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An HLA-Presented Fragment of Macrophage Migration Inhibitory Factor Is a Therapeutic Target for Invasive Breast Cancer

Oriana Hawkins,* Bhavna Verma,† Stanley Lightfoot,‡ Rinki Jain,§ Amit Rawat,‡ Sandy McNair,§ Shannon Caseltine,§ Aleksandar Mojsilovic,* Priyanka Gupta,§ Francisca Neethling,§ Othon Almanza,* William Dooley,‖ William Hildebrand,* and Jon Weidanz†,§

This report describes a novel HLA/peptide complex with potential prognostic and therapeutic roles for invasive breast cancer. Macrophage migration inhibitory factor (MIF) mediates inflammation and immunity, and MIF overexpression is observed in breast cancer. We hypothesized that the HLA class I of cancerous breast epithelial cells would present MIF-derived peptides. Consistent with this hypothesis, the peptide FLSELTQQL (MIF19–27) was eluted from the HLA-A*0201 (HLA-A2) of breast cancer cell lines. We posited that if this MIF19–27/HLA-A2 complex was exclusively found in invasive breast cancer, it could be a useful prognostic indicator. To assess the presentation of MIF peptides by the HLA of various cells and tissues, mice were immunized with the MIF19–27/HLA-A2 complex. The resulting mAb (RL21A) stained invasive ductal carcinoma (IDC) but not ductal carcinoma in situ, fibroadenoma, or normal breast tissues. RL21A did not stain WBCs (total WBCs) or normal tissues from deceased HLA-A2 donors, substantiating the tumor-specific nature of this MIF/HLA complex. As this MIF/HLA complex appeared specific to the surface of IDC, RL21A was tested as an immunotherapeutic for breast cancer in vitro and in vivo. In vitro, RL21A killed the MDA-MB-231 cell line via complement and induction of apoptosis. In an in vivo orthotopic mouse model, administration of RL21A reduced MDA-MB-231 and BT-20 tumor burden by >5-fold and by >2-fold, respectively. In summary, HLA-presented MIF peptides show promise as prognostic cell surface indicators for IDC and as targets for immunotherapeutic intervention. The Journal of Immunology, 2011, 186: 6607–6616.

Cells undergo numerous changes during the process of tumorigenesis, including genetic mutation, alterations in gene expression, and changes in protein processing. Some of these changes result in the ready availability of tumor-specific targets. For example, tumors overexpressing HER-2/neu on the outer surface of the cell’s plasma membrane are readily targeted by Abs such as trastuzumab (1). Unfortunately, the display of tumor Ags at the cell surface is a rare exception, and the vast majority of cellular changes associated with tumorigenesis are intracellular and not accessible to Abs.

Even if they are not intact, intracellular proteins distinct to cancerous cells can serve as targets for the immune system if they are displayed on the cell surface. The HLA class I molecules display a sampling of peptides from intracellular proteins on the cell’s plasma membrane. In this way, HLA class I molecules act as “nature’s proteome scanning chip” to provide the immune system with a panoramic view of the inner workings of the cell. Several intracellular systems, including proteasomal degradation and the rapid processing of defective ribosomal products, provide HLA class I molecules with peptides derived from virtually all cellular functions and compartments (2, 3). Indeed, a cornerstone of tumor immunology is recognition of cancer-specific, class I HLA-presented peptides by CD8+ CTLs (4–6). Class I HLA-presented peptides distinguish the surface of cancerous cells and promote their recognition by the adaptive immune system.

Because HLA class I molecules are able to reveal altered expression of intracellular proteins in tumors, a number of studies have searched for HLA/peptide complexes derived from cancer-associated Ags. Proteins such as hTERT, NY-ESO-1, and MAGE provide peptides for HLA class I presentation (7, 8), and these HLA/peptide complexes are being developed as immune therapies for cancer (9–11). One potential source for breast cancer-specific HLA/peptide complexes is macrophage migration inhibitory factor (MIF), a pleiotropic cytokine produced by several cell types, including epithelial cells and cells of the innate and adaptive immune system (12–14). In addition to roles in cell-mediated immunity, immune regulation, and inflammation, substantial data show that MIF has a broad impact on breast cancer progression (15–20). Several groups have explored MIF as a cancer...
diagnostic and therapeutic tool, and MIF secretion was initially used as a breast cancer prognostic in the leukocyte migration test (21). Serum MIF levels have likewise been proposed as a biomarker for prostate (22), colorectal (23), and breast cancer (24). Additionally, MIF has drawn a great deal of attention in cancer and autoimmune disease as a potential therapeutic target for neutralizing Abs, small interfering RNA, and small molecule inhibitors (25–27). To date, the results of these various applications have been mixed, and MIF remains a promising yet underdeveloped breast cancer biomarker and therapeutic target.

The initial objective of this study was to search MIF-producing cells for MIF-derived peptides presented by the class I HLA of cancerous breast cells but not healthy cells (28). A MIF-derived peptide was successfully eluted from the HLA class I of model breast cancer cell lines (28), and a mouse mAb (RL21A) specific for the MIF19–27 peptide in complex with HLA-A2 was generated. RL21A was used to screen for MIF19–27 presentation on a variety of cancerous breast cells and normal tissues. The resulting data demonstrate that the MIF19–27/HLA-A2 complex distinguishes invasive breast cancer tissue from normal breast tissue. Additionally, initial testing of the MIF19–27/HLA-A2 complex as an in vivo and in vitro target for the RL21A Ab provides encouraging data that support further development of immunotherapeutics directed toward MIF/HLA complexes.

