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Immunosensitization of Melanoma Tumor Cells to Non-MHC Fas-Mediated Killing by MART-1-Specific CTL Cultures

P. J. Frost,*† L. H. Butterfield,‡ V. B. Dissette,‡ J. S. Economou,‡ and B. Bonavida**

The discovery of human melanoma rejection Ags has allowed the rational design of antitumor immunotherapeutic strategies. One such Ag, MART-1, is expressed on >90% of human melanomas, and CTL generated against MART-1 27–35 kill most HLA A2.1+ melanoma cells. However, variant tumor cells, which do not express MART-1, down-regulate MHC, or become resistant to apoptosis, will escape killing. Cytotoxic lymphocytes kill by two main mechanisms, the perforin/granzyme degranulation pathway and the TNF/Fas/TNF-related apoptosis-inducing ligand superfamily of apoptosis-inducing ligands. In this study, we examined whether cis-diaminedichloroplatinum (II) cisplatin (CDDP) sensitizes MART-1/HLA A2.1+ melanoma and melanoma variant tumor cells to non-MHC-restricted, Fas ligand (FasL)-mediated killing by CTL. MART-1 27–35-specific bulk CTL cultures were generated by pulsing normal PBL with MART-1 27–35 peptide. These CTL cultures specifically kill M202 melanoma cells (MART-1+, HLA A2.1+, FasR+), and MART-1 27–35 peptide-pulsed T2 cells (FasR+), but not M207 melanoma cells (MART-1+, HLA A2.1−, FasR−), FLU28–66 peptide-pulsed T2 cells, or DU145 and PC-3 prostate cells (MART-1−, HLA A2.1−, FasR+). CDDP (0.1–10 μg/ml) sensitized non-MART-1 27–35 peptide-pulsed T2 to the CD8+ subset of bulk MART-1-specific CTL, and killing was abolished by neutralizing anti-Fas Ab. Furthermore, CDDP up-regulated FasR expression and FasL-mediated killing of M202, and sensitized PC-3 and DU145 to killing by bulk MART-1-specific CTL cultures. These findings demonstrate that drug-mediated sensitization can potentiate FasL-mediated killing by MHC-restricted CTL cell lines, independent of MHC and MART-1 expression on tumor cells. This represents a novel approach for potentially controlling tumor cell variants found in primary heterogeneous melanoma tumor cell populations that would normally escape killing by MART-1-specific immunotherapy. The Journal of Immunology, 2001, 166: 3564–3573.

Various immunotherapeutic strategies have been devised to generate specific antitumor responses by inducing or modifying the specific immune response of cytotoxic lymphocytes (1–3). For example, the discovery of human melanoma rejection Ags has allowed the rational design of antitumor CTL strategies, which kill melanoma tumor cells expressing the appropriate Ag and MHC class I (4, 5). Several human melanoma Ags are recognized by CTL, including MART-1/Melan-A (6, 7), gp100 (8), tyrosinase (9, 10), and the melanoma Ag-encoding gene family of Ags (11, 12). Epitopes from MART-1/Melan-A and gp100 are presented to CTL in the context of the HLA A2.1 molecule, which is expressed in a high proportion of individuals (~50% of the Caucasian population) (4, 13). Because MART-1/Melan-A is found in the majority of melanoma tumor cells (6, 9), it has been proposed as a prime target for generating specific antitumor immune responses (4).

Two major factors have limited the effectiveness of tumor-specific therapy: selection and activation of a significant population of tumor-reactive CTL and the generation of tumor variants that are not recognized by these specific CTL (14, 15). MART-1 peptide-pulsed PBMCs were used as stimulators to generate a MART-1 peptide-based bulk CTL culture similar to several antitumor immunotherapies currently being tested in the clinic (16, 17). This strategy would be expected to stimulate different CD8+ T cells expressing TCRs of varying affinity for MART-1-expressing tumor cells. MART-1-specific immunotherapy will be expected to kill all MART-1+ melanoma cells, as long as the Ag induces a significant and long-lasting immune response, and most, if not all, of the tumor cells express MHC/peptide complexes at sufficient levels to be recognized, thus activating CTL (18, 19). In addition, the melanoma cells must be sensitive to CTL-mediated killing pathways, such as Fas ligand (FasL)3 or perforin/granzyme (20). For example, low expression of MART-1 on tumor cells may not be sufficient to be recognized by MART-1-specific TCRs or the level of expression may be insufficient to trigger CTL-mediated killing (14, 21). Furthermore, these melanoma tumor cell variants may trigger CTL-mediated killing pathways (e.g., FasL or perforin) for which they are resistant (22, 23). Thus, melanoma variants that either down-regulate MHC and/or Ag or generate resistance to CTL-mediated cytotoxic pathways may avoid immune clearance, thereby limiting the effectiveness of melanoma-specific immunotherapy.

Killing of tumor cells by cytotoxic lymphocytes is mediated through two main pathways, the degranulation perforin/granzyme pathway and the TNF superfamily of apoptosis-inducing ligands (TNF, FasL, TNF-related apoptosis-inducing ligand (TRAIL)) (24–27). How these cytotoxic pathways are activated and whether certain targets preferentially trigger one and/or the other pathway is not clear. However, it has been shown that many melanoma

3 Abbreviations used in this paper: FasL, Fas ligand; CDDP, cis-diaminedichloroplatinum (II) cisplatin; LAK, lymphokine-activated killer cell(s); TIL, tumor-infiltrating lymphocyte(s); TRAIL, TNF-related apoptosis-inducing ligand.

