*Human Cancer Biology*

Clinical Cancer Research

# Accessory Cells of the Microenvironment Protect Multiple Myeloma from T-Cell Cytotoxicity through Cell Adhesion-Mediated Immune Resistance

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## Abstract

**Purpose:** Cellular immunotherapy frequently fails to induce sustained remissions in patients with multiple myeloma, indicating the ability of multiple myeloma cells to evade cellular immunity. Toward a better understanding and effective therapeutic modulation of multiple myeloma immune evasion mechanisms, we here investigated the role of the tumor microenvironment in rendering multiple myeloma cells resistant to the cytotoxic machinery of T cells.

**Experimental Design:** Using a compartment-specific, bioluminescence imaging-based assay system, we measured the lysis of luciferase-transduced multiple myeloma cells by $CD4^+$ or $CD8^+$ CTLs in the presence versus absence of adherent accessory cells of the bone marrow microenvironment. We simultaneously determined the level of CTL activation by measuring the granzyme B release in culture supernatants.

**Results:** Bone marrow stromal cells from patients with multiple myeloma and healthy individuals, as well as vascular endothelial cells, significantly inhibited the lysis of multiple myeloma cells in a cell–cell contact-dependent manner and without substantial T-cell suppression, thus showing the induction of a cell adhesion-mediated immune resistance (CAM-IR) against CTL lysis. Further analyses revealed that adhesion to accessory cells downregulated Fas and upregulated the caspase-3 inhibitor survivin in multiple myeloma cells. Reconstitution of Fas expression with bortezomib enhanced the CTL-mediated lysis of multiple myeloma cells. Repressing survivin with the small-molecule YM155 synergized with CTLs and abrogated CAM-IR *in vitro* and *in vivo*.

**Conclusion:** These results reveal the cell adhesion-mediated induction of apoptosis resistance as a novel immune escape mechanism and provide a rationale to improve the efficacy of cellular therapies by pharmacologic modulation of CAM-IR. *Clin Cancer Res; 19(20); 5591–601. ©2013 AACR.*

## Introduction

Multiple myeloma (MM) has long been the paradigmatic model for investigating the role of the microenvironment in blood cancers (1). The natural niche of multiple myeloma, the bone marrow, is a milieu of growth factors and cytokines that provides optimal conditions for multiple myeloma cell proliferation and survival. Furthermore, the bone marrow microenvironment contributes to resistance against various therapies. Interaction of multiple myeloma cells with accessory cells of the bone marrow microenvironment induces pleiotropic antiapoptotic mechanisms, thereby rendering multiple myeloma cells resistant to established therapeutic regimens (e.g., glucocorticoids, DNA-damaging chemotherapy; ref. 2), as well as investigational anti-multiple myeloma agents (3, 4). This concept, generally known as cell adhesion-mediated drug resistance (CAM-DR), is considered as one of the major obstacles hindering successful treatment (5–7). Next to these well-documented roles on multiple myeloma cell survival, growth, and drug resistance, recent evidence indicates that the bone marrow microenvironment facilitates multiple myeloma cells to escape the immune system (8). This is particularly important, because despite the therapeutic potential of cellular immunotherapies, such as allogeneic stem cell transplantation or donor lymphocyte infusions (9–11), the majority of patients with multiple myeloma receiving these therapies

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## Translational Relevance

Durable tumor regression and even cures can be achieved in patients with multiple myeloma by immunotherapeutic approaches, such as allogeneic stem cell transplantation and donor lymphocyte infusion. However, low rate of sustained remissions impedes the success of this therapeutic approach. Currently, much emphasis is placed on the role of the tumor microenvironment in tumor cell immune escape. We here describe a previously underappreciated mechanism of immune escape induced by the microenvironment: cell adhesion-mediated immune resistance. To modulate this resistance, we provide a rational combination of molecularly targeted and immunologic strategy, by combining CTLs and two clinically tested therapeutics. These findings add a new perspective to microenvironment-induced immune escape, which lead to better understanding of mechanisms involved in immune evasion and can guide clinical testing of potential drug-immunotherapy combinations toward the development of the next-generation innovative cancer immunotherapy.

do not achieve sustained remissions (12, 13). Several immunosuppressive factors such as IDO, TGF-β, or VEGF, have been shown to hamper T-cell activation or proliferation in the tumor microenvironment (14). Regulatory T cells (15), tolerogenic dendritic cells, or immunosuppressive macrophages can also suppress immunity in the microenvironment. On the other hand, it is currently unknown whether other mechanisms of immune escape exist in the tumor microenvironment. On the basis of the evidence that multiple myeloma cells in contact with accessory cells become resistant to apoptotic drugs and based on the fact that tumor cells can develop resistance to CTL-mediated cytotoxic pathways (16, 17), we postulated that the accessory cells of the bone marrow microenvironment could also confer multiple myeloma cells resistant against CTL killing mechanisms. Such an immune resistance may have an additional negative impact on the efficacy of several currently applied immunotherapeutic strategies. To investigate this, we used a compartment-specific bioluminescence imaging (CS-BLI)—based *in vitro* model, which enables us to specifically quantify the lysis of multiple myeloma cells by antigen-specific and HLA-restricted $CD4^+$ and $CD8^+$ CTLs in the presence versus absence of various accessory cells. To distinguish immune resistance of the multiple myeloma cells from immune suppression, we simultaneously measured CTL-mediated multiple myeloma lysis and CTL activation in the presence versus absence of accessory cells. We show that bone marrow stromal cells (BMSC) from patients with multiple myeloma and healthy individuals as well as vascular endothelial cells significantly inhibit the CTL-mediated lysis of multiple myeloma cells, without overt CTL suppression and thus predominantly due to the induction of immune resistance. This resistance is induced by cell–cell adhesion and therefore referred to as cell adhesion-mediated immune resistance (CAM-IR). We identified Fas downregulation and upregulation of survivin/ Mcl-1 as potential mechanisms of CAM-IR. We could counteract the Fas downregulation by bortezomib. Furthermore, repression of survivin/Mcl-1 with the small-molecule YM155 showed the improvement of CTL-mediated lysis of multiple myeloma cells *in vitro* and *in vivo* in presence of accessory cells.

## Materials and Methods

### Cell culture

The luciferase (Luc)-transduced human MM cell lines, U266 and UM9, were maintained in RPMI-1640 (Invitrogen), supplemented with 10% FBS (Integro BV) and antibiotics (penicillin/streptomycin; Life Technologies) as previously described (18). The authenticity was confirmed by partial HLA typing carried out maximal 6 months before the most recent experiment. The BMSC line HS-5 and BMSCs from patients with multiple myeloma (pBMSC) or from healthy individuals (hBMSC) were cultured in Dulbecco's modified Eagle medium (Invitrogen) with 10% FBS and antibiotics. BMSC were used in experiments until passage six. All bone marrow samples were obtained after informed consent and approved by the institutional medical ethical committee. Human umbilical vein derived endothelial cells (HUVEC) were obtained from Lonza and cultured in EGM-2 medium (Lonza).

The HLA-DP4 restricted, minor histocompatibility antigen (mHag)-specific $CD4^+$ CTL clone 3AB11; the HLA-A2–restricted mHag-specific $CD8^+$ CTL clone HA-1; the HLA-A2–specific $CD8^+$ CTL clone 1E4; and the HLA-A2–restricted WT-1–specific T-cell receptor transferred polyclonal CTLs were previously described in detail with respect to their antigen-specific and HLA-restricted cytotoxic capacity against the relevant HLA-matched and antigen-positive multiple myeloma cells (18–21). CTLs were expanded using a feeder cell–cytokine mixture and cryopreserved until use as described (19).

