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Recruitment of CTL Activity by Tumor-Specific Antibody-Mediated Targeting of Single-Chain Class I MHC-Peptide Complexes

Avital Lev, Hila Novak, Dina Segal, and Yoram Reiter

The MHC class I-restricted CD8 CTL effector arm of the adaptive immune response is uniquely equipped to recognize tumor cells as foreign and consequently initiates the cascade of events resulting in their destruction. However, tumors have developed sophisticated strategies to escape immune effector mechanisms; their most well-known strategy is down-regulation of MHC class I molecules. To overcome this and develop new approaches for immunotherapy, we have constructed a recombinant molecule in which a single-chain MHC is specifically targeted to tumor cells through its fusion to cancer-specific recombinant Ab fragments. As a model we used a single-chain HLA-A2 molecule genetically fused to the variable domains of an anti-IL-2Rα subunit-specific humanized Ab, anti-Tac. The construct, termed B2M-aTac(dsFv), was expressed in Escherichia coli, and functional molecules were produced by in vitro refolding in the presence of HLA-A2-restricted antigenic peptides. Flow cytometry studies revealed the ability to decorate Ag-positive, HLA-A2-negative human tumor cells with HLA-A2-pepptide complexes in a manner that was entirely dependent upon the specificity of the targeting Ab fragment. Most importantly, the B2M-aTac(dsFv)-mediated coating of the target tumor cells made them susceptible for efficient and specific HLA-A2-restricted, melanoma gp100 peptide-specific CTL-mediated lysis. These results demonstrate the concept that Ab-guided, Ag-specific targeting of MHC-peptide complexes on tumor cells can render them susceptible and more receptive and thus potentiate CTL killing. This type of approach may open the way for the development of new immunotherapeutic strategies based on Ab targeting of natural cognate MHC ligands and CTL-based cytotoxic mechanisms. The Journal of Immunology, 2002, 169: 2988–2996.

There is strong evidence that tumor progression in cancer patients is governed by the immune system. This is based on the fact that tumor progression is often associated with the secretion of immune suppressive factors and/or the down-regulation of MHC class I Ag presentation functions (1–5). Thus, we can infer that tumors have elaborate strategies to circumvent an apparently effective immune response. Importantly, a tumor-specific immune response can be detected in individuals, demonstrated by the ability to generate tumor-specific CD8+ CTLs in vitro from peripheral blood, draining lymph nodes, and metastatic tumor deposits of human cancer patients (6–8).

The apparent inefficiency of antitumor immune responses, which resulted in the failure to successfully combat the disease, laid the foundation for current concepts of immunotherapy. It is suggested that boosting the antitumor immune response by deliberate vaccination or by other immunotherapeutic approaches may increase the potential benefits of immune-based therapies (6, 9–11).

The MHC class I-restricted CD8 CTL effector arm of the adaptive immune response is uniquely equipped to recognize the tumor as foreign and consequently initiates the cascade of events resulting in its destruction (12, 13). Therefore, the most attractive approach to cancer immunotherapy is based on vaccination strategies designed to enhance the CTL arm of the antitumor response and consequently to overcome the mechanisms of tumor escape from CTL (9–11).

One of the most studied escape mechanisms by which tumor cells evade immune attack is by down-regulation of the MHC class I molecules, which are the Ags recognized by CTLs (1–5, 14).

Mutations along the class I presentation pathway should be the simplest way for tumors to escape CTL-mediated elimination, because mutations can be achieved by one or two mutational events (two mutations to inactivate both alleles or one mutation to create a dominant negative inhibitor) (1–3).

Down-regulation of MHC class I expression is frequently observed in human tumors and is particularly pronounced in metastatic lesions (3, 14–17). This is circumstantial but nevertheless compelling evidence of the role of CTL in controlling tumor progression in cancer patients. MHC class I expression has been mainly analyzed in surgically removed tumors using immunohistochemical methods (14–15). A partial reduction or a complete loss of MHCs has been reported, encompassing all MHC molecules or limited to particular alleles (14–15). MHC loss can be seen in some but not all lesions of a patient. Down-regulation of MHC class I expression has been attributed to mutations in β2−m, a transporter associated with Ag-presentation proteins, or the proteosomal latent membrane protein-2 and latent membrane protein-7 (2, 18–21). Additional evidence implicating the loss of MHC class I expression as a mechanism for tumor escape from CTL-mediated killing comes from a longitudinal study of a melanoma patient. Tumor cells, removed during initial surgery, presented nine different Ags that were restricted to

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four separate HLA class I alleles to CTL clones established from the patient (1). The patient remained disease free for 5 years, after which a metastasis was detected. Notably, a cell line established from the metastatic lesion lost all four alleles that had previously been shown to present melanoma Ags.