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tumor cells express Fas and TNF receptors, yet are resistant to killing via these pathways (20, 28, 29). If these resistant tumor cells are unable to trigger the perforin/granzyme pathway, or preferentially trigger the FasL/TNF cytolitic pathways, it is reasonable to assume that such tumors will be unaffected by tumor-specific CTL.

Activation of cytotoxic lymphocytes (CTL, NK, lymphokine-activated killer cells (LAK)), either by MHC/TCR interactions, IL-2, or anti-CD3 Abs, results in the up-regulation of FasL. (30, 31). Current strategies designed to activate and expand melanoma-specific CTL, which use these activation modalities, may be expected to up-regulate FasL expression on both melanoma-specific CTL and nonmelanoma-specific CTL. Because FasL-mediated killing is independent of MHC/TCR interactions, we hypothesized that FasL-expressing CTL could kill Fas-sensitive melanoma cells, irrespective of their MHC/TCR specificity. Thus, strategies to sensitize melanoma cells to Fas-mediated killing could enhance immunotherapy to all FasL-expressing CTL and other effector cells, regardless of MHC specificity. Furthermore, Fas-sensitive, non-MART-1/non-MHC-expressing melanoma cell variants would also be expected to be killed by these FasL-expressing CTL. Altogether, we hypothesize that presensitizing resistant melanoma cells to FasL-mediated killing will enhance killing of both MART-1/MHC-expressing and variant tumor cell lines.

Treatment of malignant melanomas with chemotherapy, in general, has been of limited success, with a response rate of only 10–20% (32). A number of studies have suggested that chemotherapeutic drugs may exert their cytotoxic effect, at least in part, through up-regulation or activation of the Fas/FasL apoptotic pathway (33–35). Therefore, drug resistance may be due to mutations or modifications of the Fas/FasL apoptotic pathway (36, 37). This also suggests that drug-resistant melanoma cells may also be cross-resistant to FasL-mediated killing, a major component of CTL-mediated apoptosis.

We have recently shown that subtoxic concentrations of chemotherapeutic drugs are able to sensitize drug- and immunoresistant tumor cells to FasL-mediated killing by cytotoxic lymphocytes (LAK and tumor-infiltrating lymphocytes (TIL)) in a non-MHC-restricted manner (20). Therefore, we investigated whether a combination of sensitizing drug and in vitro generated CTL and (2) resulted in killing of non-MART-1/non-HLA A2.1-expressing malignant melanoma cells, irrespective of their MHC/TCR specificity. Finally, strategies to sensitize melanoma cells to Fas-mediated killing could enhance immunotherapy to all FasL-expressing CTL and other effector cells, regardless of MHC specificity. Furthermore, Fas-sensitive, non-MART-1/non-MHC-expressing melanoma cell variants would also be expected to be killed by these FasL-expressing CTL. Altogether, we hypothesize that presensitizing resistant melanoma cells to FasL-mediated killing will enhance killing of both MART-1/MHC-expressing and variant tumor cell lines.

Materials and Methods

Cell lines

The human melanoma lines, M202 (MART-1, HLA A2.1), M207 (MART-1, HLA A2.1), and M238 (MART-1, HLA A2.1), were generated from surgical specimens and have been described previously (38). Peter Cresswell (Yale University School of Medicine, New Haven, CT) generously provided T2 cells (HLA A2.1). The human hormone-independent prostatic carcinoma cell lines, DU145 and PC-3 (HLA A2.1, MART-1), were obtained from Dr. Arie Belledgeun (Department of Urology, Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA (UCLA)). The PMMI murine CTL hybridoma was derived from BALB/PEL mice and is specific for the H-2b thymoma EL-4, and was obtained from William Clark (Department of Biology, UCLA) (39). All cell lines were cultured in 10% heat-inactivated FCS (Atlanta Biologicals, Norcross, CA) added to RPMI 1640 (Life Technologies, Grand Island, NY) with 1% sodium-pyruvate (Life Technologies), 1% nonessential amino acids (Life Technologies), and 1% Fungi-bact solution (Irvine Scientific, Santa Ana, CA), which contains 10,000 U/ml penicillin G, 10 mg/ml streptomycin, and 25 µg/ml Fungizone, and grown in a humidified atmosphere at 37°C and 5% CO2.

Generation of MART-1-specific CTL bulk culture

Generation of MART-1-specific peptide-specific bulk CTL was based on the published protocol of Plebanski (40). Briefly, normal HLA A2.1 donor (as tested by HLA-A2 (BB7.2) Ab and subsequent HLA-A2 DNA subtyping) PBMC were pulsed with MART-1-27–35 peptide (AAGIGILTV, AAPGIGILTV, prepared by the Peptide Synthesis Facility at UCLA) at 3 × 105 cells/ml suspended in IMDM (Life Technologies) at room temperature for 90 min. The cells were rinsed and plated in a 24-well plate (Costar, Cambridge, MA) at 3 × 104 cells/well, 1.5 ml/well total volume, in 10% autologous serum/RPMI 1640 + 1% Fungi-bact with 10 ng/ml IL-7 (Biosource, Camarillo, CA) and 5 µ g/ml keyhole limpet hemocyanin (Sigma, St. Louis, MO). Every week, the nonadherent cells were restimulated with fresh, autologous, peptide-pulsed, and irradiated PBMC at a 1:1 ratio and given IL-2 (10 U/ml) (Hoffman-LaRoche, Nutley, NJ) every 3–4 days. Non-MART-1-specific, LAK-like bulk CTL were generated as described above, except without peptide pulsing.

