2 to confer the Qa-1^b-mediated protection was further suggested by the ability of exogenously added bc1/bc2 signal peptide octamer (bcSP) to protect Qa-1^b/CHO cells from NKG2A$^+$ NK cells, although bcSP was not as potent as Qdm. It was demonstrated that the position (p) 4P, p5R, and p8L residues

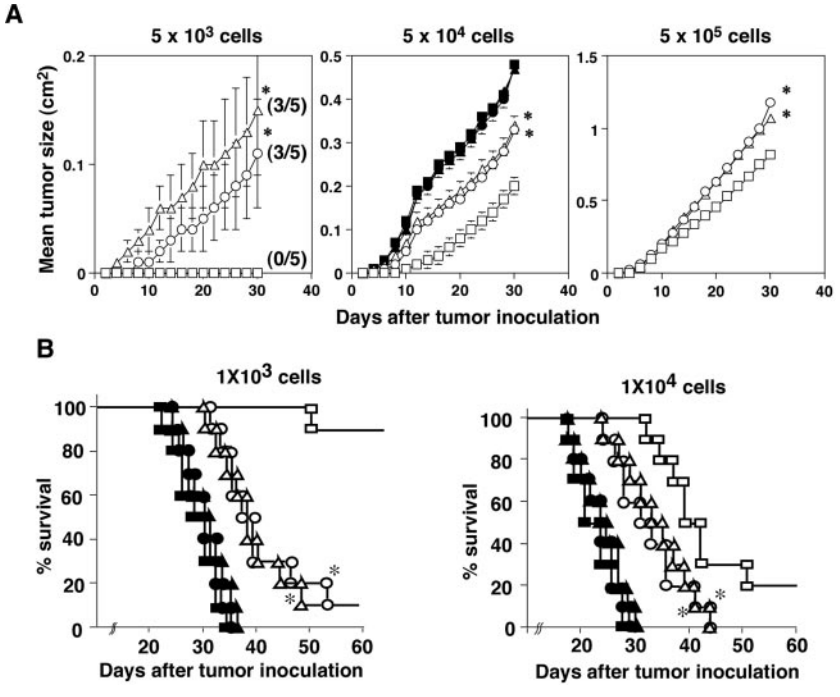

FIGURE 5. Protection of bc1- or bc2-transfected RMA-S cells from NK cell-mediated rejection in vivo. *A*, s.c. tumor formation. The indicated numbers of mock (□, ■), bc1 (○, ●)-, or bc2 (△, ▲)-transfected RMA-S cells were inoculated s.c. into untreated (□, ○, △) or anti-asialo-GM1 Ab-treated (■, ●, ▲) B6 mice, and tumor size was measured every 2 days. Data are indicated as the mean ± SD of three to five tumor-bearing mice in each group. In the left panel, frequency of tumor take is indicated in the parentheses. In the middle and right panels, all five mice developed the tumor. Similar results were obtained in three independent experiments. *, $p < 0.05$ compared with the mock transfectant. *B*, Survival curves after i.p. inoculation. The indicated numbers of mock (□, ■), bc1 (○, ●)-, or bc2 (△, ▲)-transfected RMA-S cells were inoculated i.p. into untreated (□, ○, △) or anti-asialo-GM1 Ab-treated (■, ●, ▲) B6 mice ($n = 10$ in each group), and survival of the recipients was monitored for 60 days. Similar results were obtained in three independent experiments. *, $p < 0.05$ compared with the mock transfectant.

in the Qdm peptide (AMA**PR**TL**L**L) were critically involved in the interaction with mouse CD94/NKG2A receptor (34, 35). Although the most critical p5R is conserved in the bcSP (MAQ**R**TLFL), the p4P is changed to Q and p8L is changed to F. This may explain why the bcSP was not as potent as the Qdm in the Qa-1^b/CHO protection assay.

Although the loading of Qa-1^b with Qdm from the MHC class Ia signal sequence was TAP dependent (29, 33), our results with the RMA-S transfectants suggested that loading with the bc1/bc2 signal peptide might be TAP independent. This observation is physiologically relevant, because the TAP deficiency has been observed both in trophoblasts (3) and frequently in tumors (8). It has been reported that the loading of HLA-E with human cytomegalovirus UL40 signal peptide was TAP independent (37). Further studies are needed to determine how the bc1/bc2 signal peptide could be loaded onto the Qa-1^b molecules in the bc1/bc2-transfected RMA-S cells.

