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*J Immunol* published online 11 January 2010
http://www.jimmunol.org/content/early/2010/01/11/jimmunol.0902414

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TCR Mimic Monoclonal Antibody Targets a Specific Peptide/HLA Class I Complex and Significantly Impedes Tumor Growth In Vivo Using Breast Cancer Models

Bhavna Verma,*,† Francisca A. Neethling,‡ Shannon Caseltine,§ Ginger Fabrizio,¶ Sherly Largo,∥ J. Andrew Duty,‡ Piotr Tabaczewski,§ and Jon A. Weidanz*†‡,*

Our laboratory has developed a process for generating mAbs with selectivity to unique peptides in the context of MHC molecules. Recently, we reported that RL4B, an mAb that we have called a TCR mimic (TCRm) because it recognizes peptide in the context of MHC, has cytotoxic activity in vitro and prevented growth of tumor cells in a prophylactic setting. When presented in the context of HLA-A2, RL4B TCRm recognizes the peptide GVLPALPQV derived from human chorionic gonadotropin (hCG)-β. In this study, we show that RL4B TCRm has strong binding affinity for the GVLPALPQV peptide/HLA-A2 epitope and fine binding specificity for cells that express endogenous hCGβ Ag and HLA-A2. In addition, suppression of tumor growth with RL4B TCRm was observed in orthotopic models for breast cancer. Using two aggressive human tumor cell lines, MDA-MB-231 and MCF-7, we provide evidence that RL4B TCRm significantly retards tumor growth, supporting a possible role for TCRm agents in therapeutic settings. Moreover, tumors in mice responded to RL4B TCRm therapy in a dose-dependent manner, eliminating tumors at the highest dose. RL4B TCRm strongly detects the hCGβ peptide/HLA-A2 epitope in human primary breast tumor tissue, but does not react or reacts weakly with normal breast tissue from the same patient. These results further illustrate the selective nature of TCRm Abs and the clinical relevance of the GVLPALPQV peptide/HLA-A2 epitope expression in tumor cells, because they provide the first evidence that Abs that mimic the TCR can be used to markedly reduce and suppress tumor growth. The Journal of Immunology, 2010, 184: 000–000.

Several groups have shown the expression of tumor-specific peptide/MHC epitopes in human melanoma cells, providing evidence for the existence of a class of biomarkers able to distinguish normal cells from cancer cells (1–5). Subsequent attempts to develop T cell-based therapies for the treatment of cancer have yielded ambiguous outcomes. A limited number of reports have described reduction of tumors, prevention of metastatic disease, or complete eradication of tumors in experimental cancer models and in a few human clinical trials (6–10). Regardless, T cell-eliciting vaccines and adoptive T cell therapies have provided proof of concept for immunotherapies that target tumor-specific peptide/MHC complexes (11–13). However, considerable obstacles will have to be overcome before CD8+ T cell-based therapies can be successfully translated into the clinic. Therapeutic Abs for cancer treatment are currently in widespread use and include trastuzumab, rituximab, bevacizumab, and cetuximab (14–20). Rituximab and cetuximab are known to mediate tumor cell death by specifically binding to their cognate Ag expressed on the surface of cancer cells and activating complement-dependent cytotoxicity (CDC) and Ab-dependent cellular cytotoxicity (ADCC) (16, 21). Trastuzumab and bevacizumab are reported to mediate antitumor activity through blocking receptor signaling and inhibiting cell proliferation, whereas rituximab has been shown to induce apoptosis of cancer cells (22–24). The demonstration that Abs can act as effective therapies for cancer has raised significant interest in identifying tumor-specific biomarkers. Unfortunately, only a limited number of tumor-specific markers expressed uniquely on the surface of tumor cells have been discovered, spurring increased efforts to identify new cancer cell-specific markers for therapeutic Ab targeting in oncology (25–28).

The MHC class I system along with the endogenous peptides presented on the cell surface are unique markers used by effector CD8+ T cells to discriminate normal cells from diseased cells. MHC class I complexes are constitutively expressed by all nucleated cells in the body. The MHC system includes Ag-processing machinery that processes and presents peptides in the context of MHC molecules onto the cell surface. Peptides derived from aberrant proteins in many tumor cells expressed as tumor-specific peptide/MHC complexes have been identified and might represent good targets accessible to Abs (29–31). Thus, Abs made against biomarkers such as peptide/MHC complexes that mediate antitumor effects could serve as a novel modality for cancer treatment. Recently, several groups including our own have generated Abs against specific peptide/MHC complexes (32–37). A number of different approaches have been used. Many methods rely on using bacteriophage display technology to generate antipeptide/MHC class I Abs from nonimmunized and immunized libraries (36, 38, 39). Our approach combines immunization with synthetic immunogen and
high throughput screening techniques to generate antipeptide/MHC Abs we have dubbed TCR mimics (TCRm) Abs (40, 41). The majority of the Abs generated by our laboratory using this process have shown high binding affinity and fine binding specificity, and they can be used for the detection and quantitation of peptide/MHC class I molecules (32, 33).