Materials and Methods

Cell lines and total WBCs

The T2 cell line (American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 (Caisson Laboratories, North Logan, UT), 10% FCS, and penicillin/streptomycin (Invitrogen, Carlsbad, CA). MDA-MB-231, BT-20, MCF-7, MCF10A, and 184B5 (American Type Culture Collection) were maintained as previously described (28). Cell lines were transfected to produce soluble HLA-A*-0201 molecules as previously described (28). The BT-20 cell line was transfected with the full-length HLA-A*-0201–encoding plasmid to produce the BT-20-A2 cell line.

Total WBCs were isolated from apheresis kits obtained from Meeck Blood Center (Abilene, TX). RBCs were depleted with ACK lysing buffer (Quality Biological, Gaithersburg, MD).

Mass spectrometric analysis

Peptides eluted from the soluble HLA-A*-0201 of transfected MDA-MB-231, MCF-7, and MCF10A were analyzed by mass spectrometry as described (28).

Immunization

Mice were immunized with an emulsion containing 50 µg purified MIF19–27/HLA-A2 complexes and Quil-A adjuvant (Sigma-Aldrich, St. Louis, MO) to produce TCR mimic (TCRm) as previously described (29, 30).

ELISA

Hybridoma supernatants were incubated with MIF19–27/HLA-A2 or irrelevant tetramer and bound Ab was detected with peroxidase-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) with ortho-phenylene diamine substrate (Sigma-Aldrich).

Preparation of protein-conjugated nanoparticles

The nanoprecipitation technique was used for the preparation of carboxylated poly[ol-(lactide-co-glycolide)] (50:50). One hundred micrograms poly[ol-(lactide-co-glycolide)] (50:50) was dissolved in 10 ml acetone and slowly added to 20 ml deionized water with constant stirring. After complete acetone removal, the obtained nanoparticles were purified by centrifugation. The nanoparticles were suspended in 1 ml deionized water. To 100 mg polymer nanoparticle suspension, 40 mg N-(methylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC, Thermo Scientific, Rockford, IL) and 12 mg N-hydroxysuccinimide (NHS; Thermo Scientific) was added and incubated for 30 min. The unreacted EDC and NHS were removed by three washes, followed by the addition of 10 mg bifunctional NH2-polyethylene glycol–COOH. The mixture was stirred slowly at 50 rpm for 4 h. The nanoparticles were washed three times and reacted with EDC and NHS for 20 min. The nanoparticles were purified by centrifugation and suspended in 1 ml PBS (pH 7.4). One hundred milligrams full-length MIF protein (ProSpec, East Brunswick, NJ), peptide/HLA, or peptide alone was added to the 5 mg activated nanoparticles and incubated overnight at 4°C to obtain the protein-conjugated pegylated nanoparticles. Any unreacted protein was removed by three washes in PBS.

Flow cytometric-based immunosorbent assay utilizing nanoparticles

The 1 µg protein equivalent of nanoparticles (50 µg) was suspended in 100 µl FACS buffer (0.5% BSA and 2 mM EDTA in PBS [pH 7.4]). In designated tubes 0.5 µg Abs IgG2a isotype control or RL21A was added and incubated at room temperature for 30 min. After washing twice at 10,000 rpm for 10 min, nanoparticles were resuspended in 100 µl FACS buffer. The suspended nanoparticles were then incubated with PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 15 min. The nanoparticles were washed three times by centrifugation, resuspended in FACS buffer, and analyzed on a FACSCanto (BD Biosciences, Franklin Lakes, NJ).

Abs and synthetic peptides

The hybridomas BB7.2 (anti-HLA-A2.1 Ab; American Type Culture Collection), W6/32 (pan-HLA specific Ab; American Type Culture Collection), and RL21A were maintained in Hybridoma-SFM (In vitrogen). Abs were purified using protein G (GE Healthcare, Piscataway, NJ). IgG2a and IgG2b isotype controls were purchased from MP Biomedicals (Solon, OH). Anti-MIF mAb (mlgG1) was purchased from Abnova (Taipei, Taiwan).

Unmodified peptides were synthesized by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center (Oklahoma City, OK).

Surface plasmon resonance

Binding affinity experiments were performed using SensQ, a dual channel surface plasmon resonance bio-sensing system (ICX Nomadics, Oklahoma City, OK).

Flow cytometry

T2 cells (1 × 10⁶) were pulsed in RPMI 1640 and 1% FCS overnight with 10-fold dilutions of MIF19–27 or 10 µM irrelevant peptide. Pulsed T2, total WBCs, and breast cancer cell lines were stained with 1 µg/ml RL21A Ab, BB7.2, or isotype controls, followed by PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Cells were analyzed on a FACSCalibur or FACSCanto (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Tissue procurement

Cryopreserved breast tumor specimens and adjacent normal tissue were obtained from the University of Oklahoma Breast Institute Tissue and Fluid Bank (Oklahoma City, OK, Institutional Review Board no. 09823) or with Institutional Review Board approval from Hendrick Medical Center (Abilene, TX). HLA-A2+ tissues were selected by high-resolution sequence-based typing (Institutional Review Board no. 14660). Table 1 presents clinicopathological parameters for these tissues. Cryopreserved normal tissues from two HLA-A2+ deceased donors were purchased from Integrated Laboratory Services (Chestertown, MD).

Immunohistochemistry

OCT-embedded tissues were sectioned at 5 µm, fixed with 5% methanol, blocked in 2.5% horse serum, and stained with 1 µg/ml RL21A, BB7.2, or isotype controls. Complexes were detected with goat anti-mouse IgG-HRP (Vector Laboratories, Burlingame, CA) and ImmPact dianamobenzidine (Vector Laboratories) substrate. Hematoxylin QS (Vector Laboratories) was used as a nuclear counterstain. Tissues were analyzed using the Nikon 80i microscope and DXM1200C camera and NIS-Elements software (Nikon, Melville, NY). MIF19–27/HLA-A*-0201 or irrelevant peptide/HLA-A*-0201 tetramers were added at 10 µg/ml as blocking agents to demonstrate specificity.

