Human Leukocyte Antigen-Presented Macrophage Migration Inhibitory Factor is a Surface Biomarker and Potential Therapeutic Target for Ovarian Cancer

Andrea M Patterson¹, Saghar Kaabinejadian¹, Curtis P McMurtry¹, Wilfried Bardet¹, Ken W Jackson¹, Rosemary E Zuna², Sanam Husain², Gregory P Adams³, Glen MacDonald³, Rachelle L. Dillon³, Harold Ames⁴, Rico Buchli⁵, Oriana E Hawkins⁴, Jon A Weidanz⁴, and William H Hildebrand¹,⁵*

Author Affiliations: ¹Department of Microbiology and Immunology, and ²Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104, ³Viventia Bio Inc., Winnipeg, MB, R3T 3Z1, ⁴Department of Immunotherapeutics and Biotechnology, Texas Tech University Health Sciences Center, Abilene, TX, 79601, ⁵Pure MHC LLC, Oklahoma City, OK, 73104

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Abbreviations List: HLA, human leukocyte antigen; MIF, macrophage migration inhibitory factor; CAR, chimeric antigen receptor; EpCAM, epithelial cell adhesion molecule; EGFR, epidermal growth factor receptor; FRα, folate receptor alpha; sHLA, soluble HLA; RP-HPLC, reverse-phase high performance liquid chromatography; MS, mass spectrometry; IHC, immunohistochemistry

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*Corresponding Author: William H. Hildebrand, PhD, Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, 975 NE 10th Street, BRC-317, Oklahoma City, OK, 73104. Phone: 405-271-1203; Fax: 405-271-3117; Email: William-Hildebrand@ouhsc.edu

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ABSTRACT

T cells recognize cancer cells via human leukocyte antigen (HLA)/peptide complexes and, when disease overtakes these immune mechanisms, immunotherapy can exogenously target these same HLA/peptide surface markers. We previously identified an HLA-A2-presented peptide derived from macrophage migration inhibitory factor (MIF) and generated antibody RL21A against this HLA-A2/MIF complex. The objective of the current study was to assess the potential for targeting the HLA-A2/MIF complex in ovarian cancer. First, MIF peptide FLSELTQQL was eluted from the HLA-A2 of the human cancerous ovarian cell lines SKOV3, A2780, OV90, and FHIOSE118hi and detected by mass spectrometry. By flow cytometry, RL21A was shown to specifically stain these four cell lines in the context of HLA-A2. Next, partially matched HLA-A*02:01+ ovarian cancer (n=27) and normal fallopian tube (n=24) tissues were stained with RL21A by immunohistochemistry to assess differential HLA-A2/MIF complex expression. Ovarian tumor tissues revealed significantly increased RL21A staining compared to normal fallopian tube epithelium (p<0.0001), with minimal staining of normal stroma and blood vessels (p<0.0001 and p<0.001 compared to tumor cells) suggesting a therapeutic window. We then demonstrated the anti-cancer activity of toxin-bound RL21A via the dose-dependent killing of ovarian cancer cells. In summary, MIF-derived peptide FLSELTQQL is HLA-A2-presented and recognized by RL21A on ovarian cancer cell lines and patient tumor tissues, and targeting of this HLA-A2/MIF complex with toxin-bound RL21A can induce ovarian cancer cell death. These results suggest that the HLA-A2/MIF complex should be further explored as a cell-surface target for ovarian cancer immunotherapy.
INTRODUCTION

Ovarian cancer is among the top five most deadly cancers in women, with an estimated 14,180 deaths in the year 2015 for the United States alone (1). The majority of cases (61%) reach an advanced stage before detection and, despite initial response to surgery and platinum-based chemotherapy, have a 5-year survival rate of only 27% (1). New approaches are pressingly needed, and today immunotherapeutic tumor targeting strategies such as monoclonal antibody therapy or adoptive transfer of cancer-directed T cells are proving increasingly effective (2-4). For example, the anti-HER2 antibody trastuzumab is significantly extending survival in breast cancer patients (5) and CD19-directed chimeric antigen receptor (CAR) T cell therapies are reaching 90% complete response rates in multiple clinical trials of B-cell acute lymphoblastic leukemia (6). These exciting breakthroughs indicate the value of immune therapies, but truly successful extension of these strategies to several cancer types, including ovarian, thus far remains elusive.