We reported previously on a TCRm Ab-specific for peptide GVLPALPQV derived from human chorionic gonadotropin (hCG)-β and presented by HLA-A*0201 (27). In a comprehensive review of hCG in cancer, Stenman et al. (42) reported hCGβ in the serum of 45–60% of patients with biliary and pancreatic cancers and 10–30% of other cancers. HCGβ overexpression is also associated with the development of breast carcinomas in mice and humans. Female transgenic mice overexpressing hCGβ-subunit under the ubiquitin C promoter develop mammary gland tumors from the age of 9 mo onward, with 90% penetrance at 12 mo, whereas normal mice do not develop mammary tumors (43, 44). In humans, hCGβ expression in mammary tumors has been shown to have prognostic value and identifies high-risk patients (45, 46).

We showed previously the IgG2a Ab RL4B (3,2G1) TCRm prevented tumor growth in a prophylactic model of human breast cancer (41). Further, the RL4B TCRm demonstrated CDC and ADCC activity against tumor cells in vitro. We now show that the RL4B TCRm can be used therapeutically to suppress and retard tumor growth using an orthotopic model for breast cancer. In addition, RL4B TCRm-stained primary human breast cancer cells supporting the view that GVL peptide/HLA-A*0201 expression is a clinically relevant tumor-specific biomarker. Collectively, these findings indicate the potential diagnostic and therapeutic benefits of using TCRm Abs for targeting specific T cell epitopes expressed on the surface of cancer cells.

Materials and Methods

Abs and synthetic peptide

Polyclonal anti-mouse IgG H chain–PE was purchased from Invitrogen Caltag (Burlingame, CA). Isotype control Abs including mouse IgG1, IgG2a, and IgG2b, were purchased from Southern Biotech (Birmingham, AL). The anti-hCGβ mAb was purchased from BioChain (Hayward, CA). HB-82, anti-HLA A2.1 mAb (BB7.2) expressing mouse cytotoxic T lymphocytes (CTL) against the murine Ay1.6 EL4 target cell line, was obtained from the European Collection of Animal Cell Cultures (ECACC). Abs and synthetic peptide sequences (5–31) were chemically synthesized by custom protocols using QED Technologies, Inc. (Palo Alto, CA).

Cell lines and culture

Human cell lines MDA-MB-231 (HTB-26, breast cancer), MCF-7 (HTB-131, breast cancer), THP-1 (TIB-82, monocytes), and T2 (CRL-1992, B lymphoblastoid) were obtained from the American Tissue Culture Collection (ATCC). All cell lines were maintained in appropriate media (20, 21).

Double staining of human tissue

Immunohistochemistry

Tumor samples from each patient were placed in Cryomold (Fisher Scientific, Pittsburgh, PA), coated in OCT media, flash frozen using isopentane on dry ice, and stored at −80°C until used. Tissue sections were made at 5-μm size and fixed using 5% methanol and stained with RL4B, IgG2a, BB7.2, and IgG2b at 1 μg/ml for 1 h in diluent containing 1.0% horse serum to prevent nonspecific staining of tissue. Detection of primary Ab binding was determined using goat anti-mouse IgG–HRP (ImmPRESS Anti-Mouse IgG peroxidase Kit, Vector), that, in the presence of substrate chromogen (3,3′ diaminobenzidine [DAB; Bio-Rad, Hercules, CA]), provides an indicator system (formation of brown precipitate) to visualize the location of Ag/Ab binding using light microscopy. Hematoxylin was used as a nuclear counterstain (Vector). H&E stains (Sigma-Aldrich, St. Louis, MO) were used to assess cell morphology and tumor cell presence in tissue. Tissue sections were analyzed using light microscopy (Nikon Eclipse TE 2000, inverted, deconvolution microscope with Simple PCI Suite software (Nikon, Melville, NY); DAPI (Vector, Burlingame, CA) was used as a counter stain for the nucleus.

Human tissue procurement

The institutional review board at Hendrick Medical Center (Abilene, TX) gave approval for patient-consented collection of normal and breast cancer tissue.

Immunocytochemistry

Tetramer competition assay

In several studies, tissue staining was performed in the presence of soluble peptide/HLA-A2 tetramer to confirm specific binding of the RL4B TCRm to GVL peptide/HLA-A2 target expressed on tumor cells in tissue. Prior to staining, RL4B TCRm and the BB7.2 mAbs at 1 μg/ml were each mixed with soluble GVL peptide/HLA-A2 or KIFS peptide/A2 tetramers (10 μg/ml), and then added to incubate with tissue as described above.
In vivo models

Athyric nude mice (Crl:NU-Foxn1nu) were obtained from Charles River (Wilmington, MA) and housed under sterile conditions in barrier cages. The first tumor model used MDA-MB-231 breast tumor cells. In this study, 50 mice were injected s.c. with $5 \times 10^5$ freshly harvested MDA-MB-231 cells at 97% viability in Matrigel (Sigma-Aldrich) in the right mammary pad. After tumor size reached a volume of $\approx 50$ mm$^3$, mice received i.p. injections of either 100 $\mu$g IgG2a isotype control Ab ($n = 15$) or 100 $\mu$g RL4B Ab ($n = 18$), followed by 50 $\mu$g/ wk for a total of 5 wk.

The second tumor model used MCF-7 breast cancer cells. In this study, mice were implanted s.c. with 60-drelelease 1.7 mg/pellet 17b-estradiol pellets (Innovative Research of America, Sarasota, FL) 7 d before s.c. injection with $5 \times 10^5$ freshly harvested MCF-7 cells at 96% viability in Matrigel in the right mammary pad. After tumor size reached a volume of $>70$ mm$^3$, mice received weekly i.p. injections of either 250 $\mu$g IgG2a isotype control Ab ($n = 4$) or 250 $\mu$g RL4B Ab ($n = 4$) for a total of 3 wk.