Separation of CD4+ /CD8+ populations from bulk CTL

After 5 wk of in vitro culture, CD4+ and CD8+–enriched populations were prepared from MART-1-specific bulk CTL by negative depletion, as follows. A total of 5 × 106 CTL was washed in PBS + 2% human AB serum (Life Technologies), divided into two tubes, and incubated with mouse anti-human CD4, CD19, and CD56/NA/LE Abs (PharMingen, San Diego, CA) plus either mouse anti-human CD4 Ab (PharMingen) (for CD4+ enrichment) or mouse anti-human CD8 Ab (PharMingen) (for CD8+ enrichment) for 30 min at 4°C. Except Ab was rinsed off in PBS + 2% human AB serum, and sheep ant-mouse IgG magnetic beads (Dynal, Oslo, Norway) were added for 30 min at 4°C. After incubation, 10 ml of PBS was added and Ab-bead conjugates were depleted. The remaining cells were washed, phenotyped, and used immediately in the cytotoxicity assay.

Reagents

cis-Diaminedichloroplatinum (II) cisplatin (CDDP), EGTA, ionomycin, propidium iodide, MgCl2, and PMA were purchased from Sigma. Na2H35O4 was purchased from Amersham (Arlington Heights, IL). The Fas-neutralizing Ab (clone ZB4, IgG subclass) and apoptotic Ab (clone CH11, IgM subclass) were purchased from PharMingen. Stock solutions of CDDP were routinely prepared in DMSO, whereas PMA and ionomycin were prepared in ethanol.

DNA staining

T2 cells were incubated for 18 h at 37°C and 5% CO2 in the presence of 1 µg/ml of the apoptosis-inducing CH11 anti-Fas Ab (IgM subclass; PharMingen) or isotype control (PharMingen). The cells were then collected, and 2 × 106 cells were washed once with 1× PBS and incubated for 1 h in 70% ethanol on ice. The cells were washed twice with 1× PBS, and 70 µl of RNase (1 mg/ml) and 140 µl propidium iodide (100 µg/ml) were added. DNA fragmentation (apoptosis) was determined by DNA hypodiploidy using an Epics-XL MCL flow cytometer (Coulter, Miami, FL).

Flow cytometry

M202, M207, M238, and T2 cells were incubated in the presence or absence of the chemotherapeutic drug, CDDP (0.1–10 µg/ml), for 18 h at 37°C and 5% CO2. Following the incubation, the cells were trypsinized for 5 min, collected, and washed three times in PBS + 10% FBS. For staining, 100 µl of 1–2 × 105 cells/ml was added to 96-well U-bottom plates (Costar). Fas expression was assayed by adding 10 µg/ml of PE-conjugated mouse anti-human Fas mAb (IgG subclass; PharMingen) or isotype control (PharMingen). The cells were then collected, and 2 × 106 cells were washed once with 1× PBS and incubated for 1 h in 70% ethanol on ice. The cells were washed twice with 1× PBS, and 70 µl of RNase (1 mg/ml) and 140 µl propidium iodide (100 µg/ml) were added. DNA fragmentation (apoptosis) was determined by DNA hypodiploidy using an Epics-XL MCL flow cytometer (Coulter, Miami, FL).

RT-PCR

Total RNA was extracted and purified from ~5 × 106 cells by a single step guanidinium thiocyanate-chloroform method with STAT 60 reagent (Tel-Test “B,” Friendswood, TX). Total RNA (1 µg) was reverse transcribed to first strand cDNA for 1 h at 42°C with SuperScript II reverse transcriptase.
(200 U) and random hexamer primers (20 μM) (Life Technologies, Bethesda, MD). Amplification of one-tenth of the cDNA product by PCR was performed using the following gene-specific primers: FasR sense (5'-ATG CTG GCC ATC TGG ACC CT-3'); FasL antisense (5'-GTC GCT GTA GCC AAA TTC GTC-3'); and G3PDH sense (5'-GGT GCT GTA GCC AAA TTC GTC-3'); FasL antisense (5'-CTG TCT ACC ATG TCT GTT GC-3'); and β-actin antisense (5'-CGTCATACTCCTGCTTCCTGAT-3'). PCR amplifications were conducted using the Hot Start/Ampliwax method as described by the supplier (Perkin-Elmer, Foster City, CA) with the following temperature cycling parameters: 94°C/45 s; 65°C/2 min for 26 cycles; and a final extension at 72°C/10 min. The amplified products were resolved by 1.5% agarose gel electrophoresis.