One element common to these and other immunoncology successes is the exploitation of effective cancer cell-surface markers for direct tumor targeting. A handful of candidate markers have been explored clinically in ovarian cancer such as epithelial cell adhesion molecule (EpCAM) or epidermal growth factor receptor (EGFR) as antibody targets, or folate receptor-α (FRα) as a target for CAR T cell therapy (7, 8). Early trials with these targets are proving somewhat efficacious, especially in combination with chemotherapies, and as immunotherapies become more refined these targets may prove increasingly effective. An important consideration in targeted therapy is the significant heterogeneity that is particularly apparent in ovarian cancer (9). Even when patient-specific, a single antigen will rarely be sufficient for eradicating ovarian
tumors; successful strategies will likely target multiple patient-relevant markers at once in an effort to prevent the escape mutants that spawn recurrence. As immunotherapy strategies continue to evolve, new cell-surface antigens are needed towards effective ovarian cancer targeting.

Numerous molecular changes distinguish cancerous cells from healthy cells, but the vast majority of these are hidden inside the plasma membrane and inaccessible to antibody or CAR T cell therapies. As such, human leukocyte antigen (HLA) molecules play a pivotal role exposing tumor cells for immune recognition. In ovarian cancer, dysregulated intracellular proteins are naturally targeted at the cell surface when T cells recognize class I HLA molecules presenting altered-self peptides; the presence of tumor infiltrating CD8+ lymphocytes corresponds with significantly longer survival for ovarian cancer patients, demonstrating this phenomenon (10-12). This is the basis for cancer vaccine strategies using peptides from tumor antigens such as NY-ESO-1, HER2, p53, or MUC-1, as well as immune checkpoint inhibitors which unleash T cells for recognition of tumor-associated HLA/peptide complexes (7, 8). In addition to vaccine elicited, chimeric, and checkpoint-released T cells, a new class of monoclonal antibodies that mimic the T cell receptor are able to single out HLA/peptide complexes that mark the surface of cancerous cells (13, 14). In this way, HLA/peptide complexes reveal a wealth of intracellular protein changes that can be accessed by monoclonal antibodies and derivative therapeutic strategies.

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine with dramatic overexpression and tumor-promoting properties in ovarian cancer (15-18). MIF production by
ovarian tumors correlates with increased macrophage infiltration and stimulates macrophage functions that in turn promote tumor invasiveness (15, 17). MIF also appears to support ovarian tumor survival and proliferation through p53 inhibition and Akt activation, and to promote angiogenesis through TNF-a, IL-6, and VEGF (17, 19). Furthermore, increased MIF expression in both classical ovarian cancer cells and in ovarian cancer stem cells suggests it may be developed as a marker for both (20, 21). Strategies targeted to MIF in various contexts have included small-molecule inhibitors, neutralizing antibodies, and small interfering RNA (22-24). However, the discovery of a MIF-derived peptide that is presented by the class I HLA of cancer cells offers the unique opportunity to target MIF in the form of a cancer cell-surface antigen (25, 26). The hypothesis tested here is that ovarian cancer will be susceptible to targeting through the HLA-presented MIF peptide. To test this hypothesis we first demonstrated proteomically that MIF peptide FLSELTQQL can be consistently eluted and sequenced from the HLA of ovarian cancer cell lines. The subsequent proficiency of the HLA/MIF-specific antibody RL21A in selectively staining ovarian cancer tissue and in mediating toxin-conjugated killing of ovarian cancer cells further implicates HLA/MIF as a cell-surface biomarker and potential target for exploitation in ovarian cancer immunotherapy.
METHODS

Cell lines

SKOV3 cells were purchased from the ATCC in 2011 and grown in DMEM/F12K medium with 10% FBS. A2780 cells were purchased from Sigma in 2012 and grown in RPMI with 10% FBS. FHIOSE-118hi cells and OV90 cells were received in 2011 as kind gifts from Dr. Patricia Kruk, University of South Florida, and were both grown in 1:1 Medium 199/MCDB 105 with 15% FBS. FHIOSE-118hi is an artificially transformed human ovarian cell line that is tumorigenic in mice (27), originating from the lab of Dr. Kruk and hereafter referred to as FHIOSE, and OV90 cells were purchased from ATCC in 2001 and sent to our lab from a low-passage frozen stock. T2 cells generated by Dr. Peter Cresswell, Duke University (28), were received as a kind gift from the Cresswell lab and grown in RPMI with 10% FBS. All cell lines were subjected to high-resolution sequence-based HLA typing (HLA-A, -B, -C, & -DRB1) immediately upon receipt and growth in our laboratory, and then again after stable transfection and subcloning to ensure authentication prior to use in data collection.