Thus, the down-regulation of class I MHC molecule is a severe limiting problem for cancer immunotherapy and the application of cancer vaccines.

In this study we suggest applying a novel strategy to retarget class I MHC-peptide complexes on the surface of tumor cells in a way that is independent of the extent of class I MHC expression by the target tumor cells. To this end, we use and genetically fuse two arms of the immune system. One arm, the targeting moiety, comprises tumor-specific recombinant fragments of Abs directed to tumor or differentiation Ags, which have been used for many years to target radiolabeled toxins, or drugs against cancer cells (22, 23). The second effector arm is a single-chain (sc)MHC molecule composed of human β2-m linked to the three extracellular domains of the HLA-A2 H chain (24). By connecting the two molecules into a single gene, we create a new mode for Ab-guided tumor-specific targeting of MHC-peptide complexes onto tumor cells.

The new molecule is expressed efficiently in Escherichia coli and is produced by in vitro refolding in the presence of HLA-A2-restricted peptides.

This targeting approach, as shown in this study, can render the target tumor cells susceptible to lysis by cytotoxic T cells regardless of their MHC expression level and thus may be used as a new approach to potentiate CTL-mediated antitumor immunity.

Our approach may lead to the development of a new type of recombinant therapeutic agents capable of selectively killing and eliminating tumor cells, by using natural cognate MHC ligands and CTL-based cytotoxic mechanisms.

Materials and Methods

Peptides

Peptides were synthesized by standard fluorenlymethoxycarbonyl chemistry and purified to >95% by reverse-phase HPLC. The tumor-associated HLA-A2-restricted peptides used are G9–209-2 M (IMDQVPFSV) and G9–280-9V (YLEPPGPVT), which are derived from the melanoma differentiation Ag gp100 and are common immunodominant epitopes (25, 27). These peptides are modified at the MHC anchor positions 2 (in G9–209-2 M) and 9 (in G9–280-9V) to improve the binding affinity to HLA-A2 (28). The HTLV-1-derived peptide (TSG300) for further purification (see Fig. 2B). The isolated and purified inclusion bodies were solubilized in 6 M guanidine HCl (pH 7.4) followed by reduction with 50 mM dithiothreitol. Solubilized and reduced inclusion bodies of the scMHC-aTacVL and aTac VH, mixed in a 1:2 molar ratio, were refolded by a 1/10 dilution into a redox-shuffling buffer system containing 0.1 M Tris, 0.5 M arginine, 0.09 mM oxidized glutathione (pH 10) in the presence of a 5- to 10-mol excess of the HLA-A2-restricted peptides. The final protein concentration in the refolding was 50 μg/ml scMHC-peptide complexes and Ab Fv-fusion proteins generated previously using this refolding protocol were found to be correctly folded and functional (24, 31, 33). After refolding, the protein was dialyzed against 100 mM Tris (pH 7.4), followed by purification of soluble scMHC-aTac(dsFv)-peptide complexes by ion-exchange chromatography on a Q Sepharose column (7.5 mm internal diameter × 60 cm; Amersham Pharmacia Biotech, Piscataway, NJ) applying a salt (NaCl) gradient. Peak fractions containing scMHC-aTac(dsFv) were then subjected to size-exclusion chromatography (TSK3000) for further purification and a buffer exchange to PBS.

ELISA

Immunoplates (Falcon) were coated with 10 μg/ml purified p55 Ag overnight at 4°C. Plates were blocked with PBS containing 2% skim milk and then incubated with various combinations of B2M-aTac(dsFv)-peptide (90 min at room temperature). Binding was detected using the anti-HLA conformational dependent Ab W6/32 (10°C) and washed, and incubated with various concentrations of B2M-aTac(dsFv)-peptide (90 min at room temperature). The reaction was developed using anti-mouse IgG-peroxidase. Rabbit anti-Tac Ab was used as a positive control, followed by anti-rabbit peroxidase.

