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TCR Mimic Monoclonal Antibodies Induce Apoptosis of Tumor Cells via Immune Effector-Independent Mechanisms

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mAbs that recognize peptides presented on the cell surface by MHC class I molecules are potential therapeutic agents for cancer therapy. We have previously demonstrated that these Abs, which we termed TCR mimic mAbs (TCRm), reduce tumor growth in models of breast carcinoma. However, mechanisms of TCRm-mediated tumor growth reduction remain largely unknown. In this study, we report that these Abs, in contrast to several mAbs used currently in the clinic, destroy tumor cells independently of immune effector mechanisms such as Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). We found that TCRm-mediated apoptosis of tumor cells was associated with selective and specific binding of these Abs to peptide/HLA class I complexes, which triggered the activation of JNK and intrinsic caspase pathways. This signaling was accompanied by the release of mitochondrial cytochrome c and apoptosis-inducing factor. TCRm-induced apoptosis in tumor cells was completely inhibited by soluble MHC tetramers loaded with relevant peptide as well as with inhibitors for JNK and caspases. Furthermore, mAbs targeting MHC class I, independent of the peptide bound by HLA, did not stimulate apoptosis, suggesting that the Ab-binding site on the MHC/peptide complex determines cytotoxicity. This study suggests the existence of mechanisms, in addition to ADCC and CDC, through which these therapeutic Abs destroy tumor cells. These mechanisms would appear to be of particular importance in severely immunocompromised patients with advanced neoplastic disease, since immune cell-mediated killing of tumor cells through ADCC and CDC is substantially limited in these individuals.

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Several mAbs have been successfully implemented for anticancer immunotherapy (1). However, the use of this approach for the treatment of cancer patients is limited by the scarcity of well-characterized antigenic epitopes that are exclusively expressed on the surface of tumor cells, as ideal therapeutic mAbs should bind to and destroy exclusively the neoplastic tissue. This hurdle can be overcome by extending the search of potential targets for therapeutic Abs to intracellular proteins (2–4). This approach increases the probability of finding molecules that are exclusively expressed in tumor cells. Intact intracellular proteins are shielded from the immune system recognition; however, peptides originating from these proteins are presented on the surface of all nucleated cells, including tumor cells, by MHC class I molecules. Thus, mAbs that recognize these complexes can become a new class of therapeutics for targeting tumor cells (5–7). We termed these Abs TCR mimic mAbs (TCRm), as T cells, similar to TCRm, recognize peptides presented by MHC class I molecules through the TCR engagement.

We have recently identified several MHC/peptide complexes that are exclusively expressed on human cancer cells. Therefore, they represent promising targets for the development of therapeutic Abs (8). Furthermore, we have designed two TCRm, RL4B and RL6A, with potent anti-tumor activity in vivo (9, 10). The RL4B is specific for the complex formed by peptide GVLPALPQV derived from human chorionic gonadotropin (hCG)-β and HLA-A*0201 (9). The RL6A recognizes peptide YLLPAIVHI from human p68 RNA helicase and presented by HLA-A*0201 (10). Although it has been shown that these mAbs induce complement-dependent cytotoxicity (CDC) and Ab-dependent cellular cytotoxicity (ADCC) in vitro (5, 10), it remains to be elucidated how these Abs kill tumor cells in vivo.

In this report, we provide evidence that TCRm also destroy tumor cells in xenograft models of breast cancer through mechanisms that are partially independent from ADCC and CDC. We found that specific and selective binding of these Abs to complexes formed by HLA-A*0201 and peptides derived either from hCG-β or human p68 RNA helicase induced tumor cell apoptosis, thereby reducing growth of established tumors. Interestingly, TCRm bound to peptide/HLA complexes were rapidly internalized through endocytosis. The binding and internalization were associated with the activation of JNK and the initiation of the intrinsic caspase-dependent death pathway.

Materials and Methods

Cell lines

The human breast carcinoma cell line MDA-MB-231 and the human acute monocytic leukemia cell line THP-1 were obtained from the American Type Culture Collection (Manassas, VA). The human melanoma cell lines 1520...
volumes were additional weeks. Mice were euthanized at end of the study or when tumor isotype IgG2a for 4 wk. Tumor volume was measured twice a week for 4

were evaluated by flow cytometry (FACSCanto II; BD Biosciences, San

were administrated i.p. with 366 g/ml RL4B or 366 g/ml F(ab’2) RL6A fragments and tumor volumes were evaluated. Additional doses of these Abs were injected on days 7 and day 14. Tumor volumes were calculated by using the following formula: volume = (L × 5/6)2, where L indicates the longest diameter and b the shortest diameter.

Aptosis assays

To determine levels of apoptosis in tumors harvested from mice, a TUNEL assay was performed in 5-μm-thick frozen sections of tumors using an in situ apoptosis detection kit (FragEL DNA fragmentation detection kit; Calbiochem, Gibbstown, NJ) according to the manufacturer’s instructions. Fractions of apoptotic MDA-MB-231, 1520, or 501A cells after treatment with 50–1000 ng/ml RL4B, RL6A, BB7.2, W6/32, or isotype control Ab were evaluated by flow cytometry (FACSCanto II; BD Biosciences, San