### Reagents

RGDw peptide (provided by Prof. P. de Groot; University Medical Center Utrecht, the Netherlands; ref. 22) was used at a concentration of 200 μmol/L. Neutralizing antibody against Fas ligand (BD Pharmingen) was used at a concentration of 10 μg/mL. Bortezomib and YM155 (both Selleck Chemicals) were dissolved in dimethyl sulfoxide at a concentration of 200 μmol/L and 1 mmol/L, respectively, and aliquoted for storage until use. Both were diluted in culture medium to the concentrations indicated in each experiment.

### Compartment-specific bioluminescence-based cytotoxicity assays

Accessory cells HS-5, pBMSC, hBMSC, or HUVEC, were plated at a density of $1 \times 10^4$ cells/well in white opaque flat-bottomed 96-well plates (Costar) in 100 μL culture medium. After an adherence period of 6 hours, luc-transduced multiple myeloma cells were added ($1 \times 10^4$ cells/well).

Where indicated, multiple myeloma cells and accessory cells were coincubated for 16 to 20 hours before the addition of CTLs. Otherwise multiple myeloma cells and CTLs were added together into the assay. The BLI signal emitted from surviving multiple myeloma cells was determined after 24 to 48 hours using a luminometer (SpectraMax, Molecular Devices) within 20 minutes after the addition of 125 μg/mL beetle luciferin (Promega). The % survival of multiple myeloma cells was calculated using the formula: % survival = (mean BLI signal in the absence of CTLs/mean BLI signal in the presence of CTLs) × 100%. In these assays, multiple myeloma cell survival is a direct reflection of T-cell–mediated lysis and correlates with classical chromium release assays (3).

### Granzyme B ELISA

The granzyme B (GzB) content of cell-free supernatants was determined using a commercial ELISA kit (Pelipair, Sanquin) according to the manufacturer's instructions.

### Transwell experiments

Accessory cells were seeded in 24-well plates at a density of $6 \times 10^4$ cells/well. After 6 hours, multiple myeloma cells were either added on adherent accessory cells or placed in the Transwell inserts with 0.4 μm pore membrane. After 16 to 20 hours, CTLs were added in the compartments in which the multiple myeloma cells were present. The % survival of multiple myeloma cells was determined after 24 hours as described above. % inhibition of lysis was calculated using the formula: % inhibition = (% survival in presence of accessory cells /% survival in absence of accessory cells) × 100%.

### Western blotting

To specifically harvest multiple myeloma cells after interaction with accessory cells, we used a previously described reverse Transwell system (23, 24). Briefly, HS-5 cells were seeded at a density of $0.5 \times 10^6$ cells at the bottom of a cell culture insert with a surface area of 452.4 $mm^2$, pore size 3.0 μm, and pore density $2 \times 10^6$ $cm^{-2}$ (Greiner Bio-One), which permits cell–cell interaction. After adhesion of the HS-5 cells overnight, the inserts were placed in a 6-well plate upside-down, leaving the adherent HS-5 cells on the lower surface. The multiple myeloma cells were then added at the top layer of the porous surface. After 24 hours, multiple myeloma cells were harvested, checked for HS-5 contamination by fluorescence-activated cell sorting analysis, washed twice with ice-cold PBS, and incubated for 15 minutes at 4°C in lysis buffer (Cell Signaling Technology). Lysates were centrifuged at 14,000 g for 10 minutes at 4°C, and the supernatant was removed and stored at −80°C until use. Protein concentrations were determined by a bicinchoninic acid protein assay (Pierce; Thermo Scientific). Samples were separated by SDS-PAGE and proteins were transferred to Immobilon-FL PVDF membrane (Millipore). After overnight blocking with Odyssey Block Buffer (Westburg), membranes were incubated with primary antibodies for 2 hours at room temperature (anti-survivin and anti-Mcl-1; 1:1000 dilution, Cell Signaling Technology). Blots were visualized by Odyssey infrared imaging (LICOR Biosciences) using Alexa-labeled antibodies according to the manufacturer's instructions. Band intensities were quantified using ImageJ software.

### Survivin downregulation by shRNA

Luciferase-transduced UM9 cells were transduced with lentiviral transduction particles TRCN0000073718, TRCN0000073719, TRCN0000073720, TRCN0000073721, TRCN00000222542 (Sigma-Aldrich) according to manufacturer's instructions.

### *In vivo* tumor targeting experiments

Hybrid scaffolds consisting of three 2- to 3-mm biphasic calcium phosphate particles coated with human mesenchymal stromal cells (MSC) and loaded with $Luc^+$ MM cell line UM9 were implanted subcutaneously into $RAG2^{-/-}\gamma_C^{-/-}$ mice, as described previously (25). As controls, uncoated scaffolds were implanted in the same mice and multiple myeloma cells were injected directly into the scaffold. Ten days after implantation, tumors growing in the scaffolds were treated with $CD4^+$ CTL 3AB11 ($5 \times 10^6$ CTLs per scaffold), by directly injecting the CTLs into the scaffold. An identical set of scaffolds was left untreated. At the day of T-cell injection, subcutaneous pumps (Alzet 1007D) were implanted which were either filled with PBS or with 2.08 μg/μL YM155 (Selleckchem) diluted in PBS delivering 1 mg/kg/ d YM155 continuously. Pumps were removed after 5 days. Bioluminescent imaging was conducted as described previously (25).

### Statistical and synergy analyses

The differences in triplicate measurements were determined by statistical tests as indicated in the figure legends. *P* values below 0.05 were considered as significant.

Testing the type of interaction (additive, agonistic, or synergistic) between YM155 and CTLs was analyzed using the Compusyn software program (Combosyn Inc.,), which is based upon the Chou–Talalay method (26). The combination index (CI) value was calculated in Compusyn with the input of CS-BLI measurements of multiple myeloma cell survival for CTL alone, YM155 alone, or the combination in a dose titration of at least 5 doses, titrated around the half maximal effective concentration ($EC_{50}$) of each individual effector. The calculated CI is a quantitative measure for the degree of treatment interaction, with a CI <1.0 indicating synergy, a CI = 1 indicating additive effects, and a CI >1 indicating antagonism.

## Results

### Accessory cells inhibit CTL-mediated lysis of multiple myeloma cells

To evaluate the effect of accessory cells on the T-cell–mediated lysis of multiple myeloma cells, we selected BMSCs and vascular endothelial cells, the two main accessory cell types in the bone marrow microenvironment. We used cloned $CD4^+$ and $CD8^+$ CTLs that are directed at