Scoring

Tissues were scored, in a blinded manner, based on intensity of staining (0, no stain; 1, low intensity; 2, medium intensity; 3, high intensity) and percentage of cells stained (1, 1–10%; 2, 11–30%; 3, 31–60%; 4, >60%). The two scores are multiplied to produce a composite score of 0–12.
isotype control Ab (for LDH release as described above. W6/32 and BB7.2 were used as positive controls. Toxicity assay (Promega, Madison, WI) was housed in accordance with Texas Tech University Health Sciences Center policies (Institutional Animal Care and Use Committee no. 08024). In a therapeutic setting, mice were implanted with 10^6 freshly harvested MDA-MB-231 cells in Matrigel in the mammary fat pad. After tumors reached a volume of 30 mm^3, mice received weekly i.p. injections of 500 μg either IgG2a isotype control Ab (n = 10) or RL21A (n = 10) for 5 wk. Tumors were measured for 8 wk in a blinded manner. In a second model, mice were implanted with 5 × 10^4 BT-20-A2 cells as above. After 48 h, mice received weekly i.p. injections of 500 μg isotype control Ab (n = 10) or RL21A (n = 10). Mice were euthanized when the study ended, tumors became ulcerated, or tumor volume was greater than 1200 mm^3. Tumor volumes were calculated as follows: volume = length × width^2, where the tumor diameter was measured in two dimensions, and L indicates the longest diameter and b indicates the shortest diameter.

**Table I. Donor tissue characteristics**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDC</td>
<td>24/32</td>
<td>75.0</td>
</tr>
<tr>
<td>DCIS</td>
<td>4/32</td>
<td>12.5</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>4/32</td>
<td>12.5</td>
</tr>
<tr>
<td>Nodular grade I</td>
<td>4/24</td>
<td>16.7</td>
</tr>
<tr>
<td>II</td>
<td>12/24</td>
<td>50.0</td>
</tr>
<tr>
<td>III</td>
<td>6/24</td>
<td>25.0</td>
</tr>
<tr>
<td>Not determined</td>
<td>2/24</td>
<td>8.3</td>
</tr>
</tbody>
</table>

**Receptor**

| ER/PR/Her2       | 7/24 | 29.2 |

| ER/PR/Her2       | 10/24 | 41.6 |
| 4+/4−/−     | 1/24 | 4.2  |
| 4/−/−/4−     | 4/24 | 16.6 |
| 4+/4+       | 1/24 | 4.2  |
| −/−/4−       | 1/24 | 4.2  |
| Not determined | 7/24 | 29.2 |

**In vivo tumor models**

Female athymic nude mice (Crl:NU-Foxn1<sup>nu</sup>) (Charles River Laboratories, Wilmington, MA) were housed in accordance with Texas Tech University Health Sciences Center policies (Institutional Animal Care and Use Committee no. 08024). In a therapeutic setting, mice were implanted with 5 × 10^4 freshly harvested MDA-MB-231 cells in Matrigel in the mammary fat pad. After tumors reached a volume of >30 mm^3, mice received weekly i.p. injections of 500 μg either IgG2a isotype control Ab (n = 10) or RL21A (n = 10) for 5 wk. Tumors were measured for 8 wk in a blinded manner. In a second model, mice were implanted with 5 × 10^4 BT-20-A2 cells as above. After 48 h, mice received weekly i.p. injections of 500 μg isotype control Ab (n = 10) or RL21A (n = 10). Mice were euthanized when the study ended, tumors became ulcerated, or tumor volume was >1200 mm^3. Tumor volumes were calculated as follows: volume = L × b^2, where the tumor diameter was measured in two dimensions, and L indicates the longest diameter and b indicates the shortest diameter.

**Ab-dependent cellular cytotoxicity**

MDA-MB-231 or MIF<sub>19–27</sub>-pulsed T2 cells (2 × 10^5) were incubated with 10 μg/ml RL21A and Low-Tox-H rabbit complement (Cedarlane Laboratories, Burlington, NC) for 4 h, then assayed for lactate dehydrogenase (LDH) release using the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI). W6/32 and BB7.2 were used as positive controls.

**Apoptosis assay**

MDA-MB-231 cells (1 × 10^5) were treated with 500 or 1000 ng/ml RL21A or isotype control Ab for 4 h. Cells were stained with annexin V-allophycocyanin and propidium iodide (eBioscience, San Diego, CA). Camptothecin (10 μM) was used as a positive control for apoptosis. Flow cytometric analysis was performed on a FACSCanto. Percentage specific apoptosis was calculated as (treatment apoptosis − basal apoptosis) × 100/(100 − control apoptosis).

**In-cell ELISA for cleaved caspase-3 and poly(ADP-ribose) polymerase**

MDA-MB-231 cells (10,000 cells/well) were cultured for 24 h in wells on 96-well plates (Corning, Lowell, MA). Cells were treated in triplicate with RL21A or an IgG2a isotype control mAb (0.017, 0.034, and 0.067 μg) for 24 h. After treatment, media was removed and relative activation of caspase-3 and poly(ADP-ribose) polymerase (PARP) was measured using a cleaved caspase-3 colorimetric in-cell ELISA kit (Thermo Scientific) and a cleaved PARP colorimetric in-cell ELISA kit (Thermo Scientific) according to the manufacturer’s standard protocol.