Jose, CA) in cells stained with annexin V and propidium iodide (PI) according to the manufacturer’s instructions (eBioscience, San Diego, CA). Cells treated with 10 μM camptothecin (cpt; Sigma-Aldrich) served as a positive control. CaspACE FITC-VAD-FMK in sit mark (Promega, Madison, WI) was used to determine the activation of caspases in MDA-MB-231, THP-1, 1520, and 501A tumor cells according to the manufacturer’s instruction. THP-1 cells were pulsed with YLL peptide (20 μM) for 4 h, washed, and then incubated with 1 μg/ml RL6A, RL21A, or isotype control mAb for 6 h prior to assaying for pan-caspase activation. Five micromolar CaspACE FITC-VAD-FMK was added to MDA-MB-231 cells prior to their incubation with TCRm, isotype control mAb, or cpt to inhibit activation of caspases. Caspase-3/7, caspase-8, and caspase-9 activities in the cleared cell lysates were measured using Caspase-Glo 3/7, Caspase-Glo 8, and Caspase-Glo 9 assays (Promega) respectively, according to the manufacturer’s instructions. Inhibition of caspase activity was determined using an ELISA reader (Synergy HT multi-mode microplate reader; BioTek, Winooski, VT). For inhibition experiments, cells were pretreated with 2 μM Z-DEVd-fmk (caspase-3/7 inhibitor) or 2 μM Z-LEHD-fmk (caspase-9 inhibitor) for 50 min (BioVision, San Francisco, CA). Cytochrome c released to cytoplasm of MDA-MB-231 cells was detected with FITC-conjugated human cytochrome c Ab (1 μg/ml) (eBioscience). Intracellular staining was quantified by flow cytometry (FACSCanto II). The expression of apoptosis-inducing factor (AIF)-1 was detected using AIF mouse mAb at 1 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) and flow cytometry analysis (FACSCanto II).

Cell binding/uptake studies and confocal microscopy

MDA-MB-231 cells were cultured on collagen-coated coverslips in 12-well plates. Cells were incubated with 10 μg/ml Alexa Fluor 546-labeled RL6A or RL6A IgG2a isotype control Ab diluted in fresh culture medium with 3% FBS for varying periods of time (15, 30, 60 min) at 37°C. For these studies, RL6A and IgG2a isotype control Abs (UPC10) were labeled with Alexa Fluor 546 using a mAb labeling kit following the manufacturer’s instructions (Invitrogen/Molecular Probes, Carlsbad, CA). After incubation with Abs, cells were fixed in 4% paraformaldehyde. For colocalization studies with early endosome Ag (EEA1)-1, the fixed cells were incubated with 1% BSA for 30 min. washed, and then incubated with 0.4 μg/ml goat polyclonal EEA1 Ab (Santa Cruz Biotechnology) for 60 min at room temperature. The bound Abs were detected with Alexa Fluor 488 chicken anti-goat IgG (Invitrogen/Molecular Probes). DRAQ5 dye (Bio-status, Shepshed, U.K.) was used to visualize cell nuclei.

Immunofluorescent staining was evaluated using a Leica TCS-SP2 spectral confocal scanner (Leica Microsystems, Heidelberg, Germany) with krypton-argon, green and red helium-neon lasers on a Leica IRE2 inverted microscope equipped with a Leica ×63 oil lens (NA 1.4). Images were acquired with identical settings at a resolution of 512 × 512 pixels and 8-bit channel depth. The original TIFF images were assembled in Adobe Photoshop CS3, and the brightness of all fluorescence images was concomitantly adjusted for accurate reproduction.

Cell viability assay

WST-1 (Roche, Indianapolis, IN), a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in viable cells, was used according to the manufacturer’s instructions to determine cell viability and cytotoxicity of TCRm.

Poly(ADP-ribose) polymerase activation assay

For detection of cleaved poly(ADP-ribose) polymerase (PARP), MDA-MB-231 cells untreated or treated with TCRm or control Ab were fixed in 4% formaldehyde, permeabilized, incubated with anti-cleaved PARP polyclonal rabbit Ab (1:2000 dilution; Thermo Scientific, Rockford, IL) and anti-rabbit-HRP-conjugated IgG followed by addition of substrate (TMB), and read at an absorbance of 450 nm using a Synergy HT multi-mode microplate reader.

JNK activation assay

Phospho–stress-activated protein kinase/JNK (Thr 183/Tyr 185) (G9) mouse mAb (Cell Signaling Technology, Boston, MA) was used to detect activated JNK in permeabilized tumor cells according to the manufacturer’s instructions. Intracellular staining was quantified by flow cytometry (FACSCanto II).

JNK inhibition studies

Inhibition of JNK activity was achieved using c-Jun N-terminal Kinase Inhibitor II (SP600125) (Calbiochem). The JNK inhibitor II blocks downstream activity of activated JNK I, II, and III. The optimal con-
centration of inhibitor for our studies was established to be 50 μM. The inhibitor at this concentration was added to tumor cells (10^5 cells/well) in 12-well plate cultures 30 min prior to the addition of either RL4B or RL6A, or isotype control mAb or ctrl. After 4 h incubation with Abs or ctrl, cells were stained with annexin V and PI and analyzed by flow cytometry (FACS Canto II).

**Generation of F(αβ)^2 fragments from whole RL4B TCRm**

RL4B TCRm was diazylzed against digestion buffer (20 mM sodium acetate trihydrate). Immobilized pepsin was equilibrated by suspending in digestion buffer. The dialyzed RL4B was mixed with equilibrated immobilized pepsin (Pierce) and incubated for 4 h at 37°C, and the resin separator was placed inside the reaction tube just above the mixture. Using the resin separator the crude digest was decanted into another tube. F(αβ)^2 fragment purification was performed using an immobilized protein A column. Dialysis was performed on the purified fragment with 50 KDa molecular mass cut-off to remove Fc fragments not captured by protein A. Generation of F(αβ)^2 fragments was confirmed by SDS-PAGE gel. The binding activity of F(αβ)^2 fragments was determined by ELISA with the use of peptide tetramer (GVL/A2) or irrelevant peptide tetramer (KVL/A2) as a control. Ab specific for the Fc domain of mouse Ab was used as an additional control to demonstrate the complete removal of the Fc domain. Purity of F(αβ)^2 was estimated to be >95%.