Cell-mediated cytotoxicity

Murine PMMI CTLs were activated in the presence of 10 ng/ml PMA and 3 μg/ml ionomycin and incubated for 3 h at 37°C and 5% CO₂ (41). The cells were then washed once in PBS and resuspended at a final concentration of 10⁶ cells/ml and used immediately in the cytotoxicity assay. MART-1-specific bulk CTL and nonspecific bulk CTL were washed three times in PBS and resuspended at a final concentration of 10⁶ cells/ml and used immediately in the cytotoxicity assay.

T2 cells were grown overnight in the presence or absence of CDDP (0.1–10 μg/ml) for 18 h at 37°C and 5% CO₂. Following incubation, cells were collected, washed once in fresh PBS, then labeled with 100 μCi of Na₂¹⁸⁵⁵CrO₄ for 1 h at 37°C and 5% CO₂, and then the presence or absence of exogenous peptide, or with MART-1₂₇₋₃₅ (50 μg/ml) or FLU₁₆₋₆₀ (GILGFVFTL) (50 μg/ml) peptide. The cells were then washed three times in medium, and 10⁴ cells were added to V-bottom 96-well culture plates (Costar), and used immediately in the cytotoxicity assay. To block FasL-mediated killing, ¹⁸⁵⁵Cr-labeled T2 cells were preincubated with 100 μg/ml of mouse anti-human Fas-neutralizing Ab (clone ZB4) (PharMingen) for 1 h at 37°C and 5% CO₂. Effector cells (100 μl) in the presence or absence of 3 mM EGTA/2 mM MgCl₂ (pH 7) were added at the indicated E:T ratio.

The melanoma cell lines, M202, M207, and M238, and the prostate carcinoma cell lines, DU145 and PC-3, were trypsinized for 5 min, collected, and washed once in PBS. The cells were incubated in 100 μCi of Na₂¹⁸⁵⁵CrO₄ for 1 h at 37°C and 5% CO₂, and washed three times in medium, and 10⁴ cells were added to flat-bottom 96-well culture plates (Costar) in the presence or absence of drug. The plates were incubated for 18 h at 37°C and 5% CO₂. At the time of the experiment, the CDDP-containing medium was removed, and 100 μl of fresh medium + 10% FBS was added to each well. Effector cells (100 μl) in the presence or absence of 3 mM EGTA/2 mM MgCl₂ were added at the indicated E:T ratio.

Plates were centrifuged and incubated for 5–7 h at 37°C and 5% CO₂. Following incubation, 100 μl of supernatant was harvested from each well and counted in a Beckman 4000 gamma counter (Beckman, Fullerton, CA). Total ¹⁸⁵⁵Cr release was determined by lysing target cells with 50 μl of 10% SDS (Sigma) and collecting 150 μl for count. Spontaneous release was determined by collecting 100 μl of supernatant from target cells from each treatment, absent effector cells. To determine the percentage of killing by effector cells, the spontaneous release of target cells treated with drug or Ab alone was subtracted from experimental values (drug treatment + effector cells). The percentage of cell-specific ¹⁸⁵⁵Cr release was determined as follows: percent ¹⁸⁵⁵Cr release = (experimental release – spontaneous release)/(spontaneous release – spontaneous release) × 100. The data are presented as the effector cell-mediated killing at each E:T ratio, minus the drug or Ab effects.

Statistical analysis

All experiments were conducted at least on three separate occasions. All values are presented as the mean ± SD of triplicate samples. ANOVA (one-way or two-way ANOVA) was used to test for significance. Pairwise analysis was performed by the Bonferroni/Dunn post hoc tests. p < 0.05 was determined to be significantly different from the control.

Results

Drug treatment up-regulates FasR expression on M202 melanoma target cells

We examined the expression of FasR and FasL on target cells to characterize the sensitivity of melanoma tumor cell lines to FasL-mediated killing. FasR is expressed on T2 (99% positive) (Fig. 1A), while only low level of FasR is expressed on M202 (11.7%), and no or very little FasL is expressed on M207 (0.4%) (Fig. 1B) or M238 (data not shown). FasR mRNA was observed in M202, M238, and T2, and at low levels in M207 and K562 (Fig. 2A). However, the surface expression of FasR on M202 (64.8%), but not M207 (5.2%), was up-regulated by overnight treatment with CDDP (0.1–10 μg/ml) (Fig. 1B). Similar results were observed in at least three separate experiments with M202 FasR up-regulation ranging from 25–65%. The MART-1-specific bulk CTL culture expressed FasL mRNA (Fig. 2). In contrast to some previous reports, which showed that cell lines derived from melanoma tumors express Fasl (42, 43), but in agreement with Chappel et al. (44),

FIGURE 1. Cell surface expression of FasR on target cells. T2 cells were stained with PE mouse isotype control (IgG) or PE mouse anti-human Fas Ab (clone ZB4). B, M202 and M207 cells were treated for 18 h in the presence and absence of 5 μg/ml CDDP, washed, then stained with PE mouse isotype control (IgG) or PE mouse anti-human Fas Ab (clone ZB4). Black-shaded histogram is for non-CDDP-treated cells, white-shaded histogram is for CDDP-treated cells, and gray-shaded area indicates region of overlap. Values indicate percentage of positive cells.
none of the target cell lines tested (T2, M202, M207, and M238) in this study expressed FasL mRNA (Fig. 2).