**Statistical analysis**

A one-way ANOVA with a Dunnett multiple comparison test was used to compare immunohistochemistry scores for normal, benign, and tumor tissues. A two-tailed unpaired t test was used to analyze stromal tissue scores. A two-way ANOVA with a Bonferroni posttest was used to analyze in vivo and in vitro effects of RL21A. Prism 5 software (GraphPad Software, La Jolla, CA) was used for graphing and statistical analysis.

**Results**

**MIF<sub>19–27</sub> is presented by the HLA-A*0201 of tumorigenic breast cell lines**

The class I molecule HLA-A*0201 (hereafter HLA-A2) was isolated from two tumorigenic breast cell lines (MDA-MB-231 and MCF-7) and the nontumorigenic cell line MCF10A. Peptides were separated from the HLA-A2 molecule, fractionated by reverse phase HPLC, and comparatively mapped on an electrospray ionization/time-of-flight mass spectrometer. Mass spectrometric analysis revealed that a peptide fragment of MIF, MIF<sub>19–27</sub> (FLSELTQQL), is presented by the HLA-A2 of MCF-7 and MDA-MB-231 cells but not by the HLA-A2 of MCF10A cells (Fig. 1A). For reference, an RLP5-derived peptide, KIYEQQVEQ, is presented by HLA-A2 at equivalent amounts by all three cell lines when data are normalized to total peptide (Fig. 1B). Amino acid sequencing by tandem mass spectrometry confirmed the presentation of the MIF<sub>19–27</sub> peptide on the MCF-7 and MDA-MB-231 lines and its absence on the MCF10A line (data not shown and Ref. 28). We and others have previously demonstrated MIF expression by the MCF10A, MCF-7, BT-20, and MDA-MB-231 cell lines used in this study (18, 28). We conclude that the MIF peptide is differentially presented by the HLA class I molecules of these different cell lines, but it is unclear whether this is due to the increased levels of MIF expression in the tumorigenic cell lines or whether targeted degradation of MIF is enabled by autocrine stimulation of the cell lines that express the CD44/CD74 MIF receptor complex.

**RL21A is specific for the MIF<sub>19–27</sub>/HLA-A2 complex**

The direct biochemical evidence from mass spectrometric analysis showed that, among breast cell lines, the MIF/HLA-A2 complex was unique to tumorigenic cells. To further characterize the cell and tissue specificity of MIF<sub>19–27</sub> presentation by HLA-A2, immunohistochemistry was used to detect the expression of MIF<sub>19–27</sub>/HLA-A2 on primary cells and tissues. First, we generated a TCRm mAb specific for the MIF<sub>19–27</sub>/HLA-A2 complex. Splenocytes were harvested from BALB/c mice immunized with MIF<sub>19–27</sub>/HLA-A2 tetramers, and hybridoma supernatants were screened by ELISA for reactivity with the MIF<sub>19–27</sub>/HLA-A2 complex. One clone, RL21A, produced an IgG2a Ab specific for the MIF<sub>19–27</sub> peptide in the context of HLA-A2 and showed no reactivity to full-length MIF protein, free peptide, HLA-A2, or irrelevant peptide/HLA-A2 complexes as measured by a flow cytometric-based immunoassay utilizing nanoparticles (Fig. 2A). The RL21A clone was selected for further characterization.

Surface plasmon resonance was used to quantitatively analyze the binding between RL21A and the MIF<sub>19–27</sub>/HLA-A2 complex. The association rate (K<sub>a</sub>) was 5.3 × 10<sup>3</sup> M<sup>−1</sup> s<sup>−1</sup> and the dissociation rate (K<sub>d</sub>) was 1.29 × 10<sup>−3</sup> M<sup>−1</sup> s<sup>−1</sup>, giving an equilibrium dissociation constant (K<sub>eq</sub>) of 24.4 nM. The complex half-life (t<sub>1/2</sub>) was measured at 535 s (8.9 min). The response curves showed a goodness-of-fit and a low residual SD of 3.6 resonance units and the maximum binding response at surface saturation of 199 resonance units (Supplemental Fig. 1). Compared to the low affinity (1–100 μM) and short t<sub>1/2</sub> (seconds) of the TCR for its...
cognate peptide/MHC complex (31, 32), RL21A TCRm mAb displays very good affinity for its Ag. To confirm RL21A specificity, the TAP1- and TAP2-deficient T2 cell line was pulsed with the MIF19–27 peptide FLSELTQQL or irrelevant peptides and stained with RL21A Ab (Fig. 2B). RL21A was specific for the MIF19–27/HLA-A2 complex and failed to recognize irrelevant peptides presented by the HLA-A2 of pulsed T2 cells. The RL21A TCRm mAb was highly sensitive, clearly detecting MIF19–27/HLA-A2 complexes at peptide concentrations between 9 nM and 10 μM. Mean fluorescence intensity decreased relative to decreasing peptide concentration, indicating that RL21A staining is dependent on target density (Fig. 2C). The anti–HLA-A*0201 Ab BB7.2 was used to stain peptide-pulsed T2 to confirm that the T2 cell line was successfully presenting HLA-A2 molecules pulsed with the experimental peptides (Supplemental Fig. 2).

RL21A staining of breast cancer cell lines confirms MIF19–27 presentation by HLA-A2

The HLA-A2–positive tumorigenic cell lines MCF-7 and MDA-MB-231, from which MIF19–27 was isolated (28), showed RL21A staining, as did the BT-20-A2 cell line, which had been transfected with a plasmid encoding full-length HLA-A2 (Fig. 2D, Supplemental Fig. 3). RL21A weakly stained the nontumorigenic HLA-A2–positive 184B5 cell line, consistent with the low level of MIF19–27 presentation by this cell line observed by mass spectrometry (Supplemental Fig. 2). As expected, RL21A did not stain the HLA-A2–negative, nontumorigenic MCF10A cell line (Fig. 2D, Supplemental Fig. 3). The observed levels of RL21A staining were consistent with the normalized amount of peptide detected by mass spectrometry and confirmed the expression of MIF19–27/HLA-A2 on tumorigenic breast cancer cell lines. Furthermore, MIF protein expression was demonstrated in cell lines MDA-MB-231, BT-20-A2, and MCF-10A (Supplemental Fig. 4A).