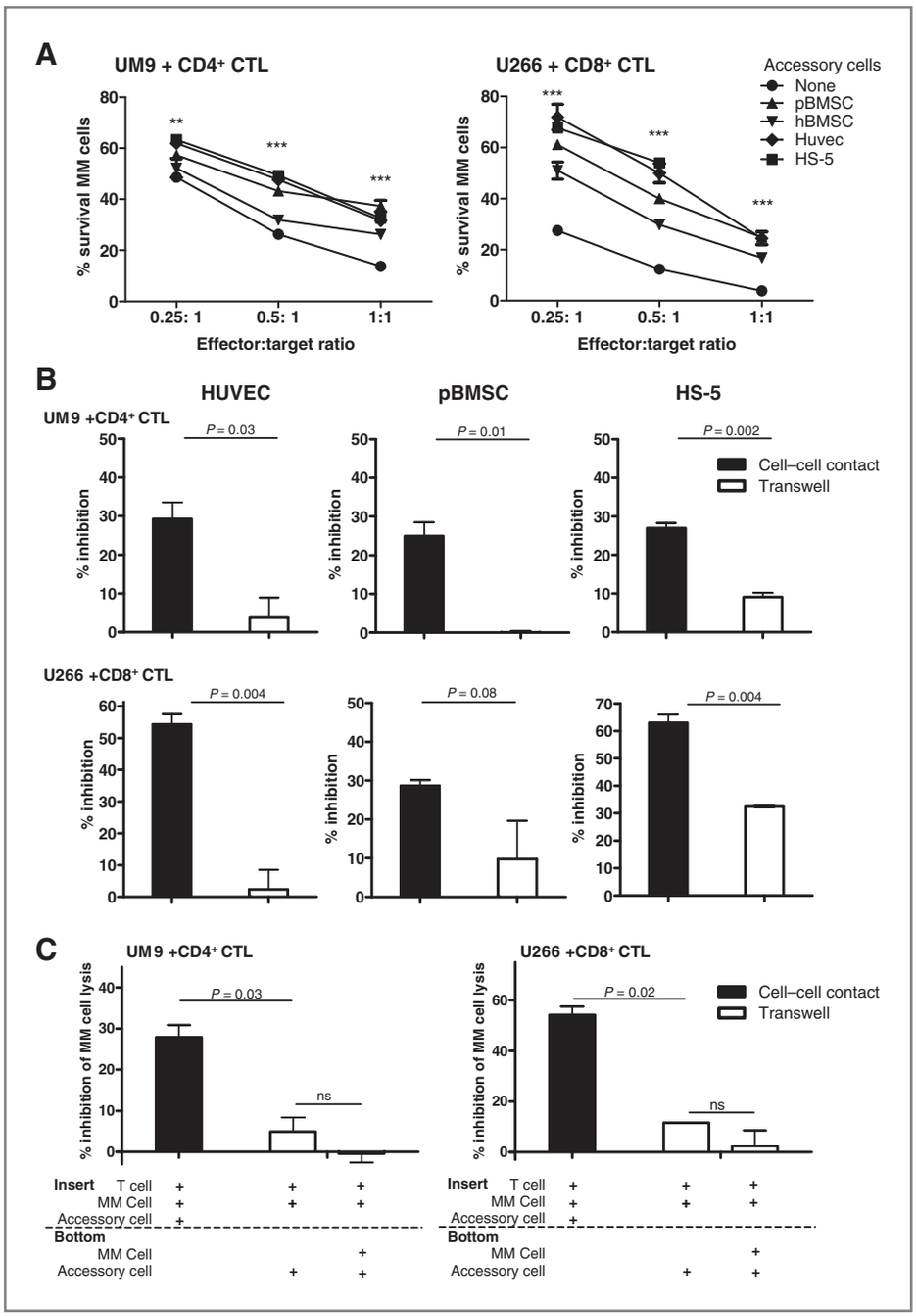


**Figure 1.** Accessory cells inhibit T-cell–mediated lysis of myeloma cells mainly through direct cell–cell contact. Luc$^+$ MM cell lines U266 and UM9 were cocultured with CD4$^+$ (A, left) or CD8$^+$ (A, right) CTLs, respectively, in the presence and absence of different accessory cells. pBMSC, patient-derived bone marrow stromal cells; hBMSC, healthy donor-derived BMSC. B, accessory cells [HUVEC (left), pBMSC (middle), or HS-5 cells (right)] were seeded at the bottom of 24-well tissue culture plates. UM9 cells (top) or U266 cells (bottom) and the relevant CTLs (CD4$^+$ CTL and CD8$^+$ CTL, respectively) were added in the wells either in direct contact with the accessory cells or separated from them by membrane inserts (Transwell). C, in extended Transwell assays, MM cells ± CD4 or CD8 TCL were cultured in the insets, whereas accessory cells alone or together with MM cells were seeded in the bottom chamber as indicated. In all assays, MM cell viability was determined 24 hours after addition of T cells by CS-BLI and was used to calculate MM cell survival (see Material and Methods). The results are depicted as the % survival or inhibition of MM cell lysis by CTLs in presence versus the absence of accessory cells at 24 hours. Error bars represent the SEM. Results represent the mean values of triplicate cultures. Differences in inhibition of T-cell lysis in the presence of accessory cells was tested by unpaired two-tailed Student *t* test. **, $P < 0.01$; ***, $P < 0.001$. Results are representative of at least three independent assays.

different mHag expressed on multiple myeloma cells, but not on accessory cells. Because the recognition of mHags is HLA restricted, these CTLs recognize only those multiple myeloma cells expressing both the relevant antigen as well as the presenting HLA molecule (18). Accordingly, to conduct our experiments, we selected UM9 as the relevant target cell for CD4$^+$ CTLs and U266 for the CD8$^+$ CTLs (Supplementary Fig. S1 A and S1B). After confirming that these CTLs are unable to lyse the accessory cells (Supplementary Fig. S1C and S1D), we tested them in compartment-specific BLI-based assays for the capacity to inhibit multiple myeloma cell survival through their cytotoxic activity (3), in the absence and presence of adherent accessory cells HS-5, pBMSCs, hBMSCs, or HUVECs (Fig. 1A). As expected, both CTLs mediated effective dose-dependent survival inhibition of the relevant MM cell line. In this experimental setting, the inhibition of survival represents multiple myeloma cell lysis by the CTLs (3), which is also confirmed by our previous findings (19, 21). Coculture with any of the accessory cells significantly inhibited this cytotoxic effect (Fig. 1A).

This inhibitory property of accessory cells was not restricted to mHag-specific CTLs because in similar assays, they also protected the multiple myeloma cells from lysis by the tumor antigen WT-1–specific polyclonal CTLs (Supplementary Fig. S2A).

### Accessory cells inhibit CTL-mediated multiple myeloma lysis in a cell–cell contact-dependent fashion

To evaluate the mechanism of the protective effect of accessory cells, we first carried out transwell experiments, in which multiple myeloma cells and accessory cells were either cocultured in direct contact, or separated by micropore membrane inserts. As expected, accessory cells significantly inhibited the multiple myeloma cell lysis by CTLs in cocultures when they were in direct contact with multiple myeloma cells (Fig. 1B). However, when accessory cells were separated by a transwell from multiple myeloma cells, the inhibitory capacity of HUVEC and pBMSC was almost completely lost and that of HS-5 diminished substantially (Fig. 1B). Finally, we carried out extended transwell assays, in which not only accessory cells but also multiple myeloma cells were cultured in the bottom chamber of the transwells (Fig. 1C). Also under these conditions, no inhibition of lysis occurred, indicating that accessory cells protected multiple myeloma cells from CTL-mediated lysis mainly via cell–cell contacts. Therefore, we next examined whether blocking the adhesion of multiple myeloma cells to accessory cells with an RGDw peptide, a soluble ligand for cell surface integrins (27), would abrogate the protection of multiple myeloma cells from CTL-mediated lysis. Preincubation of HS-5 or HUVECs with RGDw peptide, but not with an irrelevant peptide, reduced the adhesion of multiple myeloma cells to HS-5 or HUVEC (Supplementary Fig. S3) and substantially decreased their capacity to protect multiple myeloma cells from CTL-mediated lysis (Fig. 2A and B). These findings emphasized the importance of cell–cell contacts for the capacity of accessory cells to protect multiple myeloma cells from CTLs. However, the inhibitory effect could not be attributed to integrin-dependent cell adhesion alone because mere binding of multiple myeloma cells to fibronectin-, vitronectin-, or laminin-coated wells did not induce an inhibitory effect on CTL-mediated lysis of multiple myeloma cells (Fig. 2C and D).