**CDDP augments FasL-mediated killing of T2 and M202 cells by PMMI CTL hybridoma**

The Fas"+ T2 cell line is sensitive to apoptosis induced by the anti-Fas Ab, CH11 (Fig. 3A). Therefore, we used the FasL-expressing murine CTL hybridoma, PMMI, to determine sensitivity to FasL-mediated killing. PMMI kill both murine and human FasR-expressing target cells exclusively via the FasR/FasL cytotoxic pathway in a non-MHC-restricted manner when activated by PMA/ionomycin (20). PMMI kill T2 cells approximately to the same level as CH11 anti-Fas Ab, and the neutralizing anti-Fas Ab, ZB4, significantly blocked PMMI-mediated lysis (p < 0.05) (Fig. 3B). In addition, killing was independent of the FLU58–66 or MART127–35 peptide used to pulse T2 cells (Fig. 3C). Finally, CDDP treatment (10 μg/ml) significantly augmented the PMMI-mediated killing of T2, also independent of peptide (p < 0.05) (Fig. 3C). Treatment of T2 cells with CDDP alone resulted in an increase in spontaneous 51Cr release equal to 25–45% of the total 51Cr release. This is compared with an increase in spontaneous release of 5–10% of the total 51Cr release in untreated control cells (data not shown). To determine the component of cell-mediated killing, the spontaneous release due to drug treatment alone was subtracted from the experimental 51Cr release (CDDP + effector cells) to determine the percentage of PMMI-mediated killing.

As expected, the FasR-negative lines, M202, M207, and M238, were resistant to PMMI FasL-mediated killing. However, following CDDP treatment, M202 cells up-regulated both FasR expression and sensitivity to killing by PMMI (Fig. 4A), while M207 (Fig. 4B) and M238 (data not shown) remained resistant.

**MART-1-specific bulk CTL kill MART-127–35-pulsed T2 cells via both FasR/FasL- and perforin-mediated cytotoxic pathways**

Normal human PBMC (HLA A2.1+) pulsed with MART-127–35 peptide were used to generate peptide-specific bulk CTL cultures (generally 30–60% CD3+/CD8-, 40–70% CD4+, 1–3% CD16+ by phenotypic analysis, data not shown) (45). These peptide-specific bulk CTL cultures efficiently killed T2 pulsed with MART-127–35 (>50–80% at even the lowest E:T ratio), but not FLU58–66 (nonspecific peptide) or T2 without exogenous peptide (data not shown) (Fig. 5). Furthermore, killing is only partially blocked by the Ca2+-chelator, EGTA/MgCl2, which blocks the Ca2+-dependent perforin-mediated pathway, but not the Ca2+-independent FasR/FasL pathway (Fig. 5). These data suggest that MART-1-specific bulk CTL cultures can kill MART-127–35-expressing T2 cells by both perforin- and nonperforin-mediated cytotoxic pathways, but are unable to kill nonspecific peptide-labeled T2 cells. Experiments were performed on at least three separate occasions, using at least three separate preparations of CTL culture with similar results.

**CDDP sensitizes non-MART-127–35 expressing T2 cells to FasR/FasL-mediated killing by CD8+ CTL**

Killing of T2 cells by PMMI is non-MHC/peptide restricted, due solely to FasR/FasL, and is augmented by CDDP. Because CTL express FasL, we tested whether CDDP could sensitize non-MART-1-pulsed T2 cells to FasL-mediated killing by our MART-1-specific bulk CTL culture (Fig. 6). Interestingly, nonpeptide-pulsed T2 cells treated with CDDP are killed by MART-1-specific bulk CTL (Fig. 6A). Cytotoxicity was independent of EGTA/MgCl2 (Fig. 6B), and almost completely blocked by the anti-Fas...
MART-1-specific bulk CTL preparations contain a significant proportion of CD4+ T cells (generally 40–70% CD4+ by phenotypic analysis, data not shown) (45). This may be significant because CD4+ T cells have been shown to kill via the FasR/FasL-mediated cytotoxic pathway (46). Therefore, we isolated CD8+ and CD4+ populations from our bulk CTL to determine which subpopulation was responsible for killing of drug-sensitized T2 targets. Only the CD8+ cells (>80% CD8+) killed MART-1-expressing T2 cells. In the absence of MART-127–35 peptide, there was no specific killing by either CD4+ (>90% CD4+) or CD8+ cells. However, CDDP significantly sensitized non-MART-127–35 peptide-pulsed T2 to CD8+-, but not CD4+-mediated killing (p < 0.05) (Fig. 7B). These findings demonstrate that CD8+ CTL are capable of killing drug-sensitized, non-MART-127–35 peptide-expressing T2 cells via the FasR/FasL, cytotoxic pathway, in a non-MHC/peptide-restricted manner.