Flow cytometry

Cells were incubated with B2M-aTac(dsFv)-peptide complexes (25 μg/ml for 60 min at 4°C in 300 μl), washed, and incubated with the anti-HLA-A2 mAb BB7.2 (10 μg/ml for 60 min at 4°C). Detection was with anti-mouse FITC. Human anti-Tac (10 μg/ml) was used as a positive control to determine the expression of the p55 Ag, followed by incubation with anti-human FITC-labeled Ab. Cells were subsequently washed and analyzed by a FACSCalibur flow cytometer (Beckman Coulter, Fullerton, CA).

CTL clones and stimulation

CTL clones specific for the melanoma gp100-derived peptides were provided by Drs. S. Rosenberg and M. Dudley (Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD). These CTL clones were generated by cloning from bulk cultures of PBMCs from patients receiving peptide immunizations (34). CTL clones were expanded by incubation with irradiated melanoma FM3D cells (as a source of Ag) and the EBV-transformed JY cells (B lymphoblasts as APCs). The stimulation mixture also contained the OKT3 Ab (30 ng/ml) and 50 IU/ml IL-2 and IL-4.
Cytotoxicity assays

Target cells were cultured in 96-well plates (2–5 × 10^4 cells per well) in RPMI plus 10% FCS. Cells were washed and incubated with methionine and serum-free medium for 4 h followed by incubation overnight with 15 μCi/ml [35S]methionine (NEN, Boston, MA). After 3 h of incubation with B2M-aTac(dsFv)-peptide complexes (10–20 μg/ml at 37°C), effector CTL cells were added at an E:T ratio as indicated and incubated for 8–12 h at 37°C. Following incubation, [35S]methionine release from target cells was measured in a 50-μl sample of the culture supernatant. All assays were performed in triplicate. The percentage of specific lysis was calculated as follows: ([experimental release – spontaneous release]/[maximum release – spontaneous release]) × 100. Spontaneous release was measured as [35S]methionine released from target cells in the absence of effector cells, and maximum release was measured as [35S]methionine released from target cells lysed by 0.1 M NaOH.

In vivo win assay

ATAC4 (1 × 10^5) were mixed with R6C12 CTL (1 × 10^6) (E:T 10:1) in the presence or absence of B2M-aTac(dsFv) (20–50 μg/ml) in 200 μl. The mixture was injected s.c. into nude mice and the appearance of tumors was observed. ATAC4 cells alone were used as controls.

Results

Design of B2M-antiTac(dsFv)

We have recently generated a construct encoding a soluble scMHC in which the human β2-m gene is linked to the three extracellular domains (α1, α2, and α3) of the HLA-A2 H chain gene (aa 1–275) through a 15-aa-long flexible linker (24, 33). These scMHC molecules are expressed in E. coli as intracellular inclusion bodies, and upon in vitro refolding in the presence of HLA-A2-restricted tumor-associated or viral peptides they form correctly folded and functional scMHC-peptide complexes and tetramers (24, 33). These scMHC-peptide complexes have been characterized in detail for their biochemical and biophysical characteristics, as well as for their biological activity, and have found to be functional (24, 33). Most importantly, they were able to bind and stain tumor-specific CTL lines and clones. Fig. 1 shows the reactivity of these scMHC-peptide complexes, in the form of scMHC tetramers, with CTLs specific for the melanoma differentiation Ag gp100 epitopes G9–209 M and G9–280V (34). These peptides are modified at the MHC anchor positions 2 (in G9–209 M) and 9 (in G9–280V) to improve the binding affinity to HLA-A2 (28). The CD8+ CTL clones (Fig. 1, A and D) R6C12 and R1E2 were stained intensively (80–95%), specifically with the G9–209 M- and G9–280V-containing scMHC tetramers, respectively (Fig. 1, B and E). As a specificity control, the G9–209 M-specific R6C12 and G9–280V-specific R1E2 CTLs were not stained by the G9–280V and G9–209 M scHLA-A2 tetramers, respectively (Fig. 1, C and F). These CTLs also reacted similarly (intensity) with the wild-type unmodified epitopes G9–209 and G9–280 (data not shown).