### Accessory cells induce immune resistance as well as immune suppression

CTLs are known to initiate target cell death via granzyme (28) or death receptor-mediated mechanisms (29), both of which require CTL activation. Hence, the accessory cell induced inhibition of CTL-mediated lysis in our assays could be either due to suppression of T cells and/or due to an acquired resistance in multiple myeloma cells against the lysis mechanisms of CTLs. To differentiate between these, we measured GzB excretion and Fas ligand (FasL) expression on CTLs as a marker for T-cell activation upon coculture with multiple myeloma cells and accessory cells. The $CD4^+$ CTL-mediated lysis of UM9 cells was inhibited by the accessory cells (Fig. 3A), without a reduction in GzB release (Fig. 3B) or change in FasL upregulation on the CTLs (Supplementary Fig. S4A). These results excluded T-cell suppression as a mechanism and revealed the development of CAM-IR in UM9 cells. For $CD8^+$ CTLs, accessory cells not only inhibited the lysis of U266 cells (Fig. 3C), but also reduced GzB production (Fig. 3D), suggesting the induction of T-cell suppression. However, the upregulation of FasL levels on the $CD8^+$ CTLs did not diminish, thus renouncing absolute T-cell suppression (Supplementary Fig. S4B). Development of immune resistance in U266 cells could

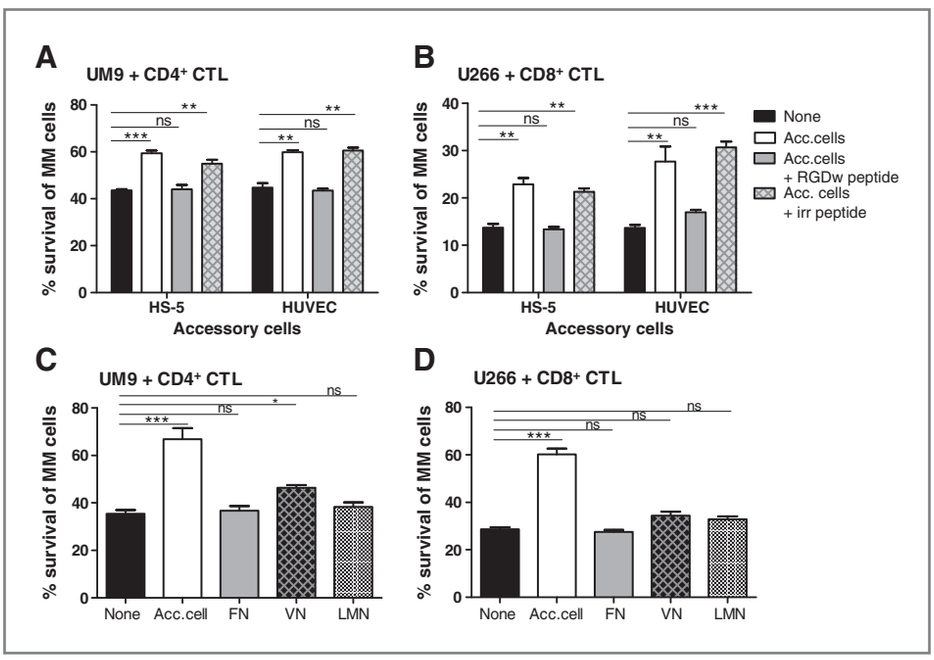


**Figure 2.** Accessory cell-mediated resistance to CTLs is abrogated by blockade of cell–cell adhesion. Accessory cells were incubated with RGDw peptide, a ligand for cell surface integrins, or an irrelevant peptide as control, 1 hour before adding the UM9 (A) or U266 (B) MM cell lines. In an accessory cell-free system UM9 (C) and U266 (D) were cultured in wells, which were coated with saturating concentrations of extracellular matrix proteins, fibronectin (FN), vitronectin (VN; both 5 μg/mL), and laminin (LMN; 50 μg/mL). Multiple myeloma cell-specific CTLs were added after 16 hours. Experimental conditions were conducted in triplicate. Survival of multiple myeloma cells was determined by CS-BLI after 24 hours of coculture with CTLs. Differences in T-cell lysis were evaluated by using unpaired two-tailed Student *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

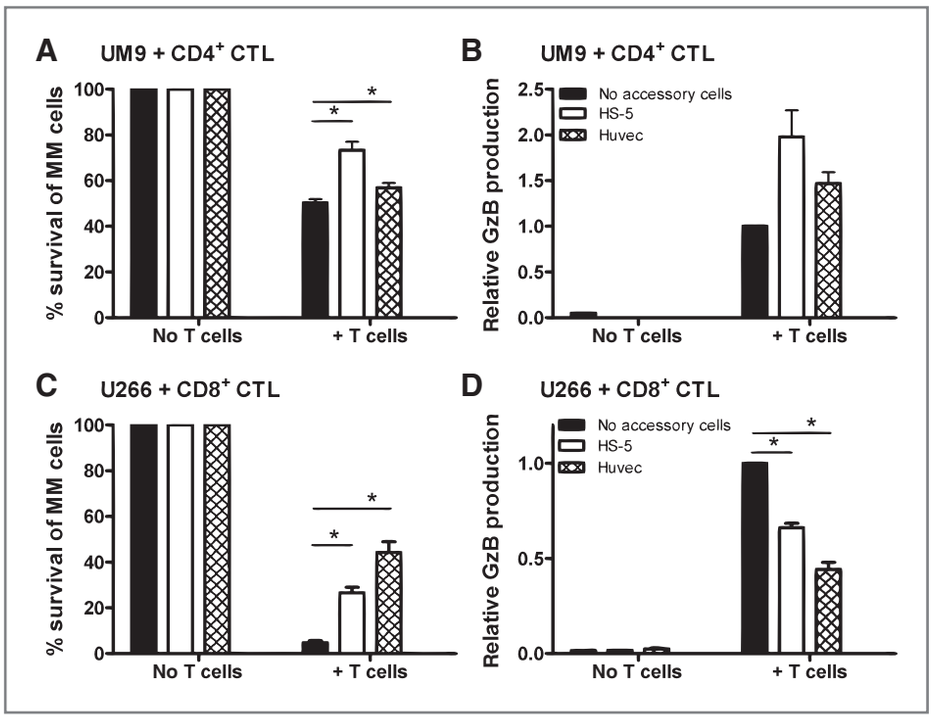


**Figure 3.** Accessory cell–tumor interaction can induce immune suppression as well as immune resistance. Luc$^+$ UM9 and U266 cells were cocultured with the CD4$^+$ CTL (A and B) and CD8$^+$ CTL (C and D), respectively, in the presence or absence of different accessory cells in triplicate (see the legend of Fig. 1 for further details). MM cell viability was determined 24 hours after addition of CTLs using CS-BLI (A and C). GzB production in the supernatant was determined by ELISA and is depicted as relative to GzB production without accessory cells (B and D). Statistical analysis involved unpaired two-tailed t test. *, $P < 0.05$. Results are representative of three independent assays.

not be excluded in these assays. Hence, we addressed this by further analyzing regulators of the apoptotic pathways in both UM9 and U266 upon interaction with accessory cells.