**MIF19–27 /HLA-A2 expression distinguishes primary invasive breast tumors**

To test the specificity of MIF19–27 presentation in primary breast tissue, 23 cryopreserved, HLA-A2–positive breast tumors and matched adjacent normal tissues were stained for HLA-A2 and MIF19–27/HLA-A2. A blind system was used to score expression levels according to intensity of staining and percentage of cells

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**FIGURE 1.** MIF19–27 is presented by HLA-A*0201 of breast cell lines. **A**, Mass spectroscopy ion map showing MIF19–27, FLSELTQQL, at a mass to charge ratio (m/z) of 539.80, followed by its isotopes. Peaks are present in MCF-7 (black)- and MDA-MB-231 (dark gray)-derived peptide pool, but absent from the MCF10A (light gray) pool. **B**, An RPL5-derived peptide, KIYEGQVEV, is presented by HLA-A*0201 at an m/z of 532.79 in equivalent amounts by all three cell lines when data are normalized.
stained in each tissue section. HLA molecules are reported to be downregulated in 30–40% of primary breast tumors (33, 34). We therefore characterized the variability in total HLA-A2 expression levels in these tissues in addition to the levels of MIF19–27/HLA-A2. Tumor and benign tissue showed no significant difference in the amount of HLA-A2 expressed (Fig. 3A). There was a significant increase in HLA-A2 staining of tumor-associated stroma versus stroma of the normal adjacent tissues (p = 0.0083; Fig. 3C). The seeming conflict between these data and prior reports of HLA downregulation in tumors may be explained by the different targets analyzed; prior reports analyzed locus-specific or pan-HLA expression in tumors, whereas this analysis characterized allele-specific expression. To our knowledge, allele-specific contributions to HLA expression in breast tissues have not been previously addressed.

For MIF19–27/HLA-A2 to serve as a therapeutic target or prognostic indicator it must distinguish invasive cells from normal and benign tissues. We found that in invasive ductal carcinoma (IDC) tissues, a significantly higher percentage of cells stained with RL21A than in normal tissues, suggesting increased expression of MIF19–27/HLA-A2 (p = 0.0008; Fig. 3B). RL21A did not notably stain either fibroadenoma or ductal carcinoma in situ (DCIS). There was a significant increase in RL21A staining in IDC-associated stroma versus stroma of the normal adjacent tissues (p = 0.0331; Fig. 3C). A high level of staining by the HLA-A2–specific Ab BB7.2 was observed for both adjacent normal and tumor tissues (Fig. 3D and 3F, respectively), whereas MIF19–27/HLA-A2 staining by RL21A was restricted to tumor tissue (Fig. 3E, 3G). The specificity of RL21A staining was confirmed by competition with tetrameric and soluble MIF19–27/HLA-A2 complexes. Tissue staining was abrogated in the presence of MIF19–27/HLA-A2 tetramer (Fig. 3I), but not irrelevant peptide/A*0201 tetramer (Fig. 3H). Additionally, RL21A-positive breast tumor tissues were found to produce MIF protein (Supplemental Fig. 4B). These data indicate that high expression of the MIF19–27/HLA-A2 complex distinguishes IDC and its associated stroma from normal and diseased noninvasive breast tissues.

**MIF19–27/HLA-A2 is not expressed on normal tissues**

The MIF protein is produced by multiple cell types and is associated with inflammation and immunity. Thus, it is conceivable that MIF19–27/HLA-A2 complexes are found on a variety of cells and tissues in healthy individuals. To test the extent of MIF19–27/HLA-A2 presentation across various tissue types, we stained total WBCs from 18 HLA-A2–positive normal donors with RL21A (Fig. 4A). Flow cytometric analysis of the 18 samples revealed that all were negative for RL21A staining, indicating that WBCs do not express MIF19–27/HLA-A2 even though WBCs express MIF protein (data not shown). We next stained a panel of 20 cryopreserved tissues each from an HLA-A2–positive male donor and an HLA-A2–positive female donor with RL21A and BB7.2 (Fig. 4B). The 40 tissues in this panel revealed little to no recognition by RL21A, supporting the view that healthy cells and tissues do not express substantial amounts of MIF19–27/HLA-A2.

**RL21A TCRm shows significant anti-tumor effect in vivo**

To examine the possibility that the RL21A TCRm could have therapeutic effects, we studied two orthotopic breast cancer models. In the first study, MDA-MB-231 tumor cells were implanted in the mammary fat pad of athymic nude mice. Treatment with 500 μg RL21A (n = 10) or IgG2a isotype control mAb (n = 10) was initiated when tumor volume reached >30 mm³. Mice were given a total of five weekly i.p. injections. By week 5, mice that had received the isotype control mAb had mean tumor