To generate the B2M-aTac(dsFv) molecule that targets the scMHC molecule to cells via an Ab Fv fragment, we fused at the C terminus of the HLA-A2 gene the L chain variable domain (V_L) of the humanized anti-CD25 (also known as Tac, p55, IL-2Rα subunit) mAb anti-Tac (35) (Fig. 2A). The H chain variable domain (V_H) is encoded by another plasmid to form a disulfide-stabilized Fv Ab fragment (dsFv) in which the V_H and V_L domains are held together and stabilized by an interchain disulfide bond engineered between structurally conserved framework residues of the Fv (Fig. 2A) (30, 31). The positions at which the cysteine residues are placed were identified by computer-based molecular modeling; because they are located in the framework of each V_H and V_L this location can be used as a general method to stabilize all Fvs without the need for further structural information. Many dsFvs have been constructed in the past few years; they have been characterized in detail and found to be extremely stable and with a binding affinity as good as other forms of recombinant Abs and often even improved (31, 36).

Construction, expression, and purification of B2M-antiTac(dsFv)

To generate the B2M-aTac(dsFv) molecule, we constructed two T7 promoter-based expression plasmids (see Materials and Methods): the scMHC molecule fused to the anti-Tac V_L domain (B2M-aTacVL) is encoded by one plasmid and the anti-Tac V_H domain is encoded by the second. In both plasmids the V_H and V_L domains contain a cysteine that was engineered, instead of a conserved framework residue, to form a dsFv fragment (31). The expression plasmid for the B2M-aTacVL was generated by an overlap extension PCR in which the HLA-A2 and V_L genes were linked by a flexible 15-aa-long linker of (Gly4-Ser)3 identical to the linker used to connect the β2-m and HLA-A2 genes in the scMHC construct (24). The construction of the expression plasmid for the anti-Tac V_H domain was described previously (30). The two plasmids were expressed separately in E. coli BL21 cells. Upon induction with isopropyl β-D-thiogalactoside, large amounts of recombinant protein accumulated in intracellular inclusion bodies.

FIGURE 1. Binding of in vitro refolded scHLA-A2 complexes to CTLs. Melanoma differentiation Ag gp100-specific CTL clones R6C12 and R1E2 were reacted with in vitro refolded purified scHLA-A2 tetramers containing the G9–209 M epitope recognized by R6C12 CTLs and the G9–280V peptide recognized by R1E2 CTLs. CTLs were stained with FITC-anti-CD8 (A and D), with PE-labeled scHLA-A2/G9–209 M tetramers (B and F), and with scHLA-A2/G9–280V tetramers (C and E). R6C12 and R1E2 CTLs were stained with the specific G9–209 M and G9–280V tetramer, respectively, but not with the control tetramer.
SDS-PAGE analysis of isolated and purified inclusion bodies demonstrated that recombinant proteins, with the correct size, constituted 80~90% of the total inclusion bodies’ protein (Fig. 2B). The inclusion bodies of each component were isolated separately, solubilized, reduced, and refolded in a renaturation buffer, which contained redox-shuffling and aggregation-preventing additives, in the presence of HLA-A2-restricted peptides derived from the melanoma differentiation Ag gp100 T cell epitopes G9−209 M and G9−280V (25–28). B2M-aTac(dsFv)/peptide molecules (complexes) were purified from the refolding solution by ion-exchange purification on a Q-Sepharose column. D. Binding of refolded B2M-aTac(dsFv)/G9−209 M to the target Ag, p55. Detection of binding was with the conformational-specific mAb w6/32.