**Statistical analysis**

In vitro results were expressed as SEM, and an unpaired Student t test, or one-way ANOVA was performed followed by Bonferroni posttest to determine significance of differences among the groups. A value of p ≤ 0.05 was considered significant. In vivo results are presented as SEM. Two-way ANOVA followed by Bonferroni posttest was used to determine significance of differences between groups using GraphPad Prism (GraphPad Software, La Jolla, CA).

**Results**

**TCRm reduce tumor growth by inducing apoptosis of tumor cells**

We have previously demonstrated that RL4B and RL6A reduce tumor growth in xenograft models of breast carcinoma (9, 10). However, the mechanisms that are responsible for this tumor growth reduction remain unclear. In accordance with our previous reports, we found that RL6A significantly reduced growth of established tumors in a xenograft model of breast carcinoma (Fig. 1A). Furthermore, administration of RL4B or RL6A Ab to mice with breast tumors improved overall survival when compared with mice that received only control IgG (Fig. 1B). The reduced tumor growth and improved survival in mice treated with TCRm were due to increased tumor cell death. Tumors from mice treated with these Abs showed enhanced apoptosis when compared with mice not treated or treated with control IgG, as demonstrated by morphological features of apoptosis such as condensation of nuclear chromatin, cell shrinkage, and apoptotic bodies (Fig. 1C). Similar results were obtained when sections were stained with TUNEL (Fig. 1C). Interestingly, tumor tissues from mice representing all experimental groups were lacking evident inflammatory infiltrate (Fig. 1C). The lack of infiltrating leukocytes was additionally confirmed by negative immunohistochemical staining for surface markers of NK cells and macrophages (data not shown). The lack of infiltrating NK cells and macrophages suggests that tumor cell death induced by TCRm was, at least partially, independent from ADCC and CDC, since NK cells and macrophages are effector cells in both mechanisms of Ab-mediated killing.

**F(αβ)^2 fragments of RL4B inhibit tumor growth**

To confirm that TCRm kill tumor cells through mechanisms independent from ADCC and CDC, nude mice inoculated with BT-20/A2 tumor cells were treated with RL4B F(αβ)^2 fragments, which do not contain the Fc fragment and consequently do not activate ADCC and CDC. This treatment resulted in the significant reduction of tumor growth when compared with mice that were administrated with control IgG2a F(αβ)^2 (Fig. 1D). However, the effect of RL4B F(αβ)^2 fragments on tumor growth was less pronounced than that observed when mice received whole RL4B (Fig. 1D). The reduced tumor growth in mice treated with RL4B F(αβ)^2 fragments indicates that tumor cell killing induced by TCRm is mediated also by mechanisms other than ADCC and CDC. Nonetheless, because whole RL4B was more efficient in reducing tumor growth, it is likely that ADCC and CDC contribute to its therapeutic effect as well.

**TCRm directly reduce tumor cell viability via activation of apoptosis**

Our in vivo studies suggested that TCRm induce apoptosis of tumor cells through mechanisms that are, at least partially, independent from ADCC and CDC. To confirm that TCRm induce tumor cell apoptosis directly without the contribution of immune cells that are involved in ADCC and CDC, we tested cytotoxic properties of TCRm in isolated in vitro settings. We found that the incubation of either RL4B or RL6A with human breast cancer cells, MDA-MB-231 (HLA-A2+/hCG-β^+^/p68^+^), or the human melanoma cells, 1520 (HLA-A2^+/hCG-β^+^/p68^+^), for 24 h resulted in a significant decrease in cell viability (Fig. 2). Cytotoxic effects of TCRm were dose-dependent with regard to both cell lines (Fig. 2), with RL4B concentrations showing almost linear inverse correlation with cell viability (Fig. 2A, 2B). Further increase in TCRm concentration did not reduce cell viability (data not shown). Specificity of TCRm in inducing cell death was confirmed by the lack of the effect of control IgG on tumor cell viability. Additionally, TCRm did not decrease the viability of BT-20 tumor cells (data not shown), which are HLA-A2^+^, confirming that the cytotoxic effect of TCRm is specifically associated with the binding of these Abs to complexes formed by peptides and HLA-A2 molecules.

Tumor cells in mice treated with TCRm died through apoptosis. We hypothesize that the similar mode of cell death is responsible for decreased viability of MDA-MB-231 cells treated with these mAbs in vitro. This hypothesis was confirmed by increased numbers of MDA-MB-231 cells that bound annexin V (early apoptosis) or bound annexin V and incorporated PI (late apoptosis) after the incubation with TCRm in comparison with MDA-MB-231 cells that were treated with control IgG (Fig. 3A, 3B). Importantly, apoptosis induced by 1 μg/ml RL6A was comparable to apoptosis induced by the treatment with 10 μM cpt, the analogs of which are currently approved for cancer chemotherapy. The degrees of apoptosis induced by both mAbs were dose-dependent, reaching their maxima at 1 μg/ml concentration (Supplemental Fig. 1A), which corresponded to the lowest viability of MDA-MB-231 cells (Fig. 2). Further increases in concentrations of Abs did not result in increased apoptosis (data not shown). The maximum apoptosis for both Abs was seen after 4 h incubation, and further increase of the incubation time did not correspond to the increased degree of apoptosis (Supplemental Fig. 1B).