A dose-response study involving RL4B TCRm was performed using nude mice implanted s.c. in the right mammary pad with $5 \times 10^5$ freshly harvested MDA-MB-231 cells at 97% viability in Matrigel. After tumors grew to $\approx 40$ mm$^3$, mice received weekly i.p. injections with 500 $\mu$g IgG2a control Ab ($n = 5$) or RL4B TCRm at 100 ($n = 4$), 250 ($n = 6$), and 500 $\mu$g ($n = 5$) for 4 wk. The RL4B TCRm and control Abs were injected into mice using a diluted of filtered sterile phosphate buffered solution. This dosage regimen followed the prophylactic setting [27] and was used in this study to see its effect in a therapeutic setting. Mice were euthanized at the end of study. Tumor volumes were calculated by assuming a spherical shape and using the following formula: volume = $(L \times B)^{3}/2$ (L is the longest diameter; b is the shortest diameter), where the mean tumor diameter was measured in two dimensions.

Detection of TCRm in tumors

Detection of RL4B TCRm Ab in tumor tissue was done by sandwich ELISA method adopted from (47) and modified as described. MDA-MB-231 cells ($5 \times 10^5$) were transplanted s.c. into the right mammary pads of four athyric nude mice. When they achieved a size of $\approx 500$ mm$^3$, i.p. injections of RL4B (500 $\mu$g for 3 d) were given to three of the four mice, whereas the remaining mouse did not receive any treatment and was used as an untreated control. On the fourth day, the mice were euthanized as per protocol by CO$_2$. The tumors were excised, rinsed in 1 PBS, transferred into a 50-ml polystyrene tube containing 2 ml 0.2% Tween 20/TBS (Fisher Scientific, Fairlawn, NJ), and sonicated. The homogenate was incubated at 37˚C, washed twice with 200 ml 0.2% Tween 20/TBS buffer (pH 7.4) (Fisher Scientific), and sonicated. The homogenate was incubated at 4˚C for 20 h for protein extraction and then centrifuged at 10,000 rpm for 30 min. Supernatant in protein concentration was collected for ELISA.

The wells of a Maxisorb 96-well plate (Nunc, Rochester, NY) were coated with relevant tetramer (GVL) and irrelevant tetramer (AIM) at a concentration of 100 ng/well in 30 mM Tris-HCl buffer (pH 8.8) in 100 ml volume for 2 h at 37˚C, washed twice with 200 ml TBS buffer (pH 7.4) (Fisher Scientific) containing 0.02% Tween 20, and blocked for 2 h at room temperature with 5% nonfat milk in 100 ml 0.02% Tween 20/TBS. The plate was then washed again twice as described previously followed by adding 100 ml of test sample prepared at a 1:1 ratio of protein extract to 0.02% Tween 20/0.5% nonfat milk/TBS. Each sample was added in triplicate for both the relevant and irrelevant wells. Purified RL4B (1000 ng/well), BB7.2A (1000 ng/well), and IgG2a (1000 ng/well) were also used as controls and incubated at room temperature for 2 h. The wells were then washed five times, and bound mAb was detected by incubating for 2 h with 4000 fold diluted horse radish peroxidase-goat conjugated F(ab)2 anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in 100 ml 0.02% Tween 20/0.5% nonfat milk/TBS. The plate was then washed five times followed by development with 50 ml one-step ABTS 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Thermo Scientific, Rockford, IL) for 20 min. The reaction was stopped by the addition of 50 ml 1X SDS solution, and the absorbance was read at 405 nm using a BIOTreater plate reader (BioTek Instruments, Winsorisk, VT).

Statistical analysis

Results are expressed as the mean $\pm$ SE. Student t tests were used to determine significance among the groups, and a value of $p < 0.001$ was considered significant. Analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

Results

The RL4B TCRm is highly specific and has a strong binding affinity for cognate peptide/HLA-A2 complex

RL4B TCRm was generated as previously described (41). The binding specificity of the TCRm was further examined using unpulsed (Fig. 1A), GVL, peptide-pulsed (Fig. 1B), and irrelevant pulsed (Fig. 1C) T2 cells at concentrations of Ab ranging from 50 to 500 ng/ml. No staining was observed for unpulsed and KIF5 irrelevant peptide-pulsed T2 cells when using the maximum concentration of 500 ng/ml (Fig. 1A, 1C). In contrast, as seen in Fig. 1B, the RL4B TCRm brightly stained T2 cells pulsed with the specific peptide GVL (20 $\mu$m) at any one of the TCRm concentrations used (50–500 ng/ml). Not shown in Fig. 1 is the absence of any staining signal to T2 cells pulsed with 10 other irrelevant peptides. These findings support the claim that RL4B TCRm binds specially to GVL peptide/HLA-A2.

