Complex Interplay of Activating and Inhibitory Signals Received by Vγ9Vδ2 T Cells Revealed by Target Cell β2-Microglobulin Knockdown

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Tumor cells often escape immunosurveillance by down-regulating MHC class I molecule expression. For human Vγ9Vδ2 T cells, a major peripheral blood T cell subset with broad antitumor reactivity, this down-regulation can affect signals transmitted by both the inhibitory and the activating MHC class I and Ib-specific NK receptors (NKRs) that these lymphocytes frequently express. To assess the overall impact of MHC down-regulation on Vγ9Vδ2 T cell activation, we used stable β2-microglobulin knockdown to generate tumor cells with a ~10-fold down-modulation of all MHC class I molecules. This down-modulation had little effect on T cell proliferation or cytokine production, but modified tumor cell killing efficiency. Ab-blocking studies identified ILT2 as an important inhibitor of tumor cell killing by Vγ9Vδ2 T cells. Down-modulation of MHC class I and Ib molecules severely reduced ILT2 inhibitory signaling, but still allowed signaling by activating CD94-based receptors. It also unveiled a frequent enhancing effect of NKG2D on tumor killing by Vγ9Vδ2 T cells. Current models suggest that activating NKRs have less affinity for their MHC ligands than homologous inhibitory NKRs. Our results show that, despite this, activating NKRs recognizing MHC class I molecules play an important role in the increased killing by Vγ9Vδ2 T cells of tumor cells with down-regulated MHC class I molecule expression, and suggest that these T cells will best lyse tumor cells combining MHC class I molecule expression down-regulation with up-regulated NKG2D ligand expression. The Journal of Immunology, 2006, 177: 6129–6136.

Cancer cells often down-regulate expression of cell surface MHC class I molecules (1–4). This helps them evade immunosurveillance mediated by conventional T lymphocytes that express αβ T cell receptors recognizing peptide/MHC complexes (5). Besides αβ T cells, γδ T cells may also be involved in tumor immunosurveillance: mice lacking these lymphocytes are highly susceptible to multiple regimens of cutaneous carcinogenesis, and γδ T cells infiltrate several tumor types (6–10).

The major human γδ T cell subset expresses a TCR with Vγ9 and Vδ2 variable regions. These Vγ9Vδ2 T cells react in vitro to a wide array of microbial agents and tumor cells (11–13). Microbial agonists for Vγ9Vδ2 T cells are small nonpeptidic phosphorylated compounds (12, 13); isopentenyl pyrophosphate, a metabolite from the mevalonate pathway of isoprenoid biosynthesis, has been identified as a tumor-derived agonist (11). How these phospho-Ags are presented to Vγ9Vδ2 T cells is not known, but presentation is MHC-independent (12), and so should not be affected by target cell MHC down-regulation. This does not imply that the down-regulation will have no effect on the Vγ9Vδ2 T cell response to tumors, because Vγ9Vδ2 T cells (and also αβ T cells) express a variety of activating and inhibitory NK receptors (NKRs) (aNKRs and iNKRs, respectively) whose ligands are MHC class I molecules (14). The iNKR ILT2 (15), for example, interacts with most if not all MHC class I molecules, because it recognizes their monomorphic α3 domain (16). Another iNKR, CD94/NKG2A, interacts only with HLA-E molecules loaded with peptides derived from HLA-I leader sequences, and has the same specificity as its activating counterpart CD94/NKG2C (17, 18).

So what effect will tumor cell MHC down-regulation have on the Vγ9Vδ2 T cell response? According to current models, aNKRs have less affinity for their ligands than homologous iNKRs (18–26). Thus, tumor cell MHC down-regulation should reduce iNKR activity, favoring recognition of altered cells, but might reduce aNKR activity even more, making it difficult for the latter to participate in the elimination of altered cells. Yet a priori, it seems more reasonable for MHC down-regulation to reduce aNKR activity less than iNKR activity, so that aNKR can still help eliminate altered cells. To clarify this, we set out to determine the effect of reducing tumor cell MHC class I molecule expression on killing by Vγ9Vδ2 T cells, and provoked a general down-modulation of target cell MHC class I molecules by β2-microglobulin (β2m) knockdown. We describe in this study the rather complex effects of this down-modulation on tumor cell killing, and conclude that aNKR can indeed play an important role in the increased killing of tumor

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*Abbreviations used in this paper: NKR, NK receptor; aNKR, activating NKR; iNKR, inhibitory NKR; β2m, β2-microglobulin; shRNA, short hairpin RNA; WT, wild-type; KIR, killer Ig-like receptor.
cells with down-regulated MHC class I expression by Vγ9Vδ2 T cells.