Interestingly, RL6A-induced apoptosis was found to be consistently greater than apoptosis caused by RL4B treatment. The better efficacy of RL6A in the induction of MDA-MB-231 cell apoptosis might be attributable to the almost 4-fold higher binding affinity of RL6A (K_d, 4.0 × 10^{-10} M) compared with RL4B (K_d, 1.5 × 10^{-9} M). We also found that RL4B and RL6A induced apoptosis of melanoma cells 1520 and 501A (Supplemental Fig. 1C), suggesting that cytotoxic effects of TCRm are not limited to one type of cancer but are rather a universal phenomenon induced by the ligation of specific MHC class I peptide complexes.

To determine whether induction of apoptosis via MHC class I signaling was restricted to tumor cells, leukocytes isolated from
FIGURE 1. TCRm improve mouse survival and reduce tumor growth by inducing tumor cell apoptosis in an orthotopic breast cancer model. A, Tumor volumes of mice treated with RL6A or isotype control mAb measured weekly after injection of MDA-MB-231 cells. *p < 0.05; two-way ANOVA. Data are representative of one experiment with n = 7 mice/group. Error bars represent SEM. B, Survival curves of mice inoculated with MDA-MB-231 cells and treated with RL4B, RL6A, or isotype control. *p < 0.05; Kaplan–Meier survival curves and χ² test. Data are representative of one experiment with n = 7 mice/group. Error bars represent SEM. C, H&E and TUNEL staining of tumor sections obtained from mice injected with MDA-MB-231 cells and administered PBS, isotype control, RL4B, or RL6A. Tumor tissue was harvested 9 wk after tumor cell inoculation. Data are representative of one experiment with n = 7 mice/group. Original magnification ×40. D, Tumor volumes of mice treated with RL4B, RL4B F(ab')2, or IgG2a F(ab')2 fragments measured on various days after BT-20/A2 tumor cell inoculation. *p < 0.05; Student t test, unpaired. Data are representative of one experiment with n = 10 mice/group.
HLA-A*0201 donors were pulsed with an unrelated peptide or with the peptide specifically recognized by each TCRm. Cells were incubated with RL4B, RL6A, or control IgG at 1 μg/ml concentration and the degree of apoptosis was evaluated. Neither RL4B nor RL6A TCRm induced cell death of unpulsed leukocytes or leukocytes pulsed with nonrelated peptide or the specific peptide recognized by each TCRm (data not shown). TCRm did not induce apoptosis in normal fibroblast and epithelial cells pulsed with specific peptide either (data not shown). Although the TCRm recognized MHC/peptide target on relevant peptide-pulsed cells, cell death was not observed, demonstrating significant differences in MHC class I/peptide signaling pathways between normal and diseased cells.

The induction of apoptosis by TCRm requires their specific binding to MHC/peptide complexes

TCRm recognize and bind to MHC class I/peptide complexes. Thus, it is likely that cytotoxic properties of these Abs are specifically dependent on their interactions with these complexes. However, it is possible that apoptosis of tumor cells mediated by TCRm is related to their interactions with other portions of MHC, including those outside the peptide groove. To test this possibility MDA-MB-231 tumor cells were incubated for 4 h with 1 μg/ml either pan MHC class I (W6/32) or anti-HLA-A2 (BB7.2) Abs. The W6/32 and BB7.2 mAbs bind to the α2/3 domain and α2 domain of HLA-A2 class I molecules, respectively (12, 13). We chose this dose and this time of incubation with these Abs, because TCRm were most efficient in inducing MDA-MB-231 cell apoptosis in this experimental setting. Both treatments did not induce an increase in apoptosis of tumor cells when compared with control IgG treatment (Supplemental Fig. 2A,2B). Even when used at 10-fold higher concentrations, neither mAb induced apoptosis of tumor cells (data not shown). These results suggest that the specific interaction between MHC class I (HLA-A2)/peptide complexes and TCRm is required for them to initiate apoptosis of tumor cells, and, in particular, TCRm docking orientation seems to be critical in this process. This hypothesis was
confirmed by the results of a competition assay utilizing soluble tetramers loaded with relevant GVL or YLL peptides or with irrelevant peptide. These data showed that the induction of apoptosis by both TCRm was significantly inhibited only in the presence of relevant peptide tetramer (Fig. 3C, 3D).

**TCRm-specific binding to MHC/peptide complexes on tumor cells leads to activation of caspases**

To demonstrate that TCRm binding to tumor cells activates signaling pathways that ultimately lead to apoptosis, we examined the activation of caspases in various tumor cells incubated with RL4B or RL6A. Both Abs induced increased caspase activity in all cell types examined, as demonstrated by the increased accumulation of FITC-conjugated Z-VAD-fmk in tumor cells treated with TCRm, when compared with control IgG-treated cells (Fig. 4A). Z-VAD-fmk is a pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases. Thus, the degree of its accumulation within cells corresponds to overall activity of caspases. To confirm that TCRm-mediated caspase activation leads to apoptosis of tumor cells, the pan-caspase inhibitor Z-VAD-fmk was added to MDA-MB-231 cells prior their incubation with RL4B or RL6A. The blockade of caspases by this inhibitor almost completely abrogated TCRm-induced apoptosis (Fig. 4B).