To characterize the binding kinetics for RL4B TCRm, surface plasmon resonance was used to examine the binding affinity ($K_d$), the rates of association and dissociation, and $t_{1/2}$ of dissociation for RL4B TCRm binding to the GVL peptide/HLA-A2 complex. The RL4B TCRm exhibited strong binding affinity ($K_d \approx 1.5 \times 10^{-9}$ M) for cognate peptide/HLA-A2 complex displaying a rapid on-rate (4.8 $\times 10^8$) and a noticeably slow off-rate (8.2 $\times 10^{-3}$). Further, RL4B TCRm binding to cognate peptide/HLA-A2 was characterized by a dissociation $t_{1/2}$ of $>8.5 \times 10^5$. These data indicate that the RL4B TCRm is endowed with favorable binding characteristics for cognate peptide/HLA-A2 complex, contributing support for the TCRm as a potentially unique tumor-targeting agent.

RL4B TCRm detects endogenously processed and presented GVL peptide/HLA-A2 complexes

The ability of RL4B TCRm Ab to detect endogenously processed peptide in the context of the HLA-A2 molecule was evaluated by immunofluorescence and flow cytometric analysis using a panel of cell lines (Fig. 2). Moreover, double staining with RL4B TCRm-PE and BB7.2-FITC (anti-HLA-A2 mAb) was performed to determine the level of colocalization of GVL peptide/HLA-A2 complexes with the expression of HLA-A2 on cells. The human cell lines used in this study included MDA-MB-231 breast cancer cells (HLA-A2$^{pos}$ and hCG$^{b}$), MCF-10A nonmammary epithelial cell lines (HLA-A2$^{neg}$ and hCG$^{b}$), and THP-1 monocytic cell lines (HLA-A2$^{neg}$/hCG$^{b}$). The MDA-MB-231 and MCF-10A cell lines were found to express hCG$^{b}$. Ag after analyzing for protein expression in detergent lysates by ELISA (data not shown). After double staining with RL4B TCRm and BB7.2, only MDA-MB-231 cells were stained simultaneously with both mAbs, indicating colocalization of the specific GVL peptide-epitope with HLA-A2. In contrast, no staining with either mAb was observed for MCF-10A cells, and only BB7.2-FITC mAb reacted with the THP-1 cells that did not express hCG$^{b}$. Collectively, these studies support the notion that the RL4B TCRm is specific for the GVL peptide/HLA-A2 complex and that the RL4B TCRm also detects endogenously processed peptide/HLA-A2 complexes.

RL4B TCRm is effective in vivo in an orthotopic breast cancer model

Previously, we reported that RL4B TCRm could inhibit tumor growth in a prophylactic model of breast cancer (41). Still, the ability of a TCRm to possess therapeutic benefits for the treatment of established tumors remains unknown. To address the potential therapeutic relevance of RL4B TCRm, an orthotopic breast cancer model was used with human breast cancer cells implanted into the mammary fat pad of athyric nude mice. Prior to tumor implantation, human breast carcinoma cells MDA-MB-231 were evaluated for GVL peptide/HLA-A2 expression using immunocytochemical analysis and immunofluorescence. As seen in Fig. 3, MDA-MB-231 cells stained with RL4B TCRm reveal a unique fluorescence pattern that possibly represents localized clusters of GVL peptide/HLA-A2 on tumor cells (Fig. 3A, inset). Further, the cell-to-cell staining intensity is variable for RL4B TCRm, suggesting that the
level of GVL peptide/HLA-A2 is widely different for each tumor cell. In agreement with flow cytometric data, BB7.2 mAb also stained MDA-MB-231 cells, with the intensity of staining appearing greater than for RL4B TCRm. Moreover, the unique cluster-like staining pattern was not as apparent when staining with BB7.2 mAb, which revealed a more uniform cellular staining pattern (Fig. 3B). The isotype control Abs did not stain the tumor cells (Fig. 3C, 3D). Based on the positive RL4B TCRm staining profile observed for MDA-MB-231 cells, two different tumor models were used.

In the first study to evaluate the antitumor properties of TCRm in vivo, athymic nude mice were implanted in the right mammary fat pad with a formulation mixture that was composed of $5 \times 10^6$ MDA-MB-231 tumor cells and Matrigel. Matrigel is a mixture of laminin, collagen IV, heparan sulfate, and entactin that creates a nutrient-rich environment to promote rapid tumor growth, resulting in palpable tumors within 4–5 wk after cell implantation (48, 49). Tumors were allowed to grow to a mean volume of approximately $50 \text{mm}^3$ before initiation of treatment with RL4B TCRm or isotype control Ab (Fig. 4A). Initial treatment dose was 100 mg given i.p. followed by subsequent weekly i.p. injections of 50 mg for both isotype control Ab and RL4B TCRm. Mice that received RL4B TCRm ($n = 18$) showed retarded or markedly delayed tumor growth up to 5 wk after treatment (week 0 tumor volume, $\sim 50 \text{mm}^3$; week 5 tumor volume, $\sim 200 \text{mm}^3$). In contrast, isotype control treated mice ($n = 15$) showed rapid uninhibited tumor growth reaching a mean tumor volume of $800 \text{mm}^3$ 5 wk after receiving five i.p. injections with isotype control Ab (Fig. 4A).