**Binding of B2M-aTac(dsFv) to target cells**

To test the ability of the B2M-aTac(dsFv) molecule to coat and target HLA-A2-peptide complexes on tumor cells, we tested its binding to HLA-A2-negative tumor cells by flow cytometry. First, we used A431 human epidermoid carcinoma cells that were stably transfected with the p55 gene (ATAC4 cells) (29) and compared the staining of transfected vs nontransfected parental cells. The binding of the B2M-aTac(dsFv) to the cells was monitored using an anti-HLA-A2 mAb BB7.2 and FITC-labeled secondary Ab. Expression of the p55 target Ag was detected by the whole anti-Tac mAb from which the dsFv fragment was derived. As shown in Fig. 3A, A431 cells do not express p55; however, the p55-transfected ATAC4 cells express high levels of the Ag (Fig. 3, B and C). Neither cell line was HLA-A2 positive (Fig. 3, D and E). As shown in Fig. 3, D and E, when testing the binding of B2M-aTac(dsFv) to these cells, the ATAC4 cells produced positive anti-HLA-A2 staining only when preincubated with B2M-aTac(dsFv) (Fig. 3, E and F) but the A431 cells were negative when preincubated with B2M-aTac(dsFv). Next, we tested the binding of B2M-aTac(dsFv) to HUT102W and CRII-2 leukemic cells, which, as shown in Fig. 4, A and D, express the p55 Ag as detected by anti-Tac but lack HLA-A2 expression (Fig. 4, B and E). As shown in Fig. 4, B and C, the ATL leukemic HUT102W cells expressing p55 produced positive anti-HLA-A2 staining when preincubated with the B2M-aTac(dsFv). Similar results were observed when leukemia (ATL) p55-positive, HLA-A2-negative CRII-2 cells were preincubated with the B2M-aTac(dsFv) molecule (Fig. 4, E and F). These results demonstrate that B2M-aTac(dsFv) can bind to its Ag, as displayed in the native form on the surface of cells. Most importantly, B2M-aTac(dsFv) could be used to coat HLA-A2-negative cells in a manner that was dependent upon the specificity of the tumor-targeting Ab fragment, rendering them HLA-A2-positive cells.

**Induction of B2M-aTac(dsFv)-mediated susceptibility to CTL lysis**

To test the ability of B2M-aTac(dsFv) to potentiate the susceptibility of HLA-A2-negative cells to CTL-mediated killing, radio-labeled target cells were first incubated with B2M-aTac(dsFv) and then tested in a [35S]methionine release assay in the presence of domain B2M molecules are difficult to separate from the B2M-dsFv molecules because, as previously shown with other dsFv-fusion proteins, V\_l fusion folding is very efficient and the product is quite soluble. However, contamination with the single-domain B2M molecules did not interfere with our subsequent analysis of the soluble B2M-aTac(dsFv) molecule. To confirm the correct formation of the dsFv fragment, we performed a reducing SDS-PAGE analysis in which the B2M-dsFv molecule was separated into its components. Fig. 2C shows the molecular form of the B2M-aTac(dsFv) after reduction, containing the B2M-aTacVL and the V\_H domains.