To determine whether the TCRm-induced caspase activity was specific, THP-1 cells (HLA-A2* and p658) (10) were pulsed with different concentrations (5–50 μM) of relevant peptide YLLPAIHVI. RL6A binds to THP-1 cells pulsed with YLLPAIHVI (relevant) peptide, with the brightest staining being observed for THP-1 cells pulsed with 50 μM YLL peptide. In contrast, no staining of irrelevant peptide-pulsed THP-1 cells was observed after their incubation with RL6A (Supplemental Fig. 3). Furthermore, RL6A specifically induced pan-caspase activation in YLL peptide-pulsed THP-1 cells, whereas mouse TCRm (RL21A-IgG2a) or IgG2a isotype control did not activate pan-caspases in these cells (Fig. 4C). Additionally, pan-caspase activity was not observed in unpulsed THP-1 cells incubated with RL6A, RL21A, or IgG2a isotype control (Fig. 4C). Thus, RL6A binds only to cells that present the specific YLL peptide in the context of HLA-A2, and this binding induces activation of caspases in these cells.

Because caspase-3 and caspase-7 are known as key executors of apoptosis and all caspase-dependent pathways of apoptosis converge at these enzymes, we examined their activities in MDA-MB-231 cells treated with TCRm. We found that caspase-3/7 activity was higher in cells treated with these Abs when compared with cells incubated with control IgG (Fig. 5A). The specificity of these findings was confirmed through the blockade of caspase-3/7 activity by the specific caspase-3/7 inhibitor Z-DEVD-fmk in cells treated with TCRm. Furthermore, we found the accumulation of PARP cleavage fragments in MDA-MB-231 cells treated with these Abs (Fig. 5B). Because PARP is a main downstream target for activated caspase-3/7 and contributes to maintaining cellular viability, these data strengthen the hypothesis that TCRm induce caspase-dependent apoptosis of tumor cells.

**TCRm induce tumor apoptosis through the intrinsic caspase-dependent pathway**

MHC class I molecules do not contain death domains or an extensive cytoplasmic tail. Additionally, the short cytoplasmic domain of the MHC molecule seems not to be essential for intracellular signaling (14). Thus, the involvement of the extrinsic caspase-dependent apoptosis pathway in TCRm-induced cell death appeared unlikely. Therefore, we examined the activation of caspase-9 in MDA-MB-231 tumor cells treated with TCRm, as this caspase is involved in an intrinsic caspase-dependent apoptosis. This treatment resulted in higher caspase-9 activity when compared with untreated and control IgG-treated cells (Fig. 6). RL4B-induced caspase-9 activation was completely inhibited in the presence of the specific caspase-9 inhibitor Z-LEHD-fmk, whereas RL6A-mediated caspase-9 activation was partially reduced by this inhibitor (Fig. 6). The specific activation of caspase-9 demonstrated by these results, as well as the lack of caspase-8 and caspase-6 activation (data not shown), points to the intrinsic apoptosis pathway as a mechanism of cell death triggered by TCRm.

**TCRm-triggered intrinsic apoptosis pathway is associated with the mitochondrial membrane damage**

Cytochrome c release from damaged mitochondria is a very early event in the intrinsic apoptosis pathway and contributes to caspase-9 activation. Therefore, we explored if this molecular mechanism contributes to apoptosis initiated by TCRm. We found that the treatment of MDA-MB-231 tumor cells with RL4B or RL6A resulted in the accumulation of cytochrome C in their cytoplasm (Fig. 7A). In addition, we identified AIF-1 in the cytoplasm of these cells (Fig. 7B). The cytoplasmic localization of AIF-1 is also a result of mitochondrial membrane damage, indicating that mitochondrial damage contributes to apoptosis initiated in tumor cells by TCRm. It is known that AIF-1 translocated into the nucleus carries out the degradation of large DNA fragments through caspase-independent mechanisms (15). Thus, these data suggest that signaling via the MHC/peptide pathway could initiate both caspase-dependent and caspase-independent tumor cell death.

**MHC class I signaling initiated by TCRm triggers JNK activation and caspase-dependent apoptosis of tumor cells**

The binding of mAbs to β2-microglobulin within MHC class I complexes induces apoptosis of tumor cells through the activation of JNK (16, 17). JNK activation leads also to apoptosis of human T cells after ligation of MHC class I molecules on their surfaces (18). Additionally, the activation of JNK in tumor cells results in cell death through a number of well-characterized pathways, including damage to the mitochondrial membrane associated with the release of cytochrome c into the cytoplasm (19, 20). Therefore, we hypothesized that TCRm trigger JNK activation in tumor cells after binding to MHC class I/peptide complexes. RL4B and RL6A induced JNK activation, as demonstrated by stress-activated protein kinase/JNK phosphorylation in MDA-MB-231 (Fig. 8A), 1520, and 501A tumor cells (data not shown). The competition assay with use of peptide-loaded tetramers showed that JNK activation was inhibited when mAbs binding to MHC class I/peptide complexes was blocked by tetramers loaded with relevant peptide (Fig. 8B).

To demonstrate that JNK activation induced by TCRm causes tumor cell apoptosis, MDA-MB-231 cells were incubated with TCRm in the presence of the JNK inhibitor SP600125. We found that apoptosis and caspase activation mediated by TCRm was significantly reduced by this inhibitor (Fig. 8C, 8D). These results confirm functional involvement of JNK in TCRm-induced apoptosis of tumor cells.