In the second tumor model, 7 d after implanting nude mice with $5 \times 10^6$ MCF-7 tumor cells and Matrigel, tumors grew 2-fold faster than MDA-MB-231 tumors in nude mice and reached $>70 \text{mm}^3$ 2 wk after MCF-7 cells were implanted. Mice received weekly i.p. injections with 250 mg of either IgG2a control Ab ($n = 4$) or RL4B TCRm ($n = 4$). Three weeks later, tumors in mice treated with isotype control Ab had grown to $>2500 \text{mm}^3$. All mice in the control group were euthanized at 3 wk owing to large tumor sizes. In contrast, RL4B TCRm-treated mice had a markedly reduced mean tumor volume $<500 \text{mm}^3$ (Fig. 4B). Moreover, the data were especially intriguing given the fact that RL4B TCRm staining intensity for MCF-7 cells was markedly less ($\sim 7.5$-fold less) than RL4B TCRm signal intensity for MDA-MB-231 cells (Fig. 4A, 4B, inset), suggesting that relatively few copies of the specific peptide/MHC complex are necessary for TCRm to mediate antitumor activity. Further, these findings demonstrate in two aggressive mouse models that TCRm targeting of specific peptide/MHC complexes has potent antitumor effects that can significantly inhibit or delay tumor growth (Fig. 4).

To determine the ability of RL4B TCRm to selectively target the tumor, MDA-MB-231 cells were transplanted s.c. into mammary pads of athymic nude mice and treated with RL4B (500 mg i.p.) for 3 d followed by euthanization and excision of the tumor. Homogenized and protein extract of the tumor was used for detection of the Ab via sandwich ELISA (47). We observed that wells coated with relevant tetramer (GVL) for RL4B showed a positive signal for all three mice (Fig. 4C) that were treated with RL4B. No signal was observed for the untreated mouse. No signal also was observed in the irrelevant tetramer coated wells for all mice (treated and untreated). As a positive control, BB7.2 was used with both relevant and irrelevant tetramer coated wells. RL4B was also used as a control to show specificity of RL4B with GVL tetramer. Mouse IgG2a was

**FIGURE 1.** Binding specificity of RL4B TCRm. A, Unpulsed T2 cells, (B) GVL peptide-pulsed cells, and (C) KIFS irrelevant peptide-pulsed T2 cells were stained with diluted RL4B TCRm. No staining for unpulsed or irrelevant peptide-pulsed T2 cells was observed, even at the highest concentration of RL4B TCRm (500 ng/ml; A and C, red line). In contrast, maximum staining of GVL peptide-pulsed (20 μM) T2 cells (B, red line) was observed with RL4B TCRm at 500 ng/ml, with decreasing RL4B staining intensity observed with titration of the TCRm. Purple fill peak represents background staining signal with IgG2a isotype control Ab.
used as a negative control to check for nonspecific binding. Based on the ELISA results, we deduced that tumor retardation observed in the in vivo models was caused by the RL4B Ab activity in the tumor.

**RL4B TCRm demonstrates dose-dependent antitumor activity in an orthotopic breast cancer model**

Next, we evaluated whether the antitumor effects for RL4B TCRm were dose-dependent. Nude mice were implanted with MDA-MB-231 tumor cells as described previously, and treatment was initiated after palpable tumors (>40 mm³) had formed. Mice (n = 5) treated with IgG2a isotype control Ab at 500 μg per injection had a mean tumor volume of 52 mm³ or a volume equivalent to the size of tumors at the start of treatment. In fact, three mice no longer had palpable tumors, suggesting that treatment with RL4B TCRm resulted in tumor elimination. These data show that the antitumor activity of RL4B TCRm in vivo is dose-dependent and demonstrate the therapeutic benefit of using a mAb with T cell-like binding specificity to eradicate established tumors in mice.

**RL4B TCRm stains primary human breast tumor tissue**

Thus far, we have focused our efforts on demonstrating the binding specificity of the TCRm using tumor cell lines and the therapeutic benefits of using RL4B TCRm to shrink tumors in treated mice. The success of RL4B TCRm at reducing or delaying tumor growth in an orthotopic model raised interest regarding the expression of the GVL peptide/HLA-A2 epitope on clinically relevant primary breast tumor tissue. To determine epitope expression, breast tissue was collected under an institutional review board protocol, flash frozen, cut into sections (5 μm thick) using a cryostat and stained with isotype control Abs, anti-HLA-A2 Ab (BB7.2 mAb), and RL4B TCRm Ab. The results are shown in Fig. 6 and demonstrate that RL4B TCRm does not stain or stains weakly HLA-A2pos normal breast tissue (Fig. 6A), but stains primary HLA-A2pos breast tumor tissue (Fig. 6B). We completed studies with breast tumor tissue from 15 HLA-A2– positive patients and detected (different percentages of cells stained at different levels of intensity) RL4B TCRm staining with each patient sample (Table I). Using an in-house grading protocol, a score was determined for 1) the proportion of cells stained in five fields, with 0 being negative and 4 representing staining of 76–100% of the cells, and 2) staining intensity that ranged from 0 (no signal) to 4 (very strong staining signal; see Materials and Methods). The 15 breast tumor tissue

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**FIGURE 2.** Colocalization of endogenous GVL peptide/HLA-A2 complexes by flow cytometric analysis and immunofluorescence staining reveal specificity of TCRm. Double staining was performed using 1000 ng/ml RL4B-PE and BB7.2-FITC with three human tumor cell lines: MDA-MB-231 (A), which expresses both hCGb and HLA-A2; MCF10A (B), which expresses hCGb but not HLA-A2; and the monocytic cell line THP-1 (C), which does not express hCGb but does express HLA-A2. MAb RL4B TCRm and BB7.2 co-stain MDA-MB-231 cells (A, quadrant 3). Neither Ab stained MCF10A cells (B, quadrant 1) and only BB7.2 stained THP-1 cells (C, quadrant 2). The background staining of the three cell lines MDA-MB-231 (A), MCF-10A (B), and THP-1 (C) was determined using IgG2a-PE and IgG2b-FITC tagged isotype control Abs.