Most importantly, we demonstrated that the bc1/bc2-transfected RMA-S cells were protected from NK cell-mediated rejection in vivo. This supports the notion that the aberrant expression of HLA-G in some tumors may be an escape mechanism from NK cell-mediated immunosurveillance (9, 10). Although this notion has recently been doubted primarily due to the failure to detect intact HLA-G proteins in tumors (19), this does not exclude the possibility that any translated isoform of HLA-G could confer the protection indirectly by up-regulating HLA-E expression.

Our present study suggested that blastocyst MHC may be a functional murine homolog of HLA-G that can protect MHC class Ia-deficient cells from NK cell-mediated lysis. An experimental mouse model for HLA-G may allow further elucidation of its physiological role in the maternal-fetal interaction and the host-tumor interaction.

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References

1. Bainbridge, D. R. 2000. Evolution of mammalian pregnancy in the presence of the maternal immune system. *Rev. Reprod. 5:67.*
2. Faulk, W. P., and A. Temple. 1976. Distribution of β_2 microglobulin and HLA in chorionic villi of human placentae. *Nature 262:799.*
3. Rodriguez, A. M., V. Mallet, F. Lenfant, J. Arnaud, M. Girr, S. Urlinger, A. Bensussan, and P. Le Bouteiller. 1997. Interferon-γ rescues HLA class Ia cell surface expression in term villous trophoblast cells by inducing synthesis of TAP proteins. *Eur. J. Immunol. 27:45.*
4. Kovats, S., E. K. Main, C. Librach, M. Stubblebine, S. J. Fisher, and R. DeMars. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science 248: 220.*
5. Lanier, L. L. 1999. Natural killer cells fertile with receptors for HLA-G? *Proc. Natl. Acad. Sci. USA 96:5343.*
6. Pazmany, L., O. Mandelboim, M. Vales-Gomez, D. M. Davis, H. T. Reyburn, and J. L. Strominger. 1996. Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science 274:792.*
7. Rouas-Freiss, N., R. E. Marchal, M. Kirszenbaum, J. Dausset, and E. D. Carosella. 1997. The alpha1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: is HLA-G the public ligand for natural killer cell inhibitory receptors? *Proc. Natl. Acad. Sci. USA 94:5249.*
8. Seliger, B., M. J. Maeurer, and S. Ferrone. 1997. TAP off—tumors on. *Immunol. Today 18:292.*
9. Paul, P., N. Rouas-Freiss, I. Khalil-Daher, P. Moreau, B. Riteau, F. A. Le Gal, M. F. Avril, J. Dausset, J. G. Guillet, and E. D. Carosella. 1998. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc. Natl. Acad. Sci. USA 95:4510.*
10. Paul, P., F. A. Cabestre, F. A. Le Gal, I. Khalil-Daher, C. Le Danff, M. Schmid, S. Mercier, M. F. Avril, J. Dausset, J. G. Guillet, and E. D. Carosella. 1999. Heterogeneity of HLA-G gene transcription and protein expression in malignant melanoma biopsies. *Cancer Res. 59:1954.*
11. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol. 16:359.*
12. Navarro, F., M. Llano, T. Bellon, M. Colonna, D. E. Geraghty, and M. Lopez-Botet. 1999. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. *Eur. J. Immunol. 29:277.*
13. Ponte, M., C. Cantoni, R. Biassoni, A. Tradori-Cappai, G. Bentivoglio, C. Vitale, S. Bertone, A. Moretta, L. Moretta, and M. C. Mingari. 1999. Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc. Natl. Acad. Sci. USA 96:5674.*
14. Rajagopalan, S., and E. O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J. Exp. Med. 189:1093.*
15. Braud, V. M., D. S. Allan, C. A. O'Callaghan, K. Soderstrom, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, L. L. Lanier, and A. J. McMichael. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature 391:795.*
16. Borrego, F., M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med. 187:813.*
17. Llano, M., N. Lee, F. Navarro, P. Garcia, J. P. Albar, D. E. Geraghty, and M. Lopez-Botet. 1998. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur. J. Immunol. 28:2854.*