The ability of the B2M-aTac(dsFv) to bind its target Ag, the α subunit of the IL-2 receptor (p55), was first tested by ELISA using purified p55. To monitor the binding of the purified B2M-aTac(dsFv) to p55-coated wells, we used the mAb w6/32, which recognizes HLA molecules only when folded correctly and which contains peptide. As shown in Fig. 2D, B2M-aTac(dsFv) binds in a dose-dependent manner to p55, which suggests that the two functional domains of the molecule, the scMHC effector domain and the Ab dsFv targeting domain, are folded correctly, indicated by the ability of the dsFv moiety to bind the target Ag and the recognition of the scMHC by the conformation-specific anti-HLA Ab.
HLA-A2-restricted melanoma gp100-peptide-specific CTL. As shown in Fig. 5A, B2M-aTac(dsFv) induced efficiently CTL-mediated lysis of p55-positive HLA-A2-negative ATAC4 cells, whereas the same B2M-aTac(dsFv) molecule did not have any effect and induced no lysis of A431 cells that do not express the Ag. ATAC4 cells alone did not exhibit any CTL-mediated lysis (Fig. 5A). Incubation of ATAC4 cells with scMHC alone, not fused to the dsFv targeting moiety, or with the anti-Tac Ab did not result in any detectable potentiation of CTL-mediated lysis (data not shown). The ability of G9–209 M-peptide-specific CTLs to kill B2M-aTac(dsFv)-preincubated ATAC4 cells (but not A431 cells) was as good as, and in many experiments better than, the efficiency of these CTLs to lyse melanoma FM3D cells that express high levels of HLA-A2 and the gp100 melanoma differentiation Ag (37) (Fig. 5B). Next, we tested B2M-aTac(dsFv)-mediated CTL lysis of p55-expressing, HLA-A2-negative leukemic cells HUT102W and CRII-2. As shown in Fig. 5E, HUT102W and CRII-2 were not susceptible to lysis by the HLA-A2-restricted CTL clones R6C12 and R1E2, specific for the G9–209 M and G9–280V gp100 peptides, respectively. However, when these p55-positive, HLA-A2-negative target cells were preincubated with the B2M-aTac(dsFv) molecule, a significant potentiation for CTL-mediated lysis was observed, which was specific for the gp100 peptide present in the B2M-aTac(dsFv) complex (Fig. 5E). B2M-aTac(dsFv)-coated HUT102W cells were efficiently killed by the G9–209 M and G9–280V peptide-specific R6C12 and R1E2 CTL clones, respectively, and CRII-2 cells were lysed by the R1E2 CTL clone. Control non-melanoma HLA-A2-positive and -negative target cells that do not express p55 did not exhibit any detectable susceptibility to lysis by the melanoma-specific CTL clones whether coated or not with the B2M-aTac(dsFv) molecule (data not shown). These results clearly demonstrate in vitro the concept that the B2M-aTac(dsFv) construct can be used efficiently for Ab-guided, Ag-specific tumor targeting of MHC-peptide complexes on tumor cells to render them susceptible or more receptive to lysis by relevant CTLs and thus potentiate antitumor immune responses.
In vivo activity of B2M-aTac(dsFv)

To initially evaluate the in vivo activity of B2M-aTac(dsFv) in a human tumor model we have performed a win-type assay in which ATAC4 cells were mixed with R6C12 CTLs specific for the G9–209 M gp100-derived peptide in the presence or absence of the B2M-aTac(dsFv) molecule. The mixture was injected s.c. to nude mice and formation of human xenografts in the animals was followed. As shown in Fig. 6, ATAC4 cells generated xenografts in nude mice 10–12 days after s.c. injection.

A mixture of ATAC4 and R6C12 CTLs did not exhibit any significant effect on tumor growth. However, when IL-2R-expressing ATAC4 cells were mixed with B2M-aTacsFv and R6C12 CTLs complete inhibition of tumor growth was observed, indicating the efficient B2M-aTacsFv-induced, CTL-mediated killing of ATAC4 target cells in vivo. In vitro results (Fig. 5) confirmed that the amount of B2M-aTacsFv and the E:T ratio used for the in vivo assay resulted in maximal lysis of ATAC4 target cells (95–100% killing). Parental IL-2R-negative A431 cells mixed with R6C12 CTLs in the presence or absence of B2M-aTacsFv generated tumors efficiently, and no effect on tumor growth was observed (data not shown).

Discussion

Tumor progression is often associated with the secretion of immune suppressive factors and/or the down-regulation of MHC class I Ag presentation functions (1–5, 14, 15). Thus, we can infer that tumors have elaborate strategies to circumvent an apparently effective immune response. Significant progress has been made toward developing vaccines that can stimulate an immune response against tumors, which involves identifying the protein Ags associated with a given tumor type. Epitope mapping of tumor Ags for HLA class I- and class II-restricted binding motifs has been performed which identified T cell epitopes (HLA-restricted peptides) that are currently being used in various vaccination programs (6, 9, 11–13). MHC class I molecules presenting the appropriate peptides are needed to provide the specific signals for recognition and killing by CTLs. However, the principal mechanism of tumor escape is the loss, down-regulation, or alteration of HLA profiles that may render the target cell unresponsive to CTL lysis, even if the cell expresses the appropriate tumor Ag. In human tumors HLA loss may be as high as 50%, suggesting that a reduction in protein levels might offer a survival advantage to the tumor cells (14, 15).