**FIGURE 3.** Results of fluorescent immunocytochemical analysis determining RL4B TCRm Ab density and distribution under controlled conditions. The breast cancer cell line MDA-MB-231 (GVL peptide-HLA-A2 positive) was stained with Abs (red fluorescence) after cytospin followed by fixation in 5% methanol. The following Abs were used at 0.5μg/ml to stain MDA-MB-231 tumor cells: RL4B TCRm (A), BB7.2 mAb (B), IgG2a isotype control mAb (C), and IgG2b isotype control mAb (D). DAPI nuclear staining is represented by a blue signal.
samples were stained with RL4B TCRm and had mean scores of 2.5 for proportion of stain cells, 3.13 for intensity of stain, and 6.1 for a total score. In comparison, RL4B TCRm used to stain adjacent normal breast tissue from five patients had mean scores of 0.6 for proportion stain, 0.6 for intensity of stain, and 1.2 for a total score. The RL4B TCRm staining differences were found to be statistically different between breast tumor and adjacent normal breast tissue in all categories scored, indicating that GVL peptide/HLA-A2 complex expression is significantly greater in breast tumor tissue than in the adjacent normal breast tissue control. These initial studies validate the use of TCRm for detection of specific peptide/HLA complexes expressed on tumor tissue and suggest that the GVL peptide epitope may be widely expressed on HLA-A2 positive breast cancer tissue.

To further confirm the specificity of RL4B TCRm, competitive binding assays were performed. Breast cancer tissue sections were stained with RL4B TCRm alone or in the presence of a specific (GVL peptide/HLA-A2) or irrelevant (KIFS peptide/HLA-A2) tetramer. The findings shown in Fig. 6C demonstrate that RL4B TCRm staining was inhibited only in the presence of the GVL peptide/HLA-A2 tetramer, providing validation for expression of the GVL peptide/HLA-A2 epitope on primary breast cancer cells. As expected, BB7.2 mAb staining of tumor tissue sections was completely blocked with both peptide/HLA-A2 tetramers (Fig. 6C). These data demonstrate that TCRm is a useful reagent for the detection of specific peptide/HLA complexes expressed on tumor tissue.

**Discussion**

This report provides proof of concept for the use of monoclonal TCRm Abs in the recognition and destruction of tumor cells via targeting specific peptide/HLA epitopes. This study significantly expands on our previously published observations that characterize the functional properties of an Ab with the type of HLA-restricted peptide specificity associated with a TCR (41). We previously showed that RL4B TCRm stained and mediated lysis of cells bearing the GVL peptide–HLA complex on their surface by both CDC and ADCC, and it protected mice from growth of an implanted human breast cancer cell line (41). In this study, we used an orthotopic model to more closely mimic the tumor microenvironment for breast cancer. In contrast to the previous report, the therapeutic effectiveness of RL4B TCRm was assessed using two different models that initiated TCRm treatment after palpable tumors had formed. For the first in vivo study, mice implanted with MDA-MB-231 breast cancer cells in the mammary fat pad with tumor volumes $\geq 40 \text{mm}^3$ were treated for 4 wk with $100 \mu g$ RL4B TCRm ($n = 4$), $250 \mu g$ ($n = 6$), and $500 \mu g$ ($n = 5$). Mice ($n = 5$) were treated weekly with $500 \mu g$ IgG2a isotype control Ab for 4 wk. Tumor size was measured twice weekly using calipers, and tumor volume was determined using the standard volume formula. Data are plotted as mean tumor volume ± SEM. Significance ($p < 0.01$) was determined using the Student $t$ test.

**FIGURE 4.** RL4B TCRm inhibits and delays tumor growth in two orthotopic breast cancer models. Athymic nude mice were implanted with $5 \times 10^7$ (A) MDA-MB-231 or (B) MCF-7 tumor cells in the right mammary fat pad. Tumor cells were implanted in the presence of Matrigel and allowed to grow until mean tumor volume reached $\geq 40 \text{mm}^3$. Mice received an i.p. injection with $100 \mu g$ of either IgG2a isotype control Ab (black line; $n = 15$) or RL4B TCRm (red line; $n = 18$) on day 0 followed by weekly i.p. injections with $50 \mu g$ of Ab for $> 5$ wk. The histogram inset in A shows staining of MDA-MB-231 tumor cells with isotype control Ab (gray fill), RL4B TCRm (red line) and BB7.2 (black line). In B, mice received weekly 4 i.p. injections with $250\mu g$ of either IgG2a isotype control Ab (black line; $n = 4$) or RL4B TCRm (red line; $n = 4$) starting on day 0. The histogram inset in B shows staining of MCF-7 tumor cells with isotype control Ab (gray fill), RL4B TCRm (red line) and BB7.2 (black line). C. RL4B TCRm mAb-treated mice showed detectable levels of the TCRm in MDA-MB-231 tumor protein extracts by ELISA. Purified RL4B TCRm and mouse IgG2a isotype Ab were used as controls. Purified BB7.2 mAb was used to assess the activity of each of the tetramers. Triplicate wells coated with relevant or irrelevant tetramer were used to measure RL4B TCRm for each mouse tumor extract. Tumor size was measured twice weekly using calipers, and tumor volume was determined using the standard volume formula. Data are plotted as mean tumor volume ± SEM. Significance ($p < 0.001$) was determined using the Student $t$ test.