CDDP sensitizes M202, and the human prostate cell lines, DU145 and PC3, to FasR/FasL-mediated killing by MART-1-specific bulk CTL

We investigated the role of CDDP in MART-1-specific bulk CTL-mediated cytotoxicity of melanoma target cells. We have previously demonstrated that concentrations of up to 10 μg/ml of CDDP are subtoxic to numerous cancer cell lines, including prostate (DU145, PC-3) and ovarian (AD10) cell lines (47, 48). In this study, both M202 and M207 were resistant up to 72-h CDDP treatment alone, as measured with 51Cr release, trypan blue exclusion dye, and propidium iodide (data not shown). Treatment with CDDP alone increased the absolute spontaneous 51Cr release by only 5–10% in comparison with untreated control cells (no CDDP), even at the highest concentration used (10 μg/ml). However, CDDP treatment did result in significant G1/G0 cell cycle arrest within 24 h (data not shown). MART-1-specific bulk CTL cultures killed M202, but not the M207 cell line, and killing was exclusively by the perforin pathway because EGTA/MgCl2 blocked nearly all cytotoxicity (Fig. 8). However, following CDDP treatment, M202 cells were killed by bulk CTL, even in the presence of EGTA/MgCl2, because M207 were not sensitized, given that they do not express FasR, nor is FasR up-regulated by CDDP in these cells (Fig. 8).

Because FasL-mediated killing is independent of MHC/TCR interactions, we hypothesized that non-MART-1-specific CTL should be able to kill drug-sensitized M202 but not M207 target cells. We generated bulk CTL culture in the absence of specific peptide pulse. These nonspecific CTL exhibited LAK-like cytotoxicity (i.e., non-MHC restricted), to T2 target cells, and killing was similar for either MART-127–35 or FLU58–66 peptide (data not shown). Additionally, both M202 and M207 were killed to an equal extent by these nonspecific CTLs, independent of MHC expression (Fig. 9A). However, in the presence of EGTA/MgCl2, only the drug-sensitized M202 cells were killed by these nonspecific CTL (Fig. 9B), supporting our hypothesis that they were sensitized to non-MHC-restricted, FasL-mediated killing.
Previously, we have shown that CDDP sensitizes human prostate cell lines, DU145 and PC3, to FasL-mediated killing by PMMI, TIL, and LAK in a non-MHC-restricted manner (20, 48). Therefore, we tested whether CDDP could also sensitize these nonmelanoma, non-MART-1-expressing target cells to killing by MART-1-specific bulk CTL cultures. Interestingly, CDDP significantly sensitized both PC-3 and DU145 prostate carcinoma cell lines to killing by MART-1-specific bulk CTL cultures, independent of EGTA/MgCl2 (p > 0.05). As expected, no killing was observed without sensitization by drugs (Fig. 10).

Discussion
The majority (>90%) of fresh melanoma tumor cells and melanoma cell lines express MART-1/Melan A, which has been proposed as a prime target for generating specific antimelanoma immune responses (4). Despite the ability to generate specific antimelanoma CTL, the ultimate efficacy of this strategy will depend solely on the ability of these CTL to kill all melanoma cells. Certainly, the demonstration that MART-1-specific CTL kill most HLA A2.1+/MART-1+ target cells in standard in vitro killing assays may not correlate with in vivo tumor clearance (49). This is especially true because HLA A2.1 and MART-1 expression on melanoma tumor cells can be highly variable, both within tumors and over time (18, 50–52). Furthermore, MART-1-specific CTL may select tumors that down-regulate MHC/peptide expression or become resistant to CTL-mediated killing pathways. Therefore, we propose a strategy to address these potential tumor escape mechanisms by sensitizing melanoma cells to FasL-mediated killing with subtoxic concentrations of CDDP. We hypothesize that FasL-expressing MART-1-specific bulk CTL, which represent in vivo response to vaccination to MART-1, should be able to kill MART-1-expressing target cells by both the FasL and perforin/granzyme cytotoxic pathways. Furthermore, we also hypothesize that subtoxic concentrations of CDDP can sensitize certain target cells to FasL-mediated killing by MART-1-specific bulk CTL, irrespective of MHC/peptide expression.

In this study, we present evidence that MART-127–35-specific bulk CTL cultures kill Fas-sensitive, HLA A2.1+/MART-1+ target cells via both the FasL and perforin pathways, and that this killing is MHC restricted. As expected, MART-1-specific bulk
CTL cultures are MART-1/HLA A2.1 restricted and have no detectable LAK-like activity. However, MART-1-specific bulk cultures can kill certain Fas-resistant target cells (M202, DU145, PC-3), independent of MHC/peptide expression, provided that these target cells are first sensitized with subtoxic concentrations of CDDP. Furthermore, the nonspecific killing by the FasR/FasL pathway is mediated primarily by the CD8+ cytotoxic subset of the bulk cultures. These findings suggest that combination sensitizing drug and immunotherapy can overcome tumor cell escape by sensitizing MART-1+/HLA A2.1+ expressing tumor cells and non-MART-1/non-HLA A2.1-expressing tumor cell variants to FasL-mediated killing by MART-1-specific bulk cytotoxic cultures. MART-1 peptide-pulsed PBMCs were used as effectors in these experiments because they represent a more realistic estimate of the in vivo immune response on tumor clearance. In addition, our bulk culture CTL preparation is similar to other, current antitumor strategies undergoing clinical trials (16, 17). However, the use of cloned MART-1-specific CTL clones in our sensitizing model will be important to determine whether specific CTL are responsible for killing sensitized non-MHC/non-MART-1 target cells. We are currently developing a number of models to test this hypothesis directly.