### Fas downregulation as a mechanism of CAM-IR

To gain insight into the mechanisms of CAM-IR in UM9 cells and to evaluate the possible development of immune resistance in U266 cells, we first investigated the expression of Fas and other death cell receptors such as TRAIL receptors 1 and 2 on multiple myeloma cells in the presence and absence of accessory cells. Incubation with HS-5 substantially downregulated Fas expression in U266, and also, but to a lesser extent, in UM9 cells, but TRAIL receptor expression was not reduced (Fig. 4A). Hence, we evaluated downregulation of Fas expression on multiple myeloma cells by accessory cells as a possible mechanism of immune resistance and used the well-known antimyeloma agent bortezomib, which can upregulate Fas in multiple myeloma cells (30). Indeed, bortezomib-treated UM9 and U266 cells upregulated Fas in the absence, but importantly, also in the presence of HS-5 (Fig. 4B). Furthermore, the addition of bortezomib significantly enhanced multiple myeloma cell lysis by CD4$^+$ and CD8$^+$ CTLs not only in the absence, but also in the presence of HS-5 (Fig. 4C). In contrast, bortezomib neither induced Fas expression in the Fas-deficient MM cell line L363, nor did it enhance the CTL-mediated lysis of L363 cells (Supplementary Fig. S5). Furthermore, FasL-blocking antibodies almost completely reverted the bortezomib-induced augmentation of CD8$^+$ CTL-mediated lysis (Fig. 4D). Altogether, these assays indicated Fas downregulation as a mechanism of immune resistance. The action of bortezomib in this system was probably not restricted to Fas modulation only, because the anti FasL antibodies did not completely block bortezomib-induced effects on CD4$^+$ CTL-mediated lysis of multiple myeloma cells (Fig. 4D).

### Downstream mechanisms of CAM-IR in multiple myeloma cells

The experiments with bortezomib indicated Fas downregulation as a possible mechanism of immune resistance, but also implicated the existence of other mechanisms. Therefore, we next investigated whether adhesion of UM9 and U266 to accessory cells altered the expression of inhibitors of apoptosis proteins and of Bcl-2 family proteins, all involved in granzyme- and death receptor-mediated apoptotic pathways used by CTLs (31). Consistent with previous reports (32), we observed a modest increase of survivin and Mcl-1 both in U266 and UM9 cells upon coculture with HS-5 (Fig. 5A). To ascertain the role of these proteins in immune resistance, we used the small-molecule YM155, which represses survivin and Mcl-1 levels (ref 33 and supplementary Fig. S6A). Indeed when we combined YM155 with CTLs in the presence or absence of accessory cells, the CTL-mediated lysis of U266 and UM9 was markedly increased (Fig. 5B) in a synergistic fashion (Supplementary Fig. S6B), whereas there was no influence of YM155 on T-cell activation (Supplementary Fig. S6C). To validate the involvement of survivin in CAM-IR, we suppressed the expression of survivin in UM9 cells by lentiviral short hairpin RNA (shRNA) transduction (Fig. 5C). Indeed, survivin shRNA-treated UM9 cells had a substantially lower capacity to induce

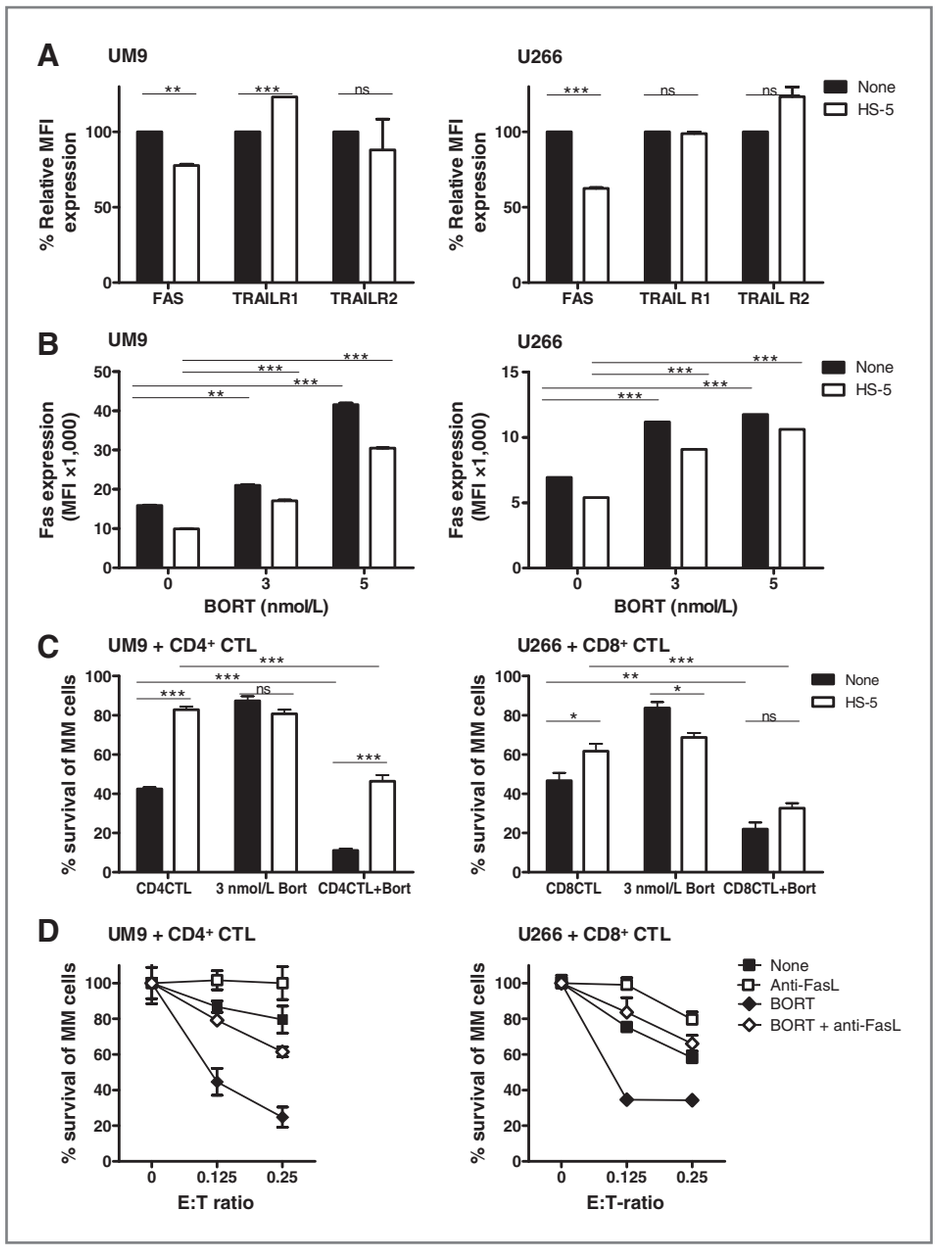


**Figure 4.** Fas downregulation is one of the mechanisms of CAM-IR in MM cells. A, MM cells were cultured in the presence or absence of accessory cells. The Mean fluorescence intensity (MFI) of Fas and TRAIL receptors 1 and 2 was measured using flow cytometry for UM9 (left) and U266 (right) cells in the presence or absence of HS-5. B, Fas expression in UM9 (left) and U266 (right) cells was assessed after a 48-hour coincubation with bortezomib or solvent control in the absence or presence of HS-5. C, % survival of UM9 (left) and U266 (right) cells was determined 48 hours after incubation with 3 nmol/L bortezomib alone, relevant CTLs (E:T = 0.25:1) alone, or the combination of bortezomib and CTLs. D, multiple myeloma cells were treated with 3 nmol/L bortezomib or solvent control in the presence or absence of Fas ligand neutralizing antibodies. Statistical analysis involved two-way ANOVA and Bonferroni *posthoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

CAM-IR (Fig. 5D), confirming the involvement of this antiapoptotic protein in CAM-IR.