Materials and Methods

Tumor cell and T cell culture

RPMI 8226, JRT, and 786.0 cells were obtained from the American Type Culture Collection. NKL cells are described in Ref. 27. All tumor cell lines were cultivated in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, Vγ9Vδ2 T cell clones G42 and G115 were as described already (28, 29). T cell clones were cultivated in RPMI 1640 supplemented with 2 mM l-glutamine, streptomycin (10 μg/ml), penicillin (100 U/ml), 10% FCS, and 300 IU/ml recombinant human IL-2 (Chiron). T cell clones were re-expanded in vitro in the above culture medium containing 8% human serum and supplemented with purified leukoagglutinin (Sigma-Aldrich; 0.5 μg/ml) and irradiated (30 Gy) allogeneic feeder cells. Vγ9Vδ2 polyclonal T cells were obtained from PBMC separated on Ficoll cushions and cultured in RPMI 1640 with 10% fetal clone-irradiated serum (HyClone) in the presence of 3 μM bromoethyl pyrophosphate (Phosphostim; Innate Pharma) and 450 IU/ml recombinant human IL-2 starting at 106 cells/ml. Polyclonal T cell preparations were used as of at least 90% Vγ9Vδ2 cells, as judged by flow cytometry analysis with anti-CD3-PE, anti-Vδ2-FITC, and pany-PC5 (Immunotech-Beckman Coulter).

Knockdown target cell generation

The βm mRNA sequence targeted by a short hairpin (shRNA) was AGUAGGCAUGGUUGUGGU. Oligonucleotides 5′-H11032 TACAGCATGTGTTGGTCAGAGCAACACATGGCATTTTTTGGAAAG-3′ and 5′-AGGTTTTCAAAAAATAGGCGATGGTGGTTGCTTTGAGCACACAACATGGCTTAC-3′ were annealed and cloned downstream from an H1 promoter, between the BgII and HindIII sites of pSUPER (30). The resulting pSUPER-βshβm was verified by sequencing. The lacZ and blasticidin resistance genes in pLentiv65V5-GW/LacZ (Invitrogen Life Technologies) were replaced by a CD90 cDNA provided by F. M. Lemoine, Paris, France, so that the CD90 cDNA was under control of the CMV promoter (resulting construct: pLenti-CD90). The H1 promoter/βm oligonucleotide cassette was excised from pSUPER-βshβm and cloned immediately upstream from the CMV promoter in pLenti-CD90 to yield pLenti-CD90-βshβm, a lentiviral vector designed to express both CD90 and shRNA-targeting luciferase mRNA. These si-luc cell lines were verified by sequencing. The lacZ was expressed at levels as the parent cell lines (Fig. 1A, upper panels). This reduction was stable over at least 3 mo in culture. We also made cell lines (named si-βm JRT, si-βm RPMI 8226, and si-βm 786.0) as compared with the parent cell lines (Fig. 1A, upper panels), and were made to distinguish effects caused by β2m knockdown from effects caused by shRNA expression or lentiviral transduction in general.