This study has presented a new approach to circumvent this problem. Ab-guided and tumor-specific targeting of class I MHC-peptide complexes onto tumor cells was shown to be an effective and efficient strategy to render HLA-A2-negative cells susceptible to lysis by the relevant HLA-A2-restricted CTLs. This new strategy of redirecting CTLs against tumor cells takes advantage of the use of recombinant Ab fragments that can localize on those malignant cells that express a tumor marker, usually associated with the transformed phenotype (such as growth factor receptors and differentiation Ags), with a relatively high degree of specificity. The tumor-targeting recombinant Ab fragments consist of the Fv variable domains, which are the smallest functional modules of Abs necessary to maintain Ag binding. This makes them especially
useful for clinical applications, not only for generating the molecule described in this study but also for making other Ab fusion proteins, such as recombinant Fv immunotoxins or recombinant Ab-cytokine fusions (38, 39), because their small size improves tumor penetration.

Structurally, the Ab-targeting fragment is fused to a scHLA molecule, which can be folded efficiently and functionally around an HLA-A2-restricted peptide. This approach can be extended to other major HLA alleles and many types of tumor specificities that are dictated by the recombinant Ab fragments, thus generating a

FIGURE 5. Potentiation of CTL-mediated lysis of HLA-A2-negative tumor cells by B2M-aTac(dsFv). Target cells coated or not with the B2M-aTac(dsFv)-peptide complexes were incubated with melanoma-reactive gp100 peptide-specific CTLs in a [35S]methionine release assay. A, A431 and p55-transfected ATAC4 HLA-A2− cells were preincubated or not with B2M-aTac(dsFv)/G9−209 M complexes followed by incubation with the G9−209 M-specific CTL R6C12 (E:T ratio, 10:1). The control are cells incubated with medium alone. B, A431 and p55-transfected ATAC4 HLA-A2− cells were preincubated with B2M-aTac(dsFv)/G9−209 M complexes followed by incubation with R6C12 CTLs. FM3D are HLA-A2− gp100+ melanoma cells. C and D, p55-transfected ATAC4 cells were preincubated with B2M-aTac(dsFv) complexes refolded with the HLA-A2-restricted peptides G9−209 M, G9−280V, and TAX followed by incubation with the G9−209 M-specific CTL clone R6C12 (C) or the G9−280V-specific CTL clone R1E2 (D). E, HUT102W and CRII-2 HLA-A2− leukemic cells were preincubated (w) or not (w/o) with B2M-aTac(dsFv) complexes containing the appropriate peptide followed by incubation with the G9−209 M-specific R6C12 CTLs or G9−280V-specific R1E2 CTLs as indicated (T, target; E, effector CTL clone).
new family of immunotherapeutic agents that may be used to augment and potentiate antitumor activities. Together with the application of mAbs for cancer therapy, this approach may be regarded as a link between antitumor Abs and cell-mediated immunotherapy.

Recombinant Abs have already been used to redirect T cells using a classical approach of bispecific Abs in which one Ab arm is directed against a tumor-specific Ag and the other arm against an effector cell-associated molecule such as CD3 for CTLs and CD16 for NK cells (40). The bispecific approach, e.g., with anti-CD3, recruit T cells independent of their specificity. The major advantage of our approach, in comparison with the bispecific Ab approach, is the ability to recruit a specific population of cytotoxic T cells that is dictated by the specificity of the peptide used to refold the complex. Thus, we can target especially very potent CTLs through the use of a very immunogenic peptide.

A major advantage of our approach is the use of a recombinant molecule that can be produced in a homogeneous form and in large quantities. Importantly, the size of the B2M-dsFv molecule, at ~65 kDa (generated with any Ab dsFv fragment), is optimal with respect to the requirements needed for good tumor penetration on one hand and a relatively long half-life and stability in the circulation on the other hand (41). A recent study describing the generation of Ab-class I MHC tetramers was recently published in which efficient CTL-mediated killing of tumor target cells was observed using Fab-streptavidin-MHC tetramer conjugates (42). The limitation of this approach, in comparison to the recombinant Ab fragment-nomonomeric scMHC fusion described in this work, is the large size of these molecules (~400 kDa) and the fact that soluble MHC tetramers can induce T cell activation themselves, whereas monomeric MHC molecules cannot induce activation unless in a relatively high local concentration (43–45).