### *In vivo* efficacy of CTL and YM155 combination therapy

Because the *in vitro* experiments with YM155 were successful in abrogating CAM-IR, we set out to test this combination therapy in a novel *in vivo* humanized mouse model, in which a human bone marrow microenvironment can be generated by subcutaneous inoculation of ceramic scaffolds coated with human BMSCs (25). Thus, we compared the CTL-mediated lysis of UM9 cells that were either seeded on humanized scaffolds or on uncoated scaffolds. Consistent with the *in vitro* data, T cells completely inhibited the outgrowth of multiple myeloma cells, which were not in the humanized microenvironment. In contrast, the outgrowth of multiple myeloma cells in the humanized scaffolds was not clearly inhibited by T cells (Fig. 6A), confirming the existence of CAM-IR *in vivo*. We next investigated whether this CAM-IR could be abrogated by combination of CTLs with YM155. Mice were thus treated with CTLs alone, YM155 alone, or both CTLs and YM155. Similar to the first experiment, CTLs were unable to eliminate multiple myeloma cells that were implanted in human MSC-coated scaffolds. YM155 therapy alone did also not show a beneficial effect on tumor growth. In striking contrast, however, the YM155 and CTL combination achieved a substantial

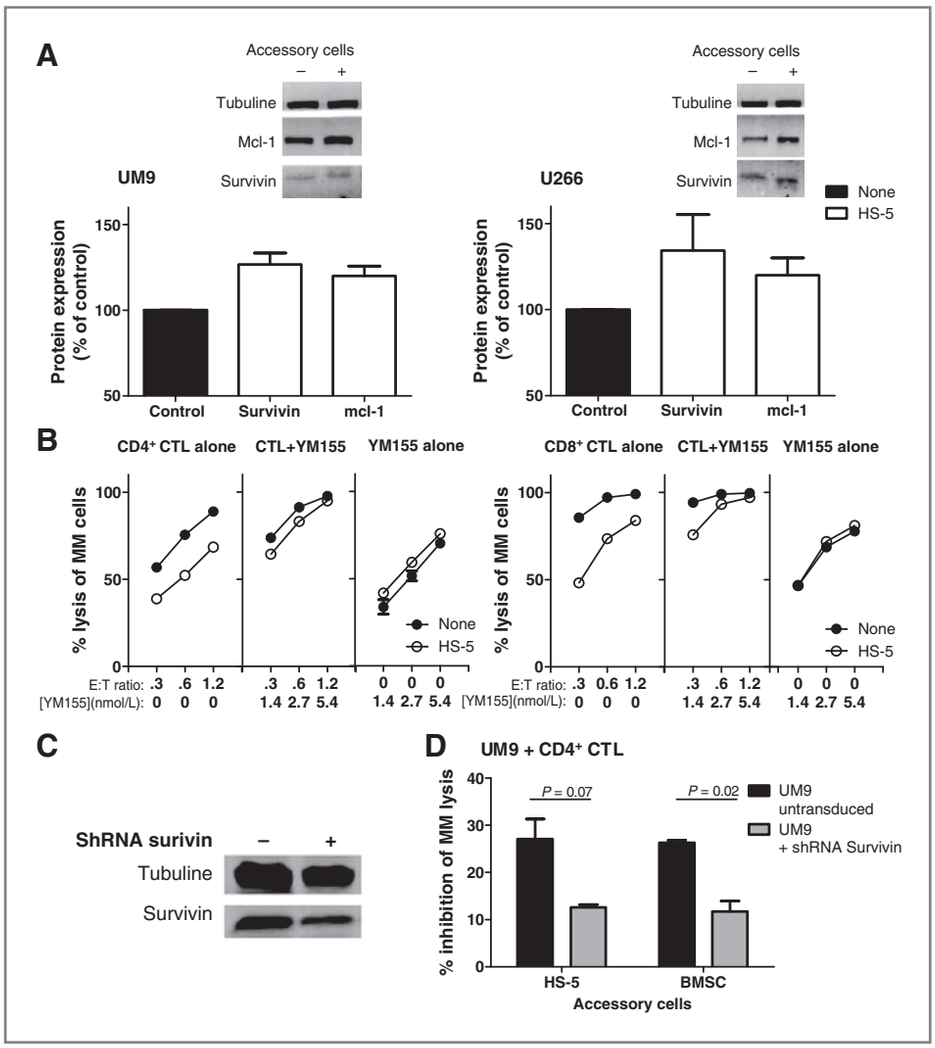


**Figure 5.** YM155 restores sensitivity to T-cell–mediated lysis in a synergistic manner. A, levels of survivin and Mcl-1 expression were visualized by Western blot analysis. Band intensities were quantified using imageJ software. The protein expression in UM9 (left) and U266 (right) cells when adhered to accessory cells are depicted relative to the control (no accessory cells) conditions. Each bar represents the mean ± SEM from three separate experiments. B, MM cell lines UM9 (left) or U266 (right) were cultured in the presence or absence of accessory cells before incubation with CTLs alone, YM155 alone, or the combination. Survival of UM9 and U266 multiple myeloma cells was assessed by CS-BLI. Results show % lysis of multiple myeloma cells after 24-hour incubation and are depicted for three doses. C, UM9 cells were transduced with shRNA for survivin and survivin protein expression was determined by Western blot analysis. D, untransduced and survivin shRNA-transduced UM9 cells were incubated with and without accessory cells and inhibition of lysis in the presence of accessory cells was assessed. Results are expressed as the mean values of triplicate cultures. Results are representative of three independent assays. Error bars represent the SEM.

antitumor effect (Fig. 6B), showing that YM155 can abrogate CAM-IR not only *in vitro*, but also *in vivo*.

## Discussion

T-cell–based immune therapies represent an important strategy for the treatment of multiple myeloma (9–11). However, their success is often hampered by various immune escape mechanisms. Development of clinically applicable strategies to overcome the immune escape is therefore critical to improve the efficacy of cellular immune therapy (34). Despite the well-established concept that the microenvironment is crucially important in the development of resistance to pharmacologic therapies (3, 35), its contribution to immune resistance, that is, rendering multiple myeloma cells resistant to the cytotoxic effect of CTLs, has received little attention until now. In this study, we focused on this question and showed that interaction of multiple myeloma cells with accessory cells of the bone marrow microenvironment can result in the development of a CAM-IR against CTLs.

We show the existence of CAM-IR mainly in an *in vitro* coculture system, originally developed to study BMSC-induced drug resistance (3). Monitoring the survival of luc-transduced multiple myeloma cells by CS-BLI offered a number of advantages over flow cytometry- or $^{51}$Cr-based cytotoxicity assays: First, it enabled us to selectively monitor the survival of multiple myeloma cells without interference of other cell compartments. Second, multiple myeloma cell lysis and T-cell activation could be measured simultaneously, which was essential to discriminate between immune suppression and immune resistance.