Abs and flow cytometry

The following Abs were used: PE-conjugated anti-CD16 and anti-CD64 (ImmunoTech-Beckman Coulter); FITC-conjugated goat F(ab′)2 anti-mouse Ig Ab (Immuno-tech-Beckman Coulter); PC5-conjugated anti-CD90 (ImmunoTech-Beckman Coulter); anti-HLA-E (MEM-E02; Serotec); anti-α-tubulin (N 356; Amershame Life Science); and anti-MIC-A, -anti-ULBP3 (R&D Systems). The following blocking Abs were used: anti-HLA class I (Sigma-Aldrich; 0.5 μg/ml) containing 8% human serum and supplemented with purified leukoagglutinin (Sigma-Aldrich; 0.5 μg/ml) and irradiated (30 Gy) allogeneic feeder cells. Vγ9Vδ2 polyclonal T cells were obtained from PBMC separated on Ficoll cushions and cultured in RPMI 1640 with 10% fetal clone-irradiated serum (HyClone) in the presence of 3 μM bromoethyl pyrophosphate (Phosphostim; Innate Pharma) and 450 IU/ml recombinant human IL-2 starting at 106 cells/ml. Polyclonal T cell preparations were used composed of at least 90% Vγ9Vδ2 cells, as judged by flow cytometry analysis with anti-CD3-PE, anti-Vδ2-FITC, and pany-PC5 (Immunotech-Beckman Coulter).

Cell culture HLA-E analysis

Cell surface proteins were biotinylated and purified using the Cell Surface Protein Biotinylation and Purification Kit (Pierce) according to the manufacturer’s instructions. Recuperated proteins were analyzed by Western blotting using Ab MEM-E02, with detection using an ECL kit (Pierce).

Chromium release assay

Cytolytic activity of T cells was measured starting at 14 days poststimulation by a standard 4-h 51Cr release assay (29). Percentage of specific target cell lysis was calculated according to the following formula: [(experimental release – spontaneous release)/maximum release – spontaneous release] × 100. Maximum and spontaneous releases were determined by respectively adding 0.1% Triton X-100 or medium to target cells in the absence of effector cells. Data are presented as the mean ± SEM of triplicate samples. In the Ab-blocking experiments, effectors were incubated with appropriate Abs or isotype controls (in general, 1 μg/ml but 10 μg/ml for Immuno 510) for 30 min at room temperature before adding target cells.

Results

Stable β2m knockdown

We worked with a representative panel of cell lines derived from hemopoietic and solid tumors recognized by Vγ9Vδ2 T cells (33–40), comprising the T leukemia-derived Jurkat (JRT) cell line, the B lymphoma-derived RPMI 8226 cell line, and the kidney adenocarcinoma-derived 786.0 cell line. We made derivatives of these lines in which β2m levels had been stably reduced, by transduction with a lentiviral vector carrying two expression cassettes. One cassette coded for a shRNA-targeting β2m mRNA, the other cassette coded for CD90, a marker protein for detection of infected cells. Approximately 80% of cells expressed CD90 after lentiviral infection, and β2m levels were significantly reduced in the cells with the highest CD90 expression (about half of the CD90-positive cells). Cell sorting was used to isolate these cells. Levels of β2m were 8- to 10-fold reduced in the sorted cell lines (named si-β2m JRT, si-β2m RPMI 8226, and si-β2m 786.0) as compared with the parent cell lines (Fig. 1A, upper panels). This reduction was stable over at least 3 mo in culture. We also made cell lines (named si-luc JRT, si-luc RPMI 8226, and si-luc 786.0) expressing a control shRNA-targeting luciferase mRNA. These si-luc cell lines were not sorted, because they expressed CD90 levels similar to those of the sorted si-β2m lines. These lines had essentially the same β2m levels as the parent cell lines (Fig. 1A, upper panels), and were made to distinguish effects caused by β2m knockdown from effects caused by shRNA expression or lentiviral transduction in general.

β2m knockdown reduces cell surface HLA class I molecule levels

FACS analyses using the W6/32 Ab, that detects all HLA class I molecules, or the B1.23.2 Ab specific for HLA-B/C, showed that β2m knockdown decreased cell surface HLA class I expression (Fig. 1A). Reductions in HLA class I expression correlated well with the β2m reductions already noted (i.e., ~10-fold). We did not have Abs suitable for FACS analysis of HLA class-E expression (Ab tested gave a stronger signal with the β2m-negative Daudi cell line than with any of our wild-type (WT) tumor lines), so cell surface proteins were biotinylated, purified, and analyzed by Western blotting. Cell surface HLA-E molecules were undetectable on Daudi cells, but were easily detected on WT JRT and RPMI 8226 cells (Fig. 1B). Levels of cell surface HLA-E molecules were ~10-fold (estimated from the scanned autoradiograms) lower in the corresponding si-β2m cell lines. Cell surface levels of all the HLA class I molecules we tested for were thus significantly reduced in our β2m knockdown lines.