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**FIGURE 2.** RL21A is specific for the MIF19–27/HLA-A2 complex. A, Nanoparticles loaded with full-length MIF protein, MIF19–27 (FLSELTOQL) peptide, MIF19–27/HLA-A2, YLLPAIVH/HLA-A2, or empty nanoparticles were stained with RL21A or isotype control. B, TAP-deficient T2 cells (1 × 10⁶) were pulsed with 10 μM MIF19–27 (black), 10 μM irrelevant peptides ILDKQKINEV (…), LLGRNSFEV (---), KVLEYVIKV (-----), or unpulsed (shaded) and stained with RL21A Ab. The irrelevant peptides shown are representative of results for six peptides tested. C, T2 cells (1 × 10⁶) were pulsed in triplicate with serial dilutions of MIF19–27 ranging from 10 μM to 9 nM, or 10 μM irrelevant peptide, and stained with RL21A Ab. D, The tumorigenic A2+ cell lines MCF-7 and MDA-MB-231, tumorigenic HLA-A2 transfectant BT-20-A2, nontumorigenic A2- MCF10A and A2+ 184B5 cell lines, and T2 pulsed with six irrelevant peptides were stained with RL21A. The relative amount of MIF19–27/HLA-A2 was determined by normalizing to total HLA-A2 as determined by BB7.2 staining.
volumes (MTV) that were >2-fold larger than mice treated with RL21A TCRm. The RL21A-treated mice continued to show reduced tumor growth even after the fifth and final week of treatment, and by week 8, the MTV was 5-fold greater in the control group than in the treated group ($p < 0.05$). These data demonstrate that RL21A can effectively reduce tumor growth and that its effects endure beyond the period of treatment (Fig. 5A).

Based on the ability of the RL21A TCRm to reduce the growth of MDA-MB-231 tumors in mice, we tested the effects of RL21A treatment on orthotopic tumors derived from a more aggressive and faster growing breast cancer cell line, BT-20-A2. Starting 48 h after BT-20-A2 cells were implanted into the mammary fat pad of athymic nude mice, 500 $\mu$g RL21A or isotype control Ab was administered weekly by i.p. injection (Fig. 5B). At day 5 palpable tumors were detectable in the control group ($n = 10$) but not in the group treated with RL21A TCRm ($n = 10$). By day 19, large tumors were observed in the control group (MTV, 723 mm$^3$), with many forming large ulcers. In contrast, significantly smaller tumors were observed in RL21A TCRm-treated mice (MTV, 360 mm$^3$; $p < 0.05$), with no evidence of ulcerated tumors. In the control group, tumor size reached volumes as large as 1700 mm$^3$ by day 21, but mice treated with RL21A had significantly smaller tumors (446 mm$^3$; $p < 0.001$). Taken together, these studies demonstrate that RL21A can significantly and effectively mediate anti-tumor responses in vivo.

**MIF$_{19–27}$/HLA-A2 sensitizes tumors to complement and cellular cytotoxicity by RL21A**

To further understand the powerful in vivo effects of targeting MIF$_{19–27}$/HLA-A2 with the RL21A TCRm, we explored the mechanisms of the anti-tumor activity of RL21A. It is known that Abs directed to tumor Ags are capable of mediating a variety of anti-tumor responses, including complement-dependent cytotoxicity (CDC). Incubation with RL21A and Low-Tox rabbit complement resulted in cytolysis of both MIF$_{19–27}$-pulsed T2 cells and MDA-MB-231 cells (Fig. 6A and 6B, respectively), indicating that the RL21A TCRm can induce CDC. Cytolysis of MDA-MB-231 cells was dose-dependent with respect to RL21A. RL21A also mediated Ab-dependent cellular cytotoxicity (ADCC) against MIF$_{19–27}$-pulsed T2 cells (Fig. 6C), as indicated by cytolysis in the presence of RL21A and PBMC. However, ADCC was not observed for MDA-MB-231 (Fig. 6D), suggesting that the number of

**FIGURE 3.** MIF$_{19–27}$ is detected on primary invasive ductal carcinoma and tumor-associated stroma. Cryopreserved, HLA-A2$^+$ IDC, DCIS, benign fibroadenoma, and matched normal adjacent tissues were stained for (A) HLA-A2 using the BB7.2 Ab and (B) MIF$_{19–27}$/HLA-A2 using RL21A. Tissues received a composite score of 0–12 based on percentage of cells stained and intensity of staining. Tumor-associated stroma and normal stroma were compared for (C) HLA-A2 and MIF$_{19–27}$/HLA-A2 staining. Representative staining is shown for (D) HLA-A2 and (E) MIF$_{19–27}$/HLA-A2 in normal adjacent tissue and for (F) HLA-A2 and (G) MIF$_{19–27}$/HLA-A2 in invasive ductal carcinoma. Specific staining with RL21A was confirmed by competing Ab with tetrameric MIF$_{19–27}$/HLA-A2 complexes. Tissue staining was abrogated in the presence of (I) MIF$_{19–27}$/HLA-A2 tetramer, but not (H) irrelevant peptide/A2 tetramer. Original magnification $\times 40$ (D–I), $\times 60$ (inset in G).
injections were initiated with 500 μg i.p. injections with 500 μg i.p.

RL21A induces apoptosis in a dose-dependent manner

TCRm mAbs to HLA/peptide complexes have been shown to directly kill tumor cells through the induction of tumor cell apoptosis (35). In this study, we investigated the ability of RL21A to mediate tumor cell death via the direct induction of apoptosis. MDA-MB-231 breast tumor cells were treated with RL21A or IgG2a isotype control mAb, and apoptosis was assessed by flow cytometric analysis. Treatment with RL21A induced apoptosis in ~25% of cells (Fig. 6F) and was dose-dependent (Fig. 6E). In comparison, isotype control mAb used at the same concentration showed significantly less apoptosis (p = 0.0381). As a positive control, camptothecin (10 μM) was used to induce cell death. We further confirmed the RL21A-mediated induction of apoptosis in a dose-dependent manner by measuring the activation of the executioner caspase-3 (Fig. 6G) and downstream cleavage of PARP (Fig. 6H) following treatment of MDA-MB-231 with RL21A compared with isotype control (p < 0.0001). The induction of MDA-MB-231 tumor cell apoptosis following Ab treatment demonstrates that in addition to CDC and ADCC, the RL21A TCRm can induce tumor death by direct ligation.