To gain insight into accessory cell–multiple myeloma cell interactions on CTL-mediated lysis, we used antigen-specific and HLA-restricted CD8$^+$ and CD4$^+$ CTLs that killed multiple myeloma cells but not the accessory cells. Although this restricted us to a limited number of T-cell clones, we hereby avoided potential bias due to bystander lysis of accessory cells. In this setting, we showed that not only BMSCs, but also vascular endothelial cells are capable of inducing CAM-IR. Although the immune resistance

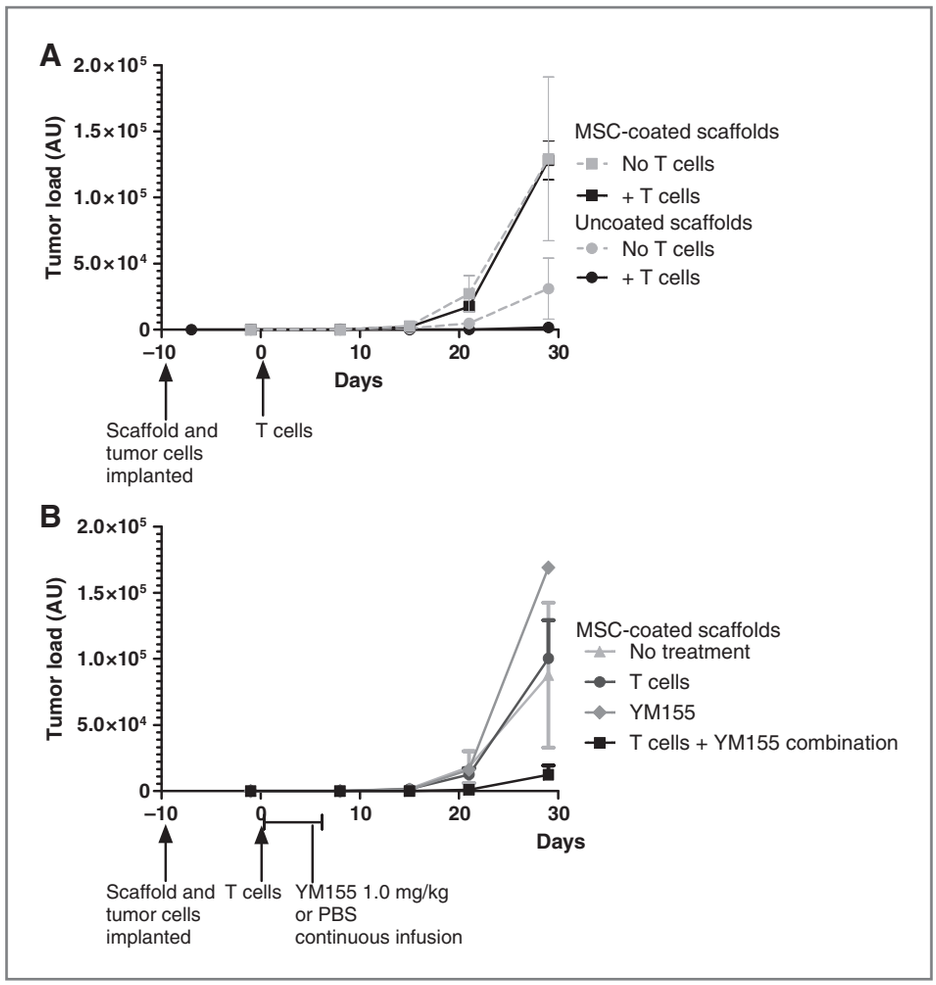


**Figure 6.** *In vivo* antitumor effect of YM155 and CTL combination therapy. In (A) hybrid scaffolds coated with human MSCs and loaded with Luc+ MM cell line UM9, or uncoated scaffolds, which were *in vivo* loaded with tumor cells, were implanted subcutaneously into RAG2$^{-/-}\gamma_C{}^{-/-}$ mice. Ten days after implantation, the scaffolds were injected with $5 \times 10^6$ CD4+ CTLs or left untreated. In (B) groups of mice ($n = 3$) were implanted with human MSC-coated and luc+ MM cell-loaded scaffolds. On day 10, $5 \times 10^6$ CD4$^+$ CTLs were injected directly into the scaffolds ("T cells" and "T cells+YM155 combination" groups), and/or a subcutaneous pump delivering YM155 at a rate 1 mg/kg/d for 5 days ("YM155" and "T cells+YM155" groups) and/or a subcutaneous pump filled with PBS ("no treatment" and "T cells" groups). Tumor load of each individual scaffold was assessed by BLI. Results are expressed as the mean tumor load in each scaffold. The error bars represent the SEM.

induction by BMSCs was in line with our expectations, the involvement of vascular endothelial cells in a similar process was intriguing, because as the main physiologic barrier between tumor cells and circulation, until now vascular endothelial cells have frequently been the subject of other types of studies addressing angiogenesis, migration, and dissemination of multiple myeloma cells (36, 37). Given the high degree of vascularization of medullary and extramedullary multiple myeloma tumors, induction of immune resistance by vascular endothelial cells may represent a hitherto neglected mechanism of immune escape which may require specific modulation toward the development of improved cellular immune therapies. These results also imply that induction of immune resistance is not limited to BMSCs, but may very well be applicable to many different types of accessory cells.

In further analyses, we observed that similar to CAM-DR (38), cell adhesion was an essential and major contributor to the induction of CAM-IR: the immune resistance could be significantly suppressed by inhibiting the adhesive interactions between accessory and multiple myeloma cells with the RGDw peptide, a known blocker of multiple integrins. However, unlike CAM-DR (39), CAM-IR could not be induced by sole binding of multiple myeloma cells to fibronectin, vitronectin, or laminin. Signals initiating CAM-IR are therefore most likely triggered by other receptor-ligand systems than the ones tested here, and require further investigation.

To differentiate immune resistance from immune suppression, we measured granzyme B excretion from and upregulation of FasL on CTLs as T-cell activation markers. Although accessory cells did not influence the activation of CD4$^+$ CTLs in both assays, the granzyme B secretion by the CD8$^+$ CTLs is diminished, indicating suppressed T-cell activation. However, the Fas upregulation was not influenced, indicating a partial or may be a split suppression. More importantly however, even in the presence of this partial T-cell suppressive environment, we could show that CAM-IR can be effectively abrogated with the use of survivin/Mcl-1 inhibitor YM155 (Fig. 5B and Supplementary Fig. S6C). In the light of these results, we postulate that modulation of CAM-IR by itself, even in an immune suppressive microenvironment, may be sufficient to improve the efficacy of cellular immune therapies.

Although the full spectrum of signaling pathways initiating and establishing CAM-IR remains to be characterized by ongoing investigation, our results already indicate that CAM-IR can develop through alterations in the activity of multiple levels of the apoptosis signaling pathways. Downregulation of Fas seems to be one of the mechanisms for CAM-IR. We have shown that this mechanism can be modulated by bortezomib, which significantly augmented the CTL-mediated lysis of multiple myeloma cells even in the presence of accessory cells. Nonetheless, it is notable that bortezomib may modulate multiple, cell-adhesion–mediated or even cell-adhesion–independent mechanisms of immune resistance, as it augmented the CTL-mediated lysis also in the absence of accessory cells. Furthermore, these effects were not completely abrogated by FasL antibodies in all CTL–multiple myeloma combinations we have tested. Indeed in our further exploration, we discovered another, more downstream mechanism of CAM-IR, which could be effectively modulated with the use of the small-molecule YM155, a known inhibitor of survivin and Mcl-1 (33). These antiapoptotic molecules control execution of cellular apoptosis; survivin at the caspase-3 activation level and Mcl-1 at the mitochondrial level (40, 41). We did not investigate the role of Mcl-1 in CAM-IR, but focused on the attribution of survivin. shRNA-mediated downregulation of survivin in UM9 cells attenuated accessory cell-induced resistance.

In conclusion we showed, to our knowledge, the first *in vitro* and *in vivo* evidence suggesting that T-cell–based immunotherapy can be hampered by the multiple myeloma cell interaction with nonmalignant cells of the local microenvironment of multiple myeloma, not only because of immune suppressive mechanisms, but also through a cell–cell contact-mediated immune resistance mechanism. These observations indicate that future preclinical studies on the impact of T-cell immunity against tumor cells should be using assays, *in vitro* and *in vivo*, which incorporate nonmalignant accessory cells of the tumor microenvironment. We also show the feasibility of modulating CAM-IR through a pharmacologic agent. This provides a rationale for further studies to evaluate the feasibility, safety, and efficacy of such CAM-IR modulating agents in combination with immune therapies.