To verify that β2m knockdown did not exert a nonspecific down-modulating effect on cell surface proteins, we compared levels of several NKG2D ligands (MIC-A, ULBP2, and ULBP3) on WT and corresponding si-β2m cell lines. As shown in Fig. 1A,
levels of these proteins were essentially unchanged by β2m knockdown: the si-β2m and WT plots overlap so completely that they cannot be distinguished.

Enhanced lysis of β2m knockdown cells by NK cells

We tested our β2m knockdown lines using a NK cell line (NKL) expressing the iNKRs ILT2 and CD94/NKG2A. This cell line lyse RPMI 8226 cells efficiently only if surface HLA class I molecules on the cells are masked by Abs (Fig. 2). However, using Abs to block ILT2, NKG2A or CD94 on NKL cells increased RPMI 8226 cell lysis only modestly (Fig. 2). Simultaneous blocking of ILT2 and the CD94/NKG2A heterodimer on NKL cells was required to increase lysis to the level obtained by Ab masking of target cell HLA class I ligands. Repression by either ILT2 alone or CD94/NKG2A alone is thus sufficient to block NKL lytic activity effectively.

In si-β2m RPMI 8226 cells, β2m knockdown increased lysis by NKL cells about as efficiently as Ab-masking of HLA class I molecules on WT cells (Fig. 2; compare si-β2m/IgG to WT/anti-HLA-I). This indicates that the increased lysis of si-β2m cells is caused by the reduced levels of available iNKR HLA class I ligands. Preincubating si-β2m cells with the W6/32 Ab led to a further increase in lysis by NKL cells. This was expected, because the levels of cell surface HLA class I molecules are not reduced to zero in the si-β2m cells (see Fig. 1).

β2m knockdown can increase or decrease killing by Vγ9Vδ2 T cells

Having established that our knockdown cells behaved as expected with NKL cells, we tested the effects of exposing our WT and β2m or control luciferase knockdown cells to Vγ9Vδ2 T cells. We used tests based on T cell proliferation, cytokine production, and cell-mediated cytotoxicity. No important differential effects on proliferation or cytokine production were observed (data not shown). However, we detected significant differences in the cytolytic activity of two different Vγ9Vδ2 T cell clones (G42 and G115) against β2m knockdown cells as compared with WT or control luciferase knockdown cells.

Different batches of these T cell clones were obtained by stimulating them, then waiting until they were incapable of lysing an
EBV-transformed B cell line (i.e., they could no longer exert nonspecific cell lysis). Batches were thus tested on tumor targets 14–30 days poststimulation. Thirty-five different experiments were conducted, in each of which the target cell line RPMI 8226 and its knockdown derivative were exposed in parallel to a particular batch of a Vγ9Vδ2 T cell clone, and cytotoxicity was measured by a chromium release assay. In all cases, results obtained with the WT target cell line and its si-luc control derivative were essentially identical (data not shown), so for simplicity only results comparing WT and β2m knockdown cells are presented below.

Typical examples of our results with the G42 clone are shown in Fig. 3A. Compared with lysis of WT cells, lysis of si-β2m cells could either be increased (first and fourth panels), unchanged (second panel), or reduced (third panel). Variability was not limited to one T cell clone or one target cell line. Similar results were obtained when the G42 clone was tested against WT and si-β2m JRT cells (Fig. 3B) or 786.0 cells (data not shown), or when the G115 clone was tested against WT and si-β2m RPMI 8226 cells (Fig. 3C), or against JRT or 786.0 cells and their si-β2m derivatives (data not shown). The varying effect of β2m knockdown was not limited to T cell clones: in RPMI 8226 cells, β2m knockdown increased susceptibility to lysis by one polyclonal Vγ9Vδ2 T cell line, SIL, while decreasing susceptibility to lysis by a second one, Sol (Fig. 3D). Note that, for a given batch of T cells, the effects of target cell β2m knockdown were similar to those of Ab-masking HLA class I molecules using A6/136 on WT target cells. This was the case whether the observed effect was a decrease (Fig. 3A, third panel), an increase (Fig. 3A, fourth panel), or little change in lysis (data not shown). Thus, the variability observed is not caused by some bizarre properties of the β2m knockdown cells unrelated to the knockdown.