Discussion

A great deal of emphasis continues to be placed on the identification of markers for diagnosis, prognosis, and targeted therapies for cancer, including breast cancer. Observations associating MIF secretion and expression with breast cancer led us to test the hypothesis that MIF-derived peptides would be presented by the class I HLA of cancerous breast epithelial cells, and thereby provide an HLA/peptide indicator of breast cancer. Because the MIF<sub>19-27</sub> peptide was eluted from the HLA of breast cancer cell lines, our next goal was to validate whether the expression of MIF<sub>19-27</sub>/HLA-A2 distinguished tumor cells from normal tissues. Differential immunohistochemical staining of IDC established MIF<sub>19-27</sub>/HLA-A2 as a possible breast cancer prognostic indicator, and in vivo testing demonstrated that this putative indicator may have therapeutic value as well. Thus, MIF<sub>19-27</sub>/HLA-A2 warrants further investigation as a promising new indicator and therapeutic target for breast cancer.

Clinically, the three most relevant prognostic and treatment-guiding markers for breast cancer are estrogen receptor (ER), progesterone receptor (PR), and the epidermal growth factor receptor 2 (HER2). Much like ER, PR, and HER2, staining with RL21A shows that MIF<sub>19-27</sub>/HLA-A2 discriminates invasive cancer from benign and normal tissues. A direct comparison has not been completed, so we cannot say whether or how MIF<sub>19-27</sub>/HLA-A2 expression corresponds to ER, PR, and HER2 on invasive samples. MIF is overexpressed in ~30% of breast carcinomas (18). Based on the observation that HLA-A*0201 is expressed by 30% of the total population and up to 50% of the Caucasian population (36), we estimate that MIF peptide will be presented by HLA-A*0201 in at least 15% of all breast tumors. Similar to HER2 overexpression/amplification, ~30% of HLA-A*0201<sup>+</sup> invasive tissue samples present the MIF<sub>19-27</sub>/HLA-A2 complex at levels that are at least two SDs above normal expression levels (37). An integrated comparison of MIF<sub>19-27</sub>/HLA-A2 complexes, ER, PR, and HER2 will be required to elucidate the full value of MIF<sub>19-27</sub>/HLA-A2 as a novel prognostic or treatment-guiding marker for invasive breast cancer.

With advances in breast cancer screening and mammography, cancer registries now indicate that DCIS represents close to 20% of the breast carcinomas diagnosed in the United States (38, 39). DCIS itself is noninvasive, but cancer recurring after DCIS treatment is often invasive. Thus, there is considerable debate...
regarding the application of breast-conserving therapy following a DCIS diagnosis as well as indicators of recurrence following such therapy. Both noninvasive and preinvasive DCIS tissues are often positive for ER, PR, and HER2, making these markers inadequate for guiding the treatment of DCIS (40–42). The data presented in this study show that the RL21A TCRm does not stain noninvasive DCIS, suggesting that the MIF19–27/HLA-A2 complex merits further investigation as a candidate indicator for the

**FIGURE 6.** RL21A kills MIF19–27/HLA-A2+ cells in vitro. A. MIF19–27- or YLLPAIVHI-pulsed T2 cells or (B) MDA-MB-231 cells were incubated with RL21A and Low-Tox rabbit complement for 4 h and then assayed for LDH release. C. MIF19–27-pulsed T2 cells or (D) MDA-MB-231 cells were incubated with RL21A and donor PBMC at an E:T ratio of 25:1 for 4 h, then assayed for LDH release. The pan–HLA-specific Ab W6/32 was used as a positive control. E. MDA-MB-231 cells were treated for 4 h with RL21A or IgG2a isotype control at 500 and 1000 ng/well, and apoptosis was detected via annexin V-allophycocyanin conjugate and propidium iodide assay. F. MDA-MB-231 cells were treated with 1000 ng/ml of TCRm RL21A or IgG2a control, and apoptosis was assessed 4 h later. Camptothecin (10 μM) was used as a positive control for apoptosis. MDA-MB-231 cells were treated with 0.017, 0.034, and 0.067 μM RL21A or isotype control for 24 h and were assayed for cleavage of (G) caspase-3 and (H) PARP. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
The dual use of HER2 as 1) a prognostic marker and 2) a therapeutic target via trastuzumab has provided a highly successful model for the treatment of breast cancer. This model served as a blueprint for these studies investigating the MIF\textsubscript{19–27}/HLA-A2 complex. RL21A treatment of two orthotopic mouse models of breast cancer resulted in a dramatic reduction in tumor burden. In the MDA-MB-231 study a 5-fold tumor reduction was observed, a result that is consistent with the in vitro ability of RL21A to kill MDA-MB-231 cells by CDC and ADCC. When the more aggressive BT-20-A2 tumor was tested in this same model, RL21A administration yielded a >2-fold tumor reduction. This magnitude of tumor reduction is consistent with the previously reported in vivo anti-tumor activity of a TCRm directed toward a p68 peptide/HLA-A2 complex (35, 43). The promising in vivo tumor reduction achieved through the targeting of HLA/peptide complexes with TCRm demonstrates that the HLA of cancerous tissues may offer a rich horizon of new complexes that can be successfully targeted by TCRm mAb.