18. Soderstrom, K., B. Corliss, L. L. Lanier, and J. H. Phillips. 1997. CD94/NKG2 is the predominant inhibitory receptor involved in recognition of HLA-G by decidual and peripheral blood NK cells. *J. Immunol. 159:1072.*
19. Bainbridge, D., S. Ellis, P. Le Bouteiller, and I. Sargent. 2001. HLA-G remains a mystery. *Trends Immunol. 22:548.*
20. Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J. Exp. Med. 188: 1841.*
21. Kurepa, Z., C. A. Hasemann, and J. Forman. 1998. Qa-1b binds conserved class I leader peptides derived from several mammalian species. *J. Exp. Med. 188:973.*
22. Sipes, S. L., M. V. Medaglia, D. L. Stabley, C. S. DeBruyn, M. S. Alden, V. Catenacci, and C. P. Landel. 1996. A new major histocompatibility complex class I b gene expressed in the mouse blastocyst and placenta. *Immunogenetics 45:108.*
23. Toyama-Sorimachi, N., Y. Taguchi, H. Yagita, F. Kitamura, A. Kawasaki, S. Koyasu, and H. Karasuyama. 2001. Mouse CD94 participates in Qa-1-mediated self recognition by NK cells and delivers inhibitory signals independent of Ly-49. *J. Immunol. 166:3771.*
24. Kawamura, T., K. Takeda, S. K. Mendiratta, H. Kawamura, L. Van Kaer, H. Yagita, T. Abo, and K. Okumura. 1998. Critical role of $NK1^+$ T cells in IL-12-induced immune responses in vivo. *J. Immunol. 160:16.*
25. Persons, D. A., J. A. Allay, E. R. Allay, R. J. Smeyne, R. A. Ashmun, B. P. Sorrentino, and A. W. Nienhuis. 1997. Retroviral-mediated transfer of the green fluorescent protein gene into murine hematopoietic cells facilitates scoring and selection of transduced progenitors in vitro and identification of genetically modified cells in vivo. *Blood 90:1777.*
26. Johnstone, R. W., K. M. Tainton, A. A. Ruefli, C. J. Froelich, L. Cerruti, S. M. Jane, and M. J. Smyth. 2001. P-glycoprotein does not protect cells against cytolysis induced by pore-forming proteins. *J. Biol. Chem. 276:16667.*
27. Kodama, T., K. Takeda, O. Shimozato, Y. Hayakawa, M. Atsuta, K. Kobayashi, M. Ito, H. Yagita, and K. Okumura. 1999. Perforin-dependent NK cell cytotoxicity is sufficient for anti-metastatic effect of IL-12. *Eur. J. Immunol. 29:1390.*
28. Vance, R. E., A. M. Jamieson, and D. H. Raulet. 1999. Recognition of the class Ib molecule Qa-1^b by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med. 190:1801.*
29. Aldrich, C. J., A. DeCloux, A. S. Woods, R. J. Cotter, M. J. Soloski, and J. Forman. 1994. Identification of a Tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell 79:649.*
30. Shastri, N., and F. Gonzalez. 1993. Endogenous generation and presentation of the ovalbumin peptide/Kb complex to T cells. *J. Immunol. 150:2724.*
31. Kärre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature 319:675.*
32. Ljunggren, H. G., and K. Kärre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J. Exp. Med. 162: 1745.*
33. Soloski, M. J., A. DeCloux, C. J. Aldrich, and J. Forman. 1995. Structural and functional characteristics of the class IB molecule, Qa-1. *Immunol. Rev. 147:67.*
34. Kraft, J. R., R. E. Vance, J. Pohl, A. M. Martin, D. H. Raulet, and P. E. Jensen. 2000. Analysis of Qa-1^b peptide binding specificity and the capacity of CD94/NKG2A to discriminate between Qa-1-peptide complexes. *J. Exp. Med. 192:613.*
35. Gays, F., K. P. Fraser, J. A. Toomey, A. G. Diamond, M. M. Millrain, P. J. Dyson, and C. G. Brooks. 2001. Functional analysis of the molecular factors controlling Qa1-mediated protection of target cells from NK lysis. *J. Immunol. 166:1601.*
36. Lee, N., A. R. Malacko, A. Ishitani, M. C. Chen, J. Bajorath, H. Marquardt, and D. E. Geraghty. 1995. The membrane-bound and soluble forms of HLA-G bind identical sets of endogenous peptides but differ with respect to TAP association. *Immunity 3:591.*
37. Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, B. P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. J. McMichael, and G. W. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science 287:1031.*