Overall, lysis of si-β2m cells was higher than that of WT cells in 18 of the 35 experiments undertaken (indicating reduced signaling by an iNKR), unchanged in 12, and decreased in 5 (indicating reduced signaling by an aNKR). This variability could originate from the tumor target cells or the effector cells, or both. Levels of the NKG2D ligands MIC-A, ULBP2 and ULBP3 on RPMI 8226 cells do not change significantly with culture conditions, however (our unpublished results). The fact that the effector cells contribute to variability is strongly suggested by our observation that Ab-masking of HLA class I molecule ligands on WT target cells can exert variable effects on cell lysis (see above). We hypothesized that some variability was caused by differences in the iNKRs and aNKRs expressed by different T cell batches, and set out to identify the NKRs in question.

**Lower signaling by the iNKR ILT2 causes increased lysis of si-β2m cells**

It has been reported previously that a fraction (varying in importance from donor to donor) of uncultured peripheral blood TCR γδ+ cells express CD94 (41). Similarly, a donor-dependent fraction of uncultured peripheral blood Vγ9Vδ2 T cells express ILT2 (44, 55, and 68% for three donors studied) and NKG2D (23, 23, and 33% for the same donors; data not shown). The G42 T cell clone expresses NKG2A, ILT2, CD94, and NKG2D, but no known killer Ig-like receptor (KIR) (data not shown). No significant effect of the CD94/NKG2A complex on target cell lysis was detected using Z199 Abs blocking NKG2A (data not shown). However, blocking ILT2 with Abs gave results showing that ILT2 is a major Vγ9Vδ2 T cell inhibitory receptor. Consider a G42 T cell clone batch that lyses WT RPMI 8226 cells less efficiently than their si-β2m derivative (Fig. 4A). Lysis of WT cells is increased by blocking ILT2, reaching a level similar to that obtained by β2m knockdown. This suggests that the main effect of β2m knockdown here is to reduce an inhibitory signal transiting via ILT2. Consistent with this, lysis of si-β2m cells is only slightly increased by blocking ILT2 (remember that, in si-β2m cells, levels of HLA class I molecules are not reduced to zero). Very similar results were obtained with the polyclonal T cell line SIL (Fig. 4B). Thus, ILT2 is the predominant inhibitory receptor on the Vγ9Vδ2 T cells tested here.

**A CD94-based aNKR is required for increased lysis of si-β2m cells**

The Sol polyclonal T cell line lyses WT RPMI 8226 cells very efficiently, and blocking ILT2 does not further augment lysis (Fig.

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**FIGURE 3. Variable effects of target cell β2m knockdown on the cytolytic activity of Vγ9Vδ2 T cells.** Cytolytic activities of two Vγ9Vδ2 T cell clones (G42 and JRT) and two polyclonal populations (SIL and Sol) against WT and β2m knockdown (si-β2m) RPMI 8226 and JRT targets are shown. Different ratios of T cell:target cell (30:1, 15:1, 3:1, and 1.5:1) were used as marked. In some cases, the effect of Ab masking of HLA class I molecules on T cell cytolytic activity against WT cells was tested (WT+anti-HLA-I). Arrows indicate the effect on cytolytic activity of target cell β2m knockdown (increase or decrease). Data are presented as the mean ± SEM of triplicate samples. WT (□); si-β2m (○); addition of IgG (—); addition of anti-HLA-I (- - -) to WT.
As noted above, β₂m knockdown reduced lysis slightly, and blocking ILT2 had little further effect on lysis of knockdown cells (Fig. 4C). However, blocking CD94 on Sol T cells with the Y9 Ab significantly decreased lysis of both WT and si-β₂m RPMI 8226 cells (Fig. 4C), suggesting that these T cells express an activatory receptor containing CD94 (called CD94/X henceforth). The alternative explanation that RPMI 8226 cells bind Y9 Abs, and that the bound Abs cross-link and activate NKG2A/CD94 leading to decreased lysis, can be ruled out. RPMI 8226 cells are CD16 and CD64 negative (data not shown), and do not bind Abs to cross-link and activate receptors (42). In line with this, Y9 treatment of RPMI 8226 cells increases lysis of NKL cells (Fig. 2), rather than decreasing it.