**TCRm are internalized by tumor cells through endocytosis**

Because mAbs targeting β2-microglobulin recruited MHC class I molecules to lipid rafts (21), we hypothesized that similar mechanisms are associated with binding of TCRm to their targets. To verify this hypothesis, we examined the cellular distribution of complexes that were composed of TCRm and MHC class I molecules.
TCRm induces the activation of pan-caspases in tumor cells. A. Pan-caspase activation induced after 4 h incubation with 1 μg/ml RL4B (top panel) or RL6A (bottom panel) in MDA-MB-231, 1520, and 501A cells. Pan-caspase activation induced by TCRm was compared with activation induced by 1 μg/ml control IgG2a or 10 μM cpt. B. RL4B (left panel), RL6A (right panel), control IgG2a, or cpt-induced apoptosis of MDA-MB-231 cells in the presence or absence of Z-VAD-fmk inhibitor. Apoptosis induced by various treatments was compared with apoptosis of untreated cells (cells). Apoptotic indexes were obtained by combining frequencies of cells stained with annexin V alone with frequencies of cells stained with both annexin V and PI. C. Pan-caspase activation in YLL pulsed (20μM) or unpulsed THP-1 cells after 6 h incubation with 1 μg/ml RL6A or control Abs RL21A TCRm (mouse IgG2a) and isotype control (IgG2a). In B, significance was calculated using an unpaired Student t test (*p < 0.05). In C, significance was calculated using one-way ANOVA (*p < 0.05). Experiments were performed three times with similar results. Data are representative of one independent experiment with three replicates per group. Error bars represent SEM.
Surprisingly, we have found that 15 min after the incubation of fluorescently labeled RL6A with MDA-MB-231 cells, red fluorescence was observable in a discontinuous manner at the edge of cells and in cytoplasmic vesicular structures, indicating that RL6A Abs after binding to HLA-A2/YLLPAIVHI peptide complexes on the surface of tumor cells were rapidly internalized. Co-staining for EEA1 suggested internalization of RL6A-HLA-A2/YLLPAIVHI peptide complexes (Fig. 9). The plasma membrane fluorescence disappeared at later time points (30 and 60 min), whereas endosomes remained stained. These results demonstrate that engagement of the cognate peptide/HLA-A2 with RL6A leads to the complete internalization of these complexes in breast tumor cells through endocytosis.

Discussion
The successful implementation of several mAbs to routine cancer therapy has proven the concept that these molecules are effective therapeutic agents, encouraging further discovery of novel anti-cancer mAbs. We have recently reported the development of a new class of anti-cancer mAbs that we termed TCRm since their specificity and binding properties resemble TCR on cytotoxic lymphocytes. Two of these mAbs, RL4B and RL6A, have shown potent anti-tumor activity in vivo (9, 10). However, mechanisms of these TCRm-mediated activities remain unclear. In this study we provide evidence that both TCRm inhibited growth of established tumors by the induction of cancer cell apoptosis through selective and direct ligation of specific MHC class I/peptide complexes. Additionally, we found that TCRm-mediated killing was partially independent from ADCC and CDC, and mechanisms of tumor cell apoptosis were associated with the activation of the caspase-dependent intrinsic pathway and JNK signaling. Intriguingly, we observed that complexes formed by TCRm with MHC class I molecules bearing peptides were rapidly internalized into endosomes of tumor cells.

Our studies in xenograft models demonstrated that tumor cells in mice treated with TCRm died through apoptosis. Interestingly, we noticed that tumor tissue from these mice lacked an obvious inflammatory infiltrate. Because the active participation of immune cells such as NK cells, monocytes, and macrophages is required for several mAbs to kill tumor cells through ADCC, these findings suggest that TCRm-mediated destruction of tumor cells is ADCC-independent. The reduction of tumor growth by F(ab′)2 fragments

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**FIGURE 5.** TCRm treatment induces activation of executioner caspases. A. Caspase-3 and caspase-7 activities in MDA-MB-231 cells incubated with 1 µg/ml RL4B, RL6A, control IgG2a, or 10 µM cpt in the absence or presence of specific caspase-3/7 inhibitor Z-DEVD-fmk. B. Cleavage of PARP in MDA-MB-231 cells after incubation with RL6A, RL4B, or control IgG2a. In A and B, significance was calculated using an unpaired Student t test (*p < 0.05). Experiments were performed two times with similar results. Data are representative of one independent experiment with three replicates per group. Error bars represent SEM.

**FIGURE 6.** TCRm treatment induces activation of the intrinsic caspase-dependent pathway. Caspase-9 activity in MDA-MB-231 cells incubated with 1 µg/ml RL4B, RL6A, control IgG2a, or 10 µM cpt in the absence or presence of specific caspase-9 inhibitor Z-LEHD-fmk. Additionally, caspase-9 activity was measured in untreated cells (cells). Significance was calculated using an unpaired Student t test (*p < 0.05). Experiments were performed two times with similar results. Data are representative of one independent experiment with three replicates per group. Error bars represent SEM.