The coating of tumor cells that had down-regulated their own MHC expression through the use of this targeting approach potentiates the cells for CTL-mediated killing while using a target on the tumor cells that is usually involved in the transformation process; most classical examples are growth factor receptors such as the IL-2R used in this study. This fact also supports the idea that by using this approach escape mutants that down-regulate the targeted receptor are not likely to have a growth advantage because the receptor is directly involved in key survival functions of the cancer cells.

Another advantage to our approach is that these new agents can be designed around the desired peptide specificity; i.e., the refolding of the B2M-Fv molecule can be performed around any appropriate HLA-restricted peptide. In this study we used HLA-A2-restricted tumor-specific CTLs recognizing T cell epitopes derived from the melanoma differentiation Ag gp100. However, the kind of Ag-reactive CTLs to be redirected to kill the tumor cells can be defined by other antigenic peptides, based on our recent knowledge of immune mechanisms in health and disease. For example, the identification of tumor-specific CTL responses in patients may suggest that these may be efficient to target. However, recent studies have demonstrated that these tumor-specific CTLs are not always optimal, because they are often present very seldom and only at very low frequencies, or even when they are present at high frequencies they may be not functional or anergic (7). Thus, a more active and promising source of CTLs can be recruited from circulating lymphocytes directed against common and very immunogenic T cell epitopes such as those derived from viruses or bacterial toxins, which can also elicit a good memory response (46, 47). It has been shown that CTL precursors directed against influenza, EBV, and CMV epitopes (peptides) are maintained at high frequencies in the circulation of cancer patients as well as healthy individuals and that these CTLs are usually active and with a memory phenotype (46, 47). Thus, these CTLs would be the source of choice to be redirected to the tumor cells through the use of a B2M-Fv molecule generated loaded with such viral-derived epitopes. The optimal agent that we are currently developing is a B2M-Fv molecule in which the antigenic peptide is also covalently linked to the complex through the use of a flexible linker connecting the peptide to the N terminus of the β2-m gene. This construct will ensure optimal stability for the scMHC complex in vivo because the stabilizing peptide is connected covalently and cannot easily leave the MHC peptide-binding groove. This type of single-chain peptide-MHC molecule was generated previously in murine and human systems for various functional and structural studies (48, 49). An additional option is to use antigenic peptide derivatives that are modified at the anchoring residues in a way that increases their affinity to the HLA binding groove (28).

There are also several options for the type of Fv fragment to be used as the targeting moiety. In addition to the dsFv type of fragment, used here, a scFv fragment can be used in which the VH and VL Ab domains are connected via a peptide linker. In such cases the B2M-Fv molecule is encoded by one plasmid, which avoids possible contamination with single-domain B2M molecules such as are encountered in this study.

Another important aspect of this study, which is supported by others, is that the coating of antigenic MHC-peptide complexes on the surface of tumor cells without transmembrane anchoring is sufficient to induce their efficient lysis by specific CTLs without knowledge about whether autologous accessorial molecules of the target tumor cells are present at all and are playing a role in such CTL-mediated killing. This observation may result from the fact that a local high concentration of coated MHC-peptide complexes displaying one particular T cell epitope (peptide) is formed on the targeted cells, which greatly exceeds the natural density of such complexes displayed on the surface of cells. Regarding the IL-2Rα subunit, several hundreds to thousands of sites per cell are present on the target cells, in comparison to very few complexes containing one particular peptide that are expected to be present on cells, which may be sufficient for effective and efficient killing even without the involvement of accessory molecules. This is without taking into consideration the down-regulation of class I MHC
expression as an escape mechanism. Further evidence of this possibility is that MHC tetramers can induce T cell activation by themselves (45), including our recent observation that CTL activation by MHC tetramers without accessory molecules can be demonstrated at the single-cell level. Another possible mode of action of our approach is the induction of Fas-Fas ligand-mediated apoptosis.

In conclusion, our results clearly demonstrate the usefulness of this approach to recruit active CTLs for tumor cell killing via cancer-specific Ab-guided targeting of scMHC-peptide complexes. These results pave the way for the development of a new immunotherapeutic approach based on naturally occurring cellular immune responses that are redirected against the tumor cells.

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