**FIGURE 5.** RL4B TCRm antitumor activity is dose-dependent in an orthotopic model of breast cancer. Female nude mice implanted with MDA-MB-231 breast cancer cells in the mammary fat pad with tumor volumes $>40 \text{mm}^3$ were treated for 4 wk with $100 \mu g$ RL4B TCRm ($n = 4$), $250 \mu g$ ($n = 6$), and $500 \mu g$ ($n = 5$). Mice ($n = 5$) were treated weekly with $500 \mu g$ IgG2a isotype control Ab for 4 wk. Tumor size was measured twice weekly using calipers, and tumor volume was determined using the standard volume formula. Data are plotted as mean tumor volume ± SEM. Significance ($p < 0.01$) was determined using the Student $t$ test.
markedly fewer copies of the GVL peptide/HLA-A2 target, yet when treated with RL4B TCRm showed significant reduction in tumor progression. These data demonstrate the ability of a TCRm Ab to inhibit tumor growth through targeting of a specific peptide/HLA class I complex.

The recent availability of soluble TCR and anti-HLA/peptide Abs has made it possible to target tumor cells via specific peptide/HLA complexes (34, 38, 50). Our approach to Ab production has led to the generation of high-affinity IgG Ab molecules that can mediate secondary immune effector functions, including CDC and ADCC (41). The $K_D$, determined using the equation $k_d/k_a = K_D$, and the dissociation $t_{1/2}$ of RL4B TCRm for cognate peptide/HLA complex was at least 10-fold greater than the binding affinity values reported for antipeptide/MHC Fab fragments isolated from phage-display libraries (36, 38, 51). Further, the bivalent nature of the TCRm IgG Ab molecules would increase binding avidity compared with a monovalent Fab fragment. Thus, the generation of Ab using an immunization/hybridoma strategy generally results in TCRm having high affinity and strong avidity for peptide/MHC. In contrast, many phage-derived Fab fragments specific for peptide-MHC may have to be molecularly engineered through modification of nucleotide bases in the hypervariable regions of the light and heavy variable chain genes, using an in vitro technique known as affinity maturation to improve binding affinity and sensitivity to enhance Ab targeting capabilities and effectiveness. Antipeptide/MHC Fabs have been engineered into tetramer complexes to increase binding avidity and detection sensitivity (52). The affinity of an Ab for its target often plays an important role in determining both its microdistribution and retention in a tumor (53). Low-affinity Abs generally show better penetration and diffuse farther into the tissue; however, this is not without associated costs. Ab uptake into the tumor is reduced, because a lower affinity interaction does not increase the amount of Ab entering tumor tissue, but actually increases the exit of free Ab. In addition, higher affinity binding Abs

Table I. RL4B tissue staining scores

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<th>Breast Tumor Tissue</th>
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Total (mean ± SEM)* 0.6 ± 0.24** 0.6 ± 0.24** 1.2 ± 0.49*** 2.7 ± 0.12*** 3.4 ± 0.16*** 6.1 ± 0.25***

* Normal adjacent proportion versus breast tumor proportion.
** Normal adjacent intensity versus breast tumor intensity.
*** Normal adjacent total versus breast tumor total.
* $p < 0.0001$ (Student t test).
NA, not applicable.
have been shown to increase tumor retention (54, 55). Moreover, therapeutic effector functions that require high receptor occupancy, such as ADCC, may be ineffective when combined with sub-saturating low-affinity binders (56).

We showed previously that RL4B TCRm activated both CDC and ADCC in vitro, and we observed the appearance of tumor-infiltrating NK cells after TCRm treatment in vivo. These observations suggest a possible role for NK cell interaction with the Fc domain of TCRm that leads to activation of ADCC and tumor cell death. We show that naked unconjugated TCRm IgG molecules have potent antitumor effects and postulate that effector mechanisms such as CDC and ADCC may be involved. In contrast, molecules lacking Fc domains, such as Ab Fab fragments and soluble TCRs, would not be expected to mediate CDC and ADCC activity because of the absence of an Fc domain. In several examples reported, antipeptide/MHC Fab fragments were effective at mediating tumor cell death only when covalently conjugated to a toxin (57–59). Moreover, Fab-Pseudomonas toxin conjugates were evaluated in cell killing assays in vitro or in tumor prevention models in vivo, but were not evaluated in more aggressive settings such as a therapeutic model as described in this study. It would seem that antipeptide/MHC Fab fragments and soluble TCR proteins that lack Fc domains would be limited to transporting payloads of drug and/or toxin to prevent tumor cell growth, a function that TCRm IgG Ab molecules could also perform. The significance of the Fc domain in mediating tumor cell destruction via targeting a specific peptide/MHC complex was shown using an in vivo tumor model and a soluble TCR protein that had been engineered into a bivalent TCR-human IgG1 chimeric molecule. The engineered fusion protein reduced metastatic disease possibly through recruitment and activation of ADCC (60). Our studies showed infiltrating NK cells in tumor lesions treated with RL4B TCRm, suggesting a possible role for ADCC on tumor growth. Further studies are being conducted by our group to investigate the mechanisms used by the TCRm in tumor killing in vivo. Nonetheless, the ability of a naked unconjugated IgG TCRm to significantly suppress and even eliminate established tumors in a dose-dependent manner in an orthotopic model of breast cancer suggests that Ab targeting of T cell epitopes could represent a novel modality for cancer treatment.