## Disclosure of Potential Conflicts of Interest

M.C. Minnema has honoraria from speakers' bureau from Jansen Cilag. No potential conflicts of interest were disclosed by the other authors.

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## References

1. Mitsiades CS, Mitsiades NS, Richardson PG, Munshi NC, Anderson KC. Multiple myeloma: a prototypic disease model for the characterization and therapeutic targeting of interactions between tumor cells and their local microenvironment. J Cell Biochem 2007;101: 950–68.
2. Noborio-Hatano K, Kikuchi J, Takatoku M, Shimizu R, Wada T, Ueda M, et al. Bortezomib overcomes cell-adhesion-mediated drug resistance through downregulation of VLA-4 expression in multiple myeloma. Oncogene 2009;28:231–42.
3. McMillin DW, Delmore J, Weisberg E, Negri JM, Geer DC, Klippel S, et al. Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. Nat Med 2010;16:483–9.
4. McMillin DW, Delmore J, Negri J, Ooi M, Klippel S, Miduturu CV, et al. Microenvironmental influence on pre-clinical activity of polo-like kinase inhibition in multiple myeloma: implications for clinical translation. PLoS ONE 2011;6:e20226.
5. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. Blood 2009;114:3367–75.
6. Meads MB, Hazlehurst LA, Dalton WS. The bone marrow microenvironment as a tumor sanctuary and contributor to drug resistance. Clin Cancer Res 2008;14:2519–26.
7. Meads MB, Gatenby RA, Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. Nat Rev Cancer 2009;9:665–74.
8. Gorgun G, Calabrese E, Soydan E, Hideshima T, Perrone G, Bandi M, et al. Immunomodulatory effects of lenalidomide and pomalidomide on interaction of tumor and bone marrow accessory cells in multiple myeloma. Blood 2010;116:3227–37.
9. Alyea E, Weller E, Schlossman R, Canning C, Webb I, Doss D, et al. T-cell–depleted allogeneic bone marrow transplantation followed by donor lymphocyte infusion in patients with multiple myeloma: induction of graft-versus-myeloma effect. Blood 2001;98:934–9.
10. Lokhorst HM, Wu K, Verdonck LF, Laterveer LL, van de Donk NW, van Oers MH, et al. The occurrence of graft-versus-host disease is the major predictive factor for response to donor lymphocyte infusions in multiple myeloma. Blood 2004;103:4362–4.
11. Verdonck LF, Lokhorst HM, Dekker AW, Nieuwenhuis HK, Petersen EJ. Graft-versus-myeloma effect in two cases. Lancet 1996;347:800–1.
12. Salama M, Nevill T, Marcellus D, Parker P, Johnson M, Kirk A, et al. Donor leukocyte infusions for multiple myeloma. Bone Marrow Transplant 2000;26:1179–84.
13. Corradini P, Cavo M, Lokhorst H, Martinelli G, Terragna C, Majolino I, et al. Molecular remission after myeloablative allogeneic stem cell

transplantation predicts a better relapse-free survival in patients with multiple myeloma. Blood 2003;102:1927–9.
14. Gajewski TF, Meng Y, Harlin H. Immune suppression in the tumor microenvironment. J Immunother 2006;29:233–40.
15. Jacobs JF, Nierkens S, Figdor CG, de Vries IJ, Adema GJ. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? Lancet Oncol 2012;13:e32–e42.
16. Chouaib S, Meslin F, Thiery J, Mami-Chouaib F. Tumor resistance to specific lysis: a major hurdle for successful immunotherapy of cancer. Clin Immunol 2009;130:34–40.
17. Begley J, Ribas A. Targeted therapies to improve tumor immunotherapy. Clin Cancer Res 2008;14:4385–91.
18. Holloway PA, Kaldenhoven N, Kok-Schoemaker HM, Dijk M, Otten HG, Tilanus M, et al. A class II-restricted cytotoxic T-cell clone recognizes a human minor histocompatibility antigen with a restricted tissue distribution. Br J Haematol 2005;128:73–81.
19. Spaapen R, van den OK, Ivanov R, Bloem A, Lokhorst H, Mutis T. Rebuilding human leukocyte antigen class II-restricted minor histocompatibility antigen specificity in recall antigen-specific T cells by adoptive T cell receptor transfer: implications for adoptive immunotherapy. Clin Cancer Res 2007;13:4009–15.
20. den Haan JM, Meadows LM, Wang W, Pool J, Blokland E, Bishop TL, et al. The minor histocompatibility antigen HA-1: a diallelic gene with a single amino acid polymorphism. Science 1998;279:1054–7.
21. Holloway PA, Kaldenhoven N, van DM, Bloem AC, de LW, van der ZR, et al. Susceptibility of malignant plasma cells to HA-1(H) specific lysis suggests a role for the minor histocompatibility antigen HA-1 in the graft-versus-myeloma effect. Leukemia 2004;18:1543–5.
22. Hantgan RR, Endenburg SC, Sixma JJ, de Groot PG. Evidence that fibrin alpha-chain RGDX sequences are not required for platelet adhesion in flowing whole blood. Blood 1995;86:1001–9.
23. Rothen-Rutishauser BM, Kiama SG, Gehr P. A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol 2005;32:281–9.
24. Takagi M, Horii K, Yoshida T. Effect of pore diameter of a porous membrane on progenitor cell content during membrane-separated coculture of hematopoietic cells and a stromal cell line. J Artif Organs 2003;6:130–7.
25. Groen RW, Noort WA, Raymakers RA, Prins HJ, Aalders L, Hofhuis FM, et al. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. Blood 2012;120:e9–e16.
26. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58:621–81.
27. Ruoslahti E. RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 1996;12:697–715.
28. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002;2:735–47.
29. Berke G. The CTL's kiss of death. Cell 1995;81:9–12.
30. Hallett WH, Ames E, Motarjemi M, Barao I, Shanker A, Tamang DL, et al. Sensitization of tumor cells to NK cell-mediated killing by proteasome inhibition. J Immunol 2008;180:163–70.
31. Sutton VR, Davis JE, Cancilla M, Johnstone RW, Ruefli AA, Sedelies K, et al. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. J Exp Med 2000;192:1403–14.
32. Wang X, Zhang Z, Yao C. Survivin is upregulated in myeloma cell lines cocultured with mesenchymal stem cells. Leuk Res 2010;34:1325–9.
33. Tang H, Shao H, Yu C, Hou J. Mcl-1 downregulation by YM155 contributes to its synergistic anti-tumor activities with ABT-263. Biochem Pharmacol 2011;82:1066–72.
34. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol 2012;12:269–81.
35. Hazlehurst LA, Landowski TH, Dalton WS. Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death. Oncogene 2003;22:7396–402.
36. Jakob C, Sterz J, Zavrski I, Heider U, Kleeberg L, Fleissner C, et al. Angiogenesis in multiple myeloma. Eur J Cancer 2006;42:1581–90.
37. Ribatti D, Nico B, Vacca A. Importance of the bone marrow microenvironment in inducing the angiogenic response in multiple myeloma. Oncogene 2006;25:4257–66.
38. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. Leukemia 2009;23:10–24.
39. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood 1999;93:1658–67.
40. Mace PD, Shirley S, Day CL. Assembling the building blocks: structure and function of inhibitor of apoptosis proteins. Cell Death Differ 2010;17:46–53.
41. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008;9:47–59.