CTL-mediated killing requires both recognition of the tumor cells through the MHC/TCR and triggering of the apoptotic pathways responsible for killing of the target cells. Tumor cells may escape CTL-mediated killing by either: 1) avoiding lymphocyte recognition by down-regulating MHC class I/peptide expression; 2) not triggering lymphocyte-mediated killing mechanisms (i.e., perforin/granzyme degranulation or FasL expression) or preferentially triggering those pathways for which they are resistant; and/or 3) down-regulating downstream apoptotic signaling components or up-regulating protective antiapoptotic factors. In these examples, tumor resistance may be independent of immune recognition and specific CTLs may have limited antitumor effects.

A second cytotoxic mechanism used by CTL to kill tumor cells after perforin/granzymes is the FasR/FasL cytotoxic pathway. The FasR/FasL pathway is important because most immunotherapeutic strategies designed to activate and/or expand specific antitumor CTL may also up-regulate FasL expression (30). In addition, numerous studies have shown that both fresh melanoma tumor cells and melanoma cell lines express FasR, but most melanoma...
cells are resistant to FasL-mediated killing, thus making target cells resistant to a major cytolytic component of CTL (53, 54). Furthermore, we and others have shown that FasR can be up-regulated on a wide variety of tumor cells (i.e., ovary, prostate, melanoma), although the exact mechanisms regulating expression remain unclear (48, 55, 56). In our study, only M202 expressed cell surface FasR, despite the fact that the other lines expressed FasR mRNA. Furthermore, additional studies need to be conducted, not only on additional melanoma cell lines, but on freshly isolated tumor cells as well. We are currently testing our hypothesis on the ability of IL-2-activated TIL cells to kill autologous, CDDP-sensitized tumor cells. It has also been proposed that FasL expressed on melanoma cells may contribute to tumor cell escape through the killing of activated, FasR-expressing CTL (42, 43, 54, 57). However, our study corroborates the findings of Chappel et al. (44), because none of the melanoma cell lines tested (M202, M207, M238) expressed FasR mRNA (Fig. 2).

TNF, anti-Fas Ab, and TRAIL have all been proposed as potential anticancer therapies, but tumor cell resistance to these factors has limited their effectiveness (27, 47, 58). It has been well established that combination of drugs and protein synthesis inhibitors can overcome resistance to killing by members of the TNF superfamily of receptors (TNFR, FasR, TRAIL receptors) (20, 27, 59). For example, Griffith et al. (27) recently showed that cycloheximide could sensitize melanoma cell lines to TRAIL-mediated killing by down-regulating the expression of TRAIL decoy receptors. Furthermore, we have demonstrated that TIL and LAK can kill drug-sensitized prostate carcinoma cell lines by the FasL-mediated pathway in a non-MHC-restricted manner (20, 48).

Most studies examining the effects of drug sensitization to overcome tumor cell resistance to Fas-mediated killing have been conducted with cytotoxic anti-Fas Abs (47, 56, 59). However, anti-Fas Ab has been shown to be toxic to mice, therefore limiting its potential therapeutic usefulness in man (60). FasL, which is expressed on activated cytotoxic lymphocytes as part of the normal immune response, offers a more effective and biologically relevant system to study sensitization of tumor cells to Fas-mediated apoptosis (20, 48). To assay the sensitivity of our target cells to FasL-mediated killing, we first used the CTL hybridoma, PMMI, which kills exclusively by the FasR/FasL, cytotoxic pathway (Fig. 3) (20, 41). We also analyzed the contribution of the perforin/granzyme- and FasR/FasL-mediated pathways, which can be differentiated, in vitro, by neutralizing anti-Fas Ab and by the Ca\(^{2+}\) chelator, EGTA/MgCl\(_2\), in CTL killing (20, 61).

Using our FasL-mediated killing model, we demonstrated that T2 cells are sensitive to killing by anti-Fas Ab (CH11), and PMMI, independent of peptide presentation, and cytotoxicity is augmented by treatment with sensitizing doses of CDDP (Fig. 3). MART-1-specific bulk CTL killing of MART-1\(_{27–35}\)-pulsed T2 was only partially blocked by EGTA/MgCl\(_2\), suggesting that both perforin- and FasR/FasL-mediated cytotoxic pathways are involved in killing (Fig. 5). However, T2 cells, either not pulsed with peptide, or pulsed with a non-specific peptide (FLU), were resistant to MART-1-specific bulk CTL. These results are surprising, because we had previously demonstrated that T2 cells are sensitive to anti-Fas Ab- and FasL-mediated killing by PMMI cells in a nonpeptide-dependent fashion (Fig. 3). One explanation may be that a negative signal in the T2 cells inhibits or blocks the MART-1-specific CTL from killing non-MART-1\(_{27–35}\)-pulsed cells, despite sensitivity to FasL (Fig. 3). It is also possible that, due to the TAP mutations, non-MART-1\(_{27–35}\)-pulsed T2 cells only weakly bind to MART-1-specific CTL. This may result in only partial or limited FasR/FasL interaction with a subsequent decrease in killing efficiency. Although it is not clear why nonsensitized T2 cells are killed by anti-Fas Ab and PMMI, but not MART-1-specific bulk CTL, CDDP overcame resistance to FasL-mediated killing by MART-1-specific bulk CTL. The killing of non-MART-1-expressing T2 cells was independent of EGTA/MgCl\(_2\) and almost totally blocked by the neutralizing anti-Fas Ab, ZB4 (Figs. 6 and 7).