We attempted in this study to establish proof of concept that a naked unconjugated TCRm IgG Ab endowed with recognition specificity for the GVL peptide/HLA-A2 epitope could target tumors and mediate antitumor therapeutic effects in vivo. The MDA-MB-231 and MCF-7 breast carcinoma cell lines were selected because both are highly invasive tumor cells, with MDA-MB-231 expressing ~4000 complexes and MCF-7 expressing ~500 complexes of GVL peptide/HLA-A2 per cell, the most and least copies of this epitope detected from tumor cell lines tested by our group. We reported previously that RL4B TCRm mediated CDC cell lysis of peptide-pulsed T2 cells correlated with epitope density (41). It is not clear whether ~500 complexes represent a therapeutic threshold for TCRm Ab to mediate tumor killing. Further evaluation of the antitumor activity of RL4B TCRm will need to be performed using tumor cells that express fewer copies of GVL peptide/HLA-A2 complexes. However, these findings are significant when compared with the high copy number of Her2/neu receptors that are required for trastuzumab to be effective at killing tumors. In fact, trastuzumab therapy is effective against tumors having Her2/neu receptor expression at >1 million copies per cancer cell (61). This finding represents a difference in receptor copy number of >2000-fold between Her2/neu receptor and GVL peptide/HLA-A2 complexes expressed on MCF-7 cells and raises the possibility that antitumor properties other than CDC and ADCC might be responsible for the significant effects observed in vivo using RL4B TCRm. Future studies are planned to investigate possible direct effects of TCRm signaling via the MHC class I pathway.

The current report has several limitations that will need to be addressed in future studies. One shortcoming of the current study is that the study did not examine the long-term effects of RL4B TCRm on the generation of tumor escape variants. Data not reported in this study show that 4 wk after termination of TCRm treatment, small tumors excised from treated mice were stained with anti-HLA-A2 and RL4B TCRm Abs, suggesting that Ag-loss variants had not been generated. Ag-loss variants have been described for several abs, including trastuzumab (62). Follow-up studies are planned to assess the potential effects of TCRm Abs on selection of Ag-loss variants and tumor escape.

A second limitation of the study is that it did not evaluate alternative routes of administering RL4B TCRm. In particular, only i.p. injections were used, which might not be the most optimal method of administration. Studies are planned to examine the effect of intratumoral and i.v. injection of RL4B TCRm on tumor progression. Future studies will be performed to evaluate the biodistribution and pharmacokinetics of RL4B TCRm in treated mice. However, in this study we directly demonstrate TCRm mAb presence in mammary tumors after i.p. injection, supporting the ability of RL4B TCRm to target tumor tissue. Finally, studies will be performed to examine the possible interactions that RL4B TCRm and CTL to the same epitope might have on one another. We recently reported that 1B10 TCRm, a mouse IgG1 mAb reactive to HLA-A2/GVL, specifically blocked the cytolytic activity of HLA-A2/GVL reactive CTL. More recently, we have shown that RL4B TCRm inhibits binding of the 1B10 TCRm and blocks CTL activity. Thus, the question of whether the therapeutic effectiveness of RL4B TCRm would be limited by CTL blockade still remains to be addressed.

The ability of RL4B TCRm to affect tumor growth via targeting peptide/HLA complexes offers a potentially novel approach to cancer treatment. Although peptide/MHC class I complexes are the natural ligands for T cells, recent data presented by our group and others suggest they are also relevant targets for Abs (32, 33, 39, 58). TCRm technology may provide an alternative strategy to adoptively transferred T cells and/or vaccines to elicit T cell responses to attack cancer cells. There are many intrinsic obstacles to overcome with strategies aimed at using or stimulating T cells to achieve antitumor responses. Some of these challenges include 1) T cells that are functionally deficient, anergic or that are unable to fully differentiate in the tumor environment; 2) a tumor environment that lacks danger signals for innate immune stimulation; and 3) the presence of active regulatory mechanisms such as CD4+CD25+ T cells that could impede any endogenous immune reaction to cancer cells. The use of TCRm Abs provides a direct strategy to target cancer cells and induce tumor death, bypassing any need to trigger the stimulation of T cells.

The results obtained using this novel TCRm further indicate that the peptide-MHC complex is a legitimate target for cancer therapy by a whole IgG Ab and the level of expression of specific complex is high enough to lead to efficient lysis. A large number of peptide Ags from tumors that are recognized by T cells have been characterized previously and now offer new targets available on the tumor surface for TCRm mAb therapy (31). These TCRms open access to a new range of targets available on the cell surface that are independent of the ultimate location of the original protein to which they are directed. The ability to create effective TCRm recognizing such peptides in the context of MHC Ags presents the opportunity to significantly expand the current repertoire of therapeutic Abs.
Acknowledgments

We thank Dr. William P. Weidanz for critical discussion of the data, Dr. Stephen Wright for assistance with the mouse models, and Dr. Kathryn Norton for tissue acquisition.

Disclosures

J.A.W. is chief scientist and founder of Receptor Logic.

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ceptors variable and joining regions recognize the same tumor epitope. Cancer Res. 62: 5611–5617.