CD94/X is also active in the G42 T cell clone: blocking CD94 with the Y9 Ab reduces lysis of WT RPMI 8226 cells by G42 cells slightly (Fig. 4A, left panel). With this batch of G42 cells, si-β₂m cells were lysed significantly more efficiently than WT cells (Fig. 4A, compare left and right panels). It is noteworthy that blocking CD94 has a much greater effect on si-β₂m cells than on lysis of WT cells (Fig. 4A, compare left and right panels). Indeed, blocking CD94 essentially eliminates the increase in cytosis obtained by MHC class I molecule down-modulation, returning the lysis level of si-β₂m cells to that of WT cells. Similar results were obtained using another batch of the G42 clone (Fig. 4D). Both masking HLA class I molecules on WT RPMI 8226 cells with the A6/136 Ab and β₂m knockdown led to an increase in lysis by the T cells. Once again, blocking CD94 on G42 cells with the Y9 Ab reduced lysis of WT RPMI 8226 cells slightly, but eliminated the increase in lysis obtained by β₂m knockdown.

The above results show that G42 cells express an activatory CD94/X receptor required for the increased lysis of si-β₂m cells relative to WT cells, and that the activity of this receptor is masked on WT cells by an inhibitory receptor responsive to HLA class I molecules. What is the identity of the X chain in CD94/X? It is not NKG2C, because the Vγ9Vδ2 T cell clones studied here have undetectable levels of NKG2C transcripts as judged by RT-PCR analysis, and no cell surface NKG2C can be detected using available Abs (data not shown). We did detect NKG2E and NKG2F transcripts by RT-PCR analysis (data not shown), but the lack of NKG2E or NKG2F-specific Abs prevented analysis of their cell surface expression. NKG2E is a better candidate for X than NKG2F, because NKG2F may not associate with CD94, or be expressed on the cell surface (43).

When we used the A6/136 Ab to mask HLA class I molecules on RPMI 8226 si-β₂m cells, we observed a marked drop in cell lysis by the G42 clone, similar to the drop obtained by blocking CD94 on the T cells (Fig. 4D). This is in complete contrast to the rise in lysis we observed for WT cells, but is readily explained by the presence of the CD94/X aNKR on G42 cells. The CD94/X receptor uses HLA class I molecules as ligands: the reduced levels of available HLA class I molecules on WT cells exposed to the A6/136 Ab or si-β₂m cells are insufficient to activate the iNKR on G42 cells, but remain sufficient to stimulate the CD94/X activatory receptor. This leads to an increase in lysis of si-β₂m cells relative to WT cells. Combining target cell β₂m knockdown with exposure to the A6/136 Ab has a cumulative effect, and there are no longer enough available HLA class I molecules to stimulate even the activatory CD94/X receptor on T cells: cell lysis now drops to a level similar to that obtained by blocking CD94.

aNKR/iNKR expression and the effect of β₂m knockdown on lysis

The experiments described above were conducted using T cell batches that lyse si-β₂m cells more efficiently than WT cells, and led us to identify ILT2 and CD94/X as major players in the reaction of Vγ9Vδ2 T cells to MHC class I molecule down-modulation. However, for some T cell batches, target cell β₂m knockdown decreased lysis or left it unchanged. This variability could reflect changes in the relative expression levels of ILT2 and CD94/X between batches, but we cannot test this hypothesis, because we have not identified the X chain. We did compare CD94 and ILT2 levels between batches, even though this gives no information about CD94/X levels, because CD94 can have a variety of partners. Although expression levels of CD94 and ILT2 do vary between different batches of T cells, the variations were not sufficiently important to permit establishment of any simple correlation.
between an increase in tumor cell lysis provoked by $\beta_m$ knockdown and high ILT2/low CD94 expression on T cells, or between a decrease in lysis and high CD94/low ILT2 expression on T cells (Fig. 5A).