**FIGURE 7.** TCRm induce release of cytochrome c and AIF into the cytoplasm of tumor cells. A. Cytochrome c accumulation in the cytoplasm of MDA-MB-231 cells after their incubation with 1 µg/ml RL4B (left panel), RL6A (right panel), or control IgG2a as determined by intracellular staining and flow cytometric analysis. B. AIF accumulation in the cytoplasm of MDA-MB-231 cells after their incubation with 1 µg/ml RL4B (left panel), RL6A (right panel), IgG2a, or 10 µM cpt, as determined by intracellular staining and flow cytometric analysis. In A and B, significance was calculated using an unpaired Student t test (*p < 0.05). Experiments were performed two times with similar results. Data are representative of one independent experiment with three replicates per group. Error bars represent SEM.
FIGURE 8. TCRm binding to specific MHC/peptide complexes leads to JNK activation. A, The magnitude of JNK phosphorylation in untreated (filled histogram) or treated MDA-MB-231 cells incubated with RL4B (left panel), RL6A (right panel), control IgG2a, or 10 μM cpt, as demonstrated by intracellular staining via flow cytometry. B, The magnitude of JNK phosphorylation, assayed as in A, in MDA-MB-231 cells incubated with RL4B (left panel), RL6A (right panel), IgG2a, or 10 μM cpt in the absence or presence of relevant (Tet+) or irrelevant (Tet−) peptide-loaded tetramers. C, RL4B (left panel), RL6A (right panel), IgG2a, or 10 μM cpt induced apoptosis of MDA-MB-231 cells in the absence or presence of JNK inhibitor. Apoptosis induced by various treatments was compared with apoptosis of untreated MDA-MB-231 cells (cells). Apoptosis indexes were obtained by combining frequencies of cells stained with annexin V alone with frequencies of cells stained with both annexin V and PI. D, RL4B (left panel), RL6A (right panel), IgG2a, or 10 μM cpt induced pan-caspase activity in MDA-MB-231 cells in the absence or presence of JNK inhibitor. In C, experiments were performed one time with three replicates per group. Error bars represent SEM. A, B, and D, Experiments were performed three times with similar results. Data are representative of one independent experiment.
from RL4B further supports this hypothesis, since these Abs do not have Fc fragments that are essential for ADCC. Additionally, Fc fragment is important for complement activation, and therefore it appears that TCRm kill tumor cells through mechanisms that are also complement-independent. Although the reduction of tumor growth by F(ab’)$_2$ fragments was significant, they were less efficient in inhibiting tumor growth when compared with the whole RL4B. Thus, we concluded that TCRm-mediated apoptosis of tumor cells is partially independent from immune effector mechanisms such as ADCC and CDC; however, both ADCC and CDC also likely contribute to anti-cancer properties of TCRm. Most currently approved therapeutic Abs kill tumor cells through these immunologic mechanisms that require proper functions of the immune system. This dependence of mAbs on an intact immune system can be perceived as disadvantageous, as patients receiving this therapy are often severely immunocompromised. Furthermore, tumor cells are shielded from complement attack by high expression of complement regulatory proteins that efficiently decrease CDC (22). Thus, the design of new mAbs that can destroy tumor cells through nonimmunologic mechanisms seems to

**FIGURE 9.** TCRm binding to MHC/peptide complexes leads to their internalization. Confocal microscopy of MDA-MB-231 cells incubated with RL6A prior to fixation. (i) Cell morphology shown by differential interference contrast, (ii) cell nuclei stained with DRAQ5, (iii) immunofluorescence detection of EEA1 expressed in endosomes, (iv) RL6A labeled with Alexa Fluor 546 in cellular cytoplasm, and (v) merged image, colocalization of EEA1 and RL6A in endosomes (yellow). Experiments were performed three times with similar results. Data are representative of one independent experiment. Scale bars, 10 μm. A 63× oil lens was used.

**FIGURE 10.** Putative mechanisms of TCRm-mediated tumor cell apoptosis. Binding of TCRm to specific MHC class I/peptide complexes on tumor cells triggers downstream activation of JNK and intrinsic caspase-dependent pathway. Additionally, this binding leads to the internalization of Ab/Ag complexes via endosomal pathway. The release of cytochrome c and AIF from mitochondria follows these initial processes. Consequently, TCRm induce death of tumor cells by activating multiple intracellular pathways leading to apoptosis.
be a promising direction. Examples of such Abs include molecules that bind to VEGF and Her-2. These Abs affect signaling pathways important for tumor growth, although anti–Her-2 Ab eliminates tumor cells through ADCC as well (1). It appears that TCRm can fit to this new category of therapeutic Abs, since they affect signaling leading to tumor cell apoptosis. Furthermore, ADCC requires high density of antigenic epitopes on the surface of target cells, as demonstrated by the correlation between trastuzumab efficacy in eliminating tumor cells and the density of Her2/neu receptor (23, 24). Therefore, TCRm that target peptide/MHC class I complexes present in low number of copies on cellular surfaces are less likely to kill tumor cells through ADCC. The high efficacy of TCRm in killing tumor cells suggests that TCRm, independently from ADCC, trigger intracellular signaling that affects the survival of tumor cells. These properties of TCRm should also be seen as another advantage, as quite often expression of tumor-specific proteins is low. Finally, in accordance with our previous report (25), we found that complexes formed by TCRm with their targets were rapidly internalized, and therefore these Abs may not be available on the cell surface long enough to activate potent ADCC responses.

TCRm-induced apoptotic signaling in tumor cells is specifically related to the interaction of these Abs with complexes formed by peptides that are positioned in the peptide groove of MHC class I molecules. In contrast, pan-MHC class I (W6/32) and anti–HLA-A2 (BB7.2) Abs that bind to α2/α3 and α2 domains of MHC class I molecules, respectively (12), did not trigger apoptosis of tumor cells in vitro, although they are capable of killing tumor cells through ADCC and CDC (data not shown). Our observations regarding W6/32 and BB7.2 Abs are in accordance with previously published reports (26, 27). In contrast to W6/32 and BB7.2 Abs, mAbs binding to the α1 or α3 domains or the β2-microglobulin protein induced death of activated B and T cells and malignant B cells (12, 16, 28–30). Thus, the net effect of mAb binding to MHC class I molecules on cell survival clearly depends on the localization of antigenic epitopes recognized by these Abs within the MHC class I molecule. Because the binding specificity of TCRm, which seems to be crucial for induction of tumor cell apoptosis, overlaps with that for TCR on cytotoxic lymphocytes, the induction of apoptotic signaling by TCRm suggests that similar signaling can be activated in tumor cells by the interaction with TCR on lymphocytes. Confirmation of this hypothesis can provide a novel mechanism of T cell-dependent cytotoxicity.