Because our preparation of bulk CTL included a significant proportion of non-CD8\(^+\) T cells, it was possible that these non-specific lymphocytes could also contribute to FasL-mediated killing. For example, it was recently shown that CD4\(^+\) cells kill melanoma cells via the FasL-mediated pathway (46). Separation of CD4\(^+\) and CD8\(^+\) populations demonstrated that only the CD8\(^+\) CTL were involved in killing of either MART-1-pulsed T2 cells or drug-sensitized T2 cells (Fig. 7B). The lack of CD4\(^+\) FasL-mediated killing may be due to the activation of effector cells using MART-1 presented only in context of MHC class I, which activate CD8\(^+\), but not CD4\(^+\) cells (15). FasL is expressed only on activated T cells; therefore, it is likely that CD4\(^+\), which do not trigger through MHC class I, would not express cell surface FasL, and will be unable to kill Fas-sensitive target cells (46).

As expected, the M202 cell line was killed by MART-1-specific CTL bulk cultures, and this killing was mediated by the perforin/granzyme pathway. However, following CDDP treatment, M202...
were sensitized to FasL-mediated killing by both MART-1-specific bulk CTL and PMMI (Figs. 4A and 8A). Thus, M202, a specific target of MART-1-specific bulk CTL, can be killed via both the perforin/granzyme pathway and the FasR/FasL pathway, following sensitization. It can be argued that only a small percentage of our bulk culture represents a MART-1-specific population of CTL, and that perhaps at least some of the response that we observed was due to nonspecific, LAK-like activity. However, non-MART-1-specific CTL, which have LAK-like properties, can kill both M202 and M207 cells by an EGTA/MgCl₂-dependent manner (Fig. 9A), but in the presence of EGTA/MgCl₂, only drug-sensitized M202 cells were killed (Fig. 9B). Drug sensitization to FasL-mediated killing has previously been reported for prostate and ovarian tumor cell lines (20, 47, 48), but this is the first report using melanoma-specific CTL. Furthermore, because FasL-mediated killing is independent of MHC/peptide expression, it is possible that non-MART-1/HLA-A2.1-expressing melanoma cells can be sensitized to MART-1-specific bulk CTL-mediated killing.

Neither of the non-MART-1/non-HLA A2.1-expressing melanoma cell lines (M207 and M238) that we tested were sensitized to Fas-mediated killing, most likely because they did not express FasR. To prove that our MART-1-specific bulk culture could kill non-MART-1/HLA A2.1-expressing target cells, we used a model that we had previously demonstrated could be sensitized to FasL-mediated killing. The ability of the prostate cell lines, DU145 and PC3, to be sensitized by CDDP to FasR/FasL-mediated killing has been well characterized by us and others (20, 48, 62). These non-HLA A2.1/non-MART-1, FasR⁺ DU145, and PC3 prostate carcinoma cell lines initially were not killed by MART-1-specific CTL, but could be sensitized to by CDDP to FasL-mediated killing (Fig. 10). These findings are in agreement with our previous results of PMMI, TIL, and LAK sensitization (20, 48). Altogether, our findings show that sensitizing chemotherapeutic drugs can redirect MHC-restricted killing of tumor cells by sensitizing some tumor cells to FasR/FasL-mediated cytotoxicity. This would allow MART-1-specific immunotherapy to circumvent the escape of variant tumor cells that down-regulate either MHC or MART-1 expression. Finally, while FasR is expressed in cells of the brain, retina, heart, and ovary, it is not clear how sensitizing drugs would have an effect, if any, on either FasR expression or sensitivity on normal, nontumor cells, in vivo.

We have previously reported that CDDP sensitizes human prostate cancer cells to both anti-Fas Ab- and FasL-mediated killing by PMMI, LAK, and TIL cell lines (20, 48). Sensitization of DU145 and PC-3 is not dependent on the de novo up-regulation of FasR, Bcl-2, Bax, and c-Myc (20, 48). Thus, it is likely that sensitization may be drug and cell specific, resulting in alterations in regulation of apoptotic genes, modification of signaling components and enzymatic pathways, DNA repair mechanisms, and mitochondrial function. Despite different upstream signaling components, FasL, TNF, and TRAIL all initiate similar apoptosis caspase cascades, and recent evidence suggests that chemotherapeutic drugs may modify activation/activity of certain caspases, such as caspase 3 and 8 (63, 64).

Despite early successes in generating melanoma-specific immunotherapy, in vivo response rates have been disappointing (65, 66). This has led to a focus on developing more effective melanoma-specific Ags that generate stronger CTL responses. However, if the failure of CTL-mediated immunotherapy is due to tumor down-regulation of MHC/peptide and/or generation of tumor cells resistance to CTL-mediated killing, then these new strategies will ultimately fail. We hypothesize that combination therapy of subtoxic concentrations of chemotherapeutic drugs, to sensitize cells to non-MHC-mediated killing, in conjunction with CTL immunotherapy, may enhance treatment by bypassing these potential tumor escape mechanisms. In this study, we show that pretreatment of certain target cells with subtoxic concentrations of CDDP can sensitize tumor cells to FasL-mediated killing in a non-MHC-restricted manner. This will be expected to both enhance and prolong melanoma-specific CTL immunotherapy.

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