This analysis is complicated by the fact that whereas NK cells such as the NKL cell line used above display tight and bright expression levels of ILT2 and CD94 (data not shown), NKR staining patterns of cultured T cell clones are heterogeneous. This is because their NK expression levels being inducible (44), they vary with their precise activation status, and all cells in a clonal population do not have the same activation status at a given moment. Note also that cells with low ILT2 expression can have either low or high CD94 levels, as indeed can cells with high ILT2 expression (Fig. 5B).

**NKG2D is also required for the increased lysis of si-$\beta_m$ cells**

We have shown above that G42 T cells express the aNKR CD94/X, but that the activity of this receptor is masked by iNKR activity. Thus, Ab blocking of CD94/X has little effect on lysis of WT cells but markedly decreases lysis of cells receiving a reduced iNKR signal (si-$\beta_m$ cells). Ab blocking of another aNKR, NKG2D, had little effect on lysis of a number of WT tumor targets we tested, and we wondered whether its activity could also be unmasked by $\beta_m$ knockdown. In our first experiment, we used one of the G42 T cell batches that had allowed detection of the important role played by CD94/X in killing of si-$\beta_m$ cells. Blocking NKG2D on the surface of these G42 T cells had little effect on lysis of WT RPMI 8226 cells, but essentially eliminated the increase in lysis provoked by $\beta_m$ knockdown (Fig. 4D). Indeed, blocking NKG2D had essentially the same effect as blocking CD94/X. The G42 cells used in this experiment thus require both the activating receptors NKG2D and CD94/X for efficient target cell lysis.

As shown in Fig. 6A, unmasking activation by NKG2D turned out to be a frequent result of reducing the T cell inhibitory signal by target cell $\beta_m$ knockdown. For several different tumor targets, and both the G42 and G115 clones, a clear effect of blocking the NKG2D signal was only apparent after $\beta_m$ knockdown. As observed above, blocking the NKG2D signal essentially eliminates the increase in cytolyis obtained by MHC class I molecule down-modulation, returning the lysis level of si-$\beta_m$ cells to that of WT cells.

The TCR is required for efficient lysis in addition to NKG2D and CD94/X, as is shown by the clear reduction in lysis of WT and si-$\beta_m$ RPMI 8226 cells obtained by Ab blocking of the TCR on G42 T cells (Fig. 6B). Notably, blocking the TCR essentially eliminates the increase in lysis uncovered by $\beta_m$ knockdown.

**Discussion**

The ~10-fold decrease in tumor cell MHC class I molecule expression we obtained by $\beta_m$ knockdown had little effect on cytokine production or proliferation of Vγ9Vδ2 T cells exposed to them. The decrease in tumor cell MHC class I molecule expression did alter the Vγ9Vδ2 T cell lytic response, however. This is in line with previous observations showing that the activation thresholds of cytokine and proliferative responses are higher than that of the lytic response (45). Although the resulting lytic response change varied with the batch of T cells tested and their culture status, the result we observed most frequently was an increase in the lytic response, doubtless reflecting the decreased activity of iNKR using MHC class I molecules as ligands.

Previous work has implicated the iNKR CD94/NKG2A in the regulation of Vγ9Vδ2 T cell activation by phosphoantigens and tumor cells (40, 42, 46). However, Ab blocking of this complex had little effect on tumor lysis by the panel of Vγ9Vδ2 cells tested.
here, in which ILT2 appeared to be the major iNKR. Thus, in most instances, Ab blocking of ILT2 significantly increased tumor lysis by V9V82 T cell clones and polyclonal cell lines, with lysis increasing to levels similar to those seen after Ab masking of tumor cell MHC class I molecules, or after tumor cell β2m knockdown. The low affinity of ILT2 for its ligands, and the variable but generally low ILT2 expression levels on T cell clones (44, 47) may have led to an underestimation of the importance of ILT2 in the past. However, the low affinity of ILT2 for MHC class I molecules may be compensated for by high expression levels of its ligands, which include both classical and nonclassical MHC class I molecules, and ILT2 is much more frequently expressed on classical MHC-restricted T cells than other iNKR. This receptor may thus play a more important role in the regulation of T cell responses than previously realized.