The significant in vivo reduction in MDA-MB-231 tumor burden was consistent with the in vitro induction of apoptosis in these cells. However, the significant in vivo reduction in BT-20-A2 tumor burden was somewhat surprising because RL21A demonstrated only a modest ability to kill these cells in vitro. We have recently demonstrated that TCRm directly activates caspases, apoptosis, and cell death by signaling through specific MHC/peptide complexes (35), and we suspect that RL21A induced in vivo killing of BT-20-A2 tumors by the same mechanism. Indeed, Verma et al. (35) showed immune effector-independent anti-tumor mechanisms in vivo using F(\text{ab})\textsubscript{2} fragments of a different TCRm in the same BT-20-A2 tumor model. Those findings and the modest ADCC and CDC activity observed in vitro for RL21A against BT-20-A2 tumor cells in the current study may point to a CDC- and ADCC-independent mechanism of action for RL21A in vivo. Also of interest is that both of the cell lines used in the present study are phenotypically triple negative (ER-, PR-, and HER-2–low), suggesting that RL21A could be valuable to a subpopulation of breast cancer patients bereft of targeted therapies. Taken together, these observations indicate that RL21A has potential as a therapeutic agent, although the in vivo mechanisms of tumor reduction warrant further investigation.

The described in vivo testing of RL21A provides a model whereby passive immunization with a TCRm specific to MIF\textsubscript{19–27}/HLA-A2 reduces tumor burden. It would also seem prudent to test the MIF\textsubscript{19–27}/HLA-A2 complex for its ability to generate a protective CTL response. Viral peptide fragments that were discovered by direct HLA peptide elution and validated as unique to infected cells by specific binding of TCRm (44) have been successfully tested in a CTL-eliciting vaccine (45). Given the recent success of a vaccine that targets T cells to HLA-presented fragments of prostatic acid phosphatase for the treatment of prostate cancer (46), testing the entire MIF Ag or additional MIF peptides in a vaccine designed to direct CTL to cancerous cells is another logical step. One outstanding question that we will address is whether HLA molecules other than HLA-A2 present fragments of MIF on the surface of invasive breast cancer cells; these further investigations will identify the MIF-derived epitopes and HLA alleles that may provide suitable targets for TCRm therapy or a CTL-eliciting anti-cancer vaccine.

A number of studies have linked MIF with cancer progression and, although it shows promise as a cancer biomarker (47), MIF has not emerged as a clinical indicator for cancer (24, 25). One difficulty in using MIF as a prognostic indicator is the current lack of understanding regarding the individual and combined roles of intracellular MIF, secreted forms of MIF, and MIF receptor signaling on overall tumor growth. Recent findings suggest that a better prognosis is linked to abundant intracellular MIF, whereas secreted MIF enhances invasiveness in the presence of its receptor (20). In this study, we demonstrate MIF\textsubscript{19–27}/HLA-A2 presentation on the MDA-MB-231 cell line, which is known to have low constitutive levels of intracellular MIF and an ability to up-regulate MIF secretion in response to autocrine stimulation (20). Furthermore, although MIF is expressed by WBCs, our results did not show RL21A staining of WBCs, suggesting poor correlation between MIF protein expression and presentation of MIF\textsubscript{19–27}/HLA-A2 in normal cells. In the past, it was presumed that HLA molecules sample every protein and that peptide/HLA density was dependent on expression levels of the protein Ag. However, our findings with Her2/neu and other recent evidence with the hTERTp540 peptide/HLA-A2 complex suggest that protein levels may not correspond to HLA/peptide complex formation (9, 29). The immunodominant presentation of the HER\textsubscript{2669–77} peptide/HLA-A2 complex does not correlate with levels of Her2/neu protein expression in tumor cells. Moreover, prediction of CTL responses correlated with levels of specific peptide/HLA complex but not protein expression. Still others have shown that although CTL directed to hTERTp540 could be generated by vaccination, hTERT-positive tumors were not lysed (9) because Ag processing did not produce this peptide (48). Because intracellular MIF levels remain low and secreted MIF levels increase due to autocrine stimulation in cell lines presenting MIF\textsubscript{19–27}, we propose the presentation of MIF\textsubscript{19–27} is linked to the availability of defective ribosomal products (49). It is possible that tumor cells having such defective ribosomal products could preferentially load MHC class I molecules with cytosolic-derived peptides, as observed in this study with MIF\textsubscript{19–27}/HLA-A2 complexes presented on tumor cells. Future studies are planned to test this hypothesis. Finally, we expect the use of the direct peptide discovery and validation techniques described herein to yield new insights pertaining to tumor-associated changes in protein processing and presentation.

In summary, a number of studies have linked MIF with cancer progression and, although it shows promise as a cancer biomarker (47), MIF has not emerged as a clinical indicator for cancer (24, 25). In this study, we identified a MIF\textsubscript{19–27}/HLA-A2 complex on the triple-negative MDA-MB-231 and BT-20-A2 cell lines, we generated an RL21A TCRm mAb specific for this peptide/HLA complex, and we distinguished invasive breast cancer from normal tissues and benign lesions via the MIF\textsubscript{19–27}/HLA-A2 complex. These data demonstrate that MIF/HLA complexes may represent prognostic indicators for invasive breast cancer. Furthermore, using Her2/herceptin as a developmental model, MIF peptide/HLA complexes were successfully targeted in vivo with the mAb RL21A. Future characterization of MIF peptide/HLA complexes to assess their utility as prognostic indicators and to ascertain their value as therapeutic targets is warranted. Finally, the data presented in this study give hope that MIF peptide/HLA complexes distinct to breast cancer can be discovered, characterized, and targeted in a tightly coordinated manner.
Disclosures

J.A.W. is the Chief Scientist and Founder of Receptor Logic, Inc. The remaining authors have no financial conflicts of interest.

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