We found that direct and specific binding of TCRm to MHC class I/peptide complexes triggers signaling in tumor cells through the activation of JNK and the intrinsic caspase-dependent death pathway. These findings extend beyond some early investigations suggesting that MHC class I molecules can initiate signaling resulting in anti-proliferative and apoptotic responses in immune cells (31–35). Triggering intracellular transduction events likely involves the participation of MHC class I molecules in transmembrane signaling, thereby suggesting receptor-like functions for these molecules. However, molecular mechanisms associated with this signaling remain unknown. Physical associations between MHC class I molecules and hormone or growth factor receptors, such as insulin receptor, insulin-like growth factor receptor, and epidermal growth factor receptor points to the hypothetical possibility that signaling initiated by ligand interacting with MHC class I molecule can be transduced through these receptors (36, 37). The role of other surface molecules and adaptor proteins in this process is not well understood.

mAbs that cross-link MHC class I complexes independently of peptide specificity have been shown to activate several intracellular signaling pathways, including 1) phosphorylation of tyrosine kinases, 2) activation of the JAK/STAT pathway, and 3) upregulation of PI3K, leading to JNK activation (17, 18, 21, 38–41). Furthermore, these cross-linking mAbs also have been reported to induce apoptosis through caspase-dependent and caspase-independent mechanisms in B and T lymphocytes and in B cell malignancies. However, effects of these Abs on epithelial malignancies have not yet been investigated. We found that TCRm induced JNK activation. Nonetheless, events leading to JNK activation and the immediate downstream signaling components that trigger caspase-dependent and -independent tumor death remain to be elucidated (Fig. 10).

The significance of our findings is also underscored by the scarcity of reports that describe anti-cancer properties of mAbs that target MHC class I molecules. Anti-proliferative and proapoptotic effects of Abs targeting β2-microglobulin within the HLA complex have been demonstrated (16, 21). However, potential therapeutic applications of these Abs are limited, as β2-microglobulin is expressed in all nucleated cells. Furthermore, levels of circulating β2-microglobulin are increased in cancer patients; therefore, large quantities of these Abs will be used at the periphery, and consequently a high dose of these Abs will be required to achieve desired therapeutic effects. In contrast, TCRm bind to targets expressed uniquely on diseased cells.

In summary, our study delineated molecular mechanisms of signaling via the MHC class I/peptide complex pathway initiated by TCRm binding. We showed that TCRm selectively and directly induce cancer cell apoptosis in epithelial malignancies leading to effective reduction of advanced tumor growth in vivo.

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Disclosures
J.A.W. is Chief Scientist and founder of Receptor Logic, Inc. The other authors have no financial conflicts of interest.

References


SUPPLEMENTAL FIGURE 1. TCRm induced apoptosis of tumor cells. (A) Apoptosis of MDA-MB-231 cells induced by various concentrations of RL4B (upper panel) or RL6A (lower panel). (B) Apoptosis of MDA-MB-231 cells induced by RL4B (upper panel), RL6A (lower panel) or IgG2a incubated for various time points. (C) Apoptosis of MDA-MB-231, 1520 and 501A cells induced by 1µg/ml of RL4B (left panel) or RL6A (right panel). For B, apoptosis indexes were obtained by combining frequencies of cells stained with Annexin-V alone with frequencies of cells stained with both Annexin-V and PI. For A and C, data were normalized against apoptosis induced by IgG2a control treatment. Experiments were performed two times with similar results. Data are representative of one independent experiment with 4 replicates per group. Error bars represent SEM.

SUPPLEMENTAL FIGURE 2. Apoptosis induction with peptide independent class I HLA antibodies. (A) Apoptosis of MDA-MB-231 cells induced by BB7.2, IgG2a or 10µM cpt. (B) Apoptosis of MDA-MB-231 cells induced by W6/32, IgG2a or 10µM cpt. Apoptotic indexes were obtained by combining frequencies of cells stained with Annexin-V alone with frequencies of cells stained with both Annexin-V and PI. Experiments were performed two times with similar results. Data are representative of one independent experiment with 3-4 replicates per group. Error bars represent SEM.
SUPPLEMENTAL FIGURE 3. Binding specificity of RL6A. THP-1 cells were loaded with different concentrations of YLL peptide (5μM - 50μM) and stained with 1μg/ml concentration of RL6A. Staining was observed with YLL pulsed THP-1 cells, no staining was observed with unpulsed or irrelevant peptide (AGL) pulsed THP-1 cells. IgG2a was used as an isotype antibody control. Data are represented as mean fluorescence intensity (MFI). Data are representative of one experiment.
Supplemental Figure 2

A

B

Apoptosis Index

Treatment

Apoptosis Index

Treatment
Supplemental figure 3

The graph shows the binding (MFI) of different samples at various concentrations. The x-axis represents different concentrations: 5 μm, 10 μm, 20 μm, 50 μm, unpbed, AGU 20 μm, and 20 μm. The y-axis represents the binding (MFI) ranging from 0 to 50. Two conditions are shown: RL6A 1 μg/ml and IgG2a 1 μg/ml.