The 10-fold decrease of MHC class I molecule expression levels provoked by tumor cell β2m knockdown prevented signaling by inhibitory ILT2 on V9V82 T cells. However, sufficient MHC class I molecules remained for signaling by yet unidentified activating CD94/X receptors, because the increase in lysis of β2m knockdown cells relative to WT cells could be eliminated by blocking CD94 with the specific mAb Y9. This Ab has been shown to prevent engagement of inhibitory CD94 receptors—see its effect on lysis by NK cells—but has never shown any agonist activity, e.g., through CD94 cross-linking; A. Moretta, unpublished observations). Consistent with this, Ab masking of the residual MHC class I molecules on β2m knockdown tumor cells inhibited their lysis to about the same extent as blocking CD94 on the effector cells.

The effects of MHC class I molecule down-regulation we observed are in apparent contradiction with current models, which propose that αNKR have less affinity for their ligands than homologous iNKR (18–26). However, for a biological effect to be obtained, receptors must not only bind their ligands, but sufficient numbers of complexes must also be formed to trigger signaling. Perhaps fewer αNKR-ligand complexes than iNKR-ligand complexes are required to trigger signaling. This would be in line with results from a study showing that activating KIR2DS2 can function at lower ligand (mimicked by a mAb agonist) concentrations than inhibitory KIR2DL2 or KIR2DL3, even though the Ab binds to all these receptors with the same affinity (48). It is also possible that the effect of HLA class I molecule down-modulation on target cell lysis by immune effectors will depend on the precise combination of αNKR and iNKR they express. In any case, our results show that αNKR using MHC class I molecule ligands make an important contribution to the increased killing by V9V82 T cells of tumor cells with down-regulated MHC class I expression.

For the T cell clones studied here, the most frequently observed result of tumor MHC class I molecule down-modulation was an increase in the V9V82 T cell lytic response. However, for some batches of a T cell clone no change in lytic efficiency was observed, and only for a few preparations was a decrease in lytic efficiency observed. This suggests that MHC class I molecule down-regulation will not allow tumor cells to escape from immunosurveillance by V9V82 T cells in general, and this is an important consideration for their possible therapeutic use (49). The different batches of the T cell clones were obtained by stimulating or restimulating T cells with a complex mixture of reagents, necessarily including different batches of feeder cells. Some variability was thus to be expected between different batches of a T cell clone, even when tested at the same time poststimulation. We hypothesized that some of this variability was linked to changes in the relative expression levels of αNKR and iNKR. Thus, batches with a relatively higher level of αNKR would have less lytic activity against MHC class I molecule down-modulated cells than against WT cells, whereas the converse would be true for preparations with a relatively higher level of iNKR. Preparations with balanced levels of αNKR and iNKR would show approximately equal lytic activity against WT and MHC class I molecule down-modulated cells. Unfortunately, it was not possible to test this hypothesis, because ILT2 levels do not vary much between different T cell clone preparations, and we cannot test for variations in CD94/X, which our work shows to be an important player in the reaction of V9V82 T cells to changes in MHC class I molecule levels: we have not been able to identify the X subunit, although we have been able to rule out its being NKG2C. NKG2E remains a possible candidate for X. CD94/NKG2 receptors are specific for HLA-E (17), so this class Ib molecule is a probable ligand for the CD94/X receptor studied here. We could not test this hypothesis, however, because Abs that block HLA-E specifically are not available.

Finally, it is interesting that target cell MHC class I molecule down-modulation unveiled a role not only for CD94/X, but also for NKG2D in the resulting increase in killing by V9V82 T cells. Indeed, this increase requires engagement of both these activating receptors on the T cells: blocking just one of the two receptors with specific Abs is sufficient to eliminate the increase. This may be of importance for limiting efficient lysis by V9V82 T cells to target cells with not only reduced expression of MHC class I molecules, but also up-regulated expression of NKG2D ligands. Because such ligands are generally up-regulated on stressed, infected, or transformed cells (16, 50–52), this would direct V9V82 T cell lytic activity preferentially toward cells showing both signs of being transformed.

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Disclosures
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