Production of soluble HLA-A*02:01 and peptide purification

Soluble HLA (sHLA) was produced and peptides purified as previously described (29). Briefly, each cell line was stably transfected with the sHLA-A*02:01 construct, subcloned, and grown at high density in hollow-fiber bioreactors. The sHLA/peptide complexes were isolated from supernatants over an immunoaffinity column using an engineered C-terminal purification tag. Peptides were removed by acid boil, purified through a 3 kDa filter, and lyophilized.
Liquid chromatography (LC) and mass spectrometry (MS)

First dimension LC: Peptides were resuspended in 10% acetic acid and fractionated over a 150 mm long C$_{18}$ column (2 mm internal diameter Gemini column; pore size, 110 Å; particle size, 5 μm; Phenomenex) for reverse-phase high-performance LC (RP-HPLC) using a Michrom BioResources Paradigm MG4 instrument. An acetonitrile (ACN) gradient was run at pH 10 using a two-solvent system. Solvent A contained 2% ACN in water and solvent B contained 95% ACN in water, each with 10mM ammonium formate. The column was pre-equilibrated at 2% solvent B. Peptide was loaded at a flow rate of 120 μl/min over an 18 minute period, and then two linear gradients were run at 160 μl/min: 4-40% B for 40 min, followed by 40-80% B for 8 min.

Second dimension LC: Forty peptide-containing first dimension HPLC fractions were dried, resuspended in 10% acetic acid and applied individually to nano-scale RP-HPLC (Eksigent nanoLC415, AB Sciex) including a 0.5 mm long C$_{18}$ trap column (350 μm internal diameter; ChromXP) with 3μm particles and 120Å pores, and a 15 cm long C$_{18}$ separation column (75 μm internal diameter; ChromXP) packed with the same medium. An ACN gradient was run at pH 2.5 using a two-solvent system. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in 95% ACN in water. The column was pre-equilibrated at 2% solvent B. Samples were loaded at 5 μl/min flow rate onto the trap column and run through the separation column at 300 nl/min with two linear gradients: 10-40% B for 70 min, followed by 40-80% B for 7 min.

MS: The column effluent was injected using the nanospray III ion source of an AB Sciex TripleTOF 5600 quadrupole time-of-flight mass spectrometer with the source voltage set to 2400 v. Information dependent analysis (IDA) of peptide ions was acquired based on a survey scan in
the TOF-MS positive-ion mode over a range of 300-1250 m/z for 0.25 sec. Following each survey scan, up to 22 ions with a charge state of 2 to 5 and intensity of at least 200 counts were subjected to collision-induced dissociation (CID) for tandem MS analysis (MS/MS) over a maximum period of 3.3 seconds. Selection of a particular ion m/z was excluded for 30 sec after 3 initial MS/MS experiments. Dynamic collision energy was utilized to automatically adjust the collision voltage based upon ion size and charge. PeakView® Software version 1.2.0.3 was used for data visualization.

Antibodies

The BB7.2 (pan-HLA-A2 monoclonal antibody) expressing mouse hybridoma cell line was purchased from the ATCC. The RL21A hybridoma was generated by PureMHC as previously described (26). BB7.2 and RL21A hybridomas were grown in Hybridoma-SFM (Invitrogen), and antibodies were purified using protein G (GE Healthcare, Piscataway, NJ). Mouse IgG2a isotype control antibody was purchased from R&D Systems (20102; Minneapolis, MN). Mouse IgG2b isotype control antibody was purchased from BioLegend (MG2b-57; San Diego, CA). Goat anti-mouse IgG AffiniPure F(ab’)_2 fragment conjugated to R-Phycoerythrin was purchased from Jackson ImmunoResearch Laboratories, Inc. (115-116-071; West Grove, PA).

Flow Cytometry

SKOV3 and A2780 cells were first transfected with a full-length HLA-A*02:01 construct as previously described (30), and the resulting SKOV3-A2 and A2780-A2 along with SKOV3, A2780, FHIOSE, and OV90 cells (5 x 10^5) were stained with 0.01 mg/ml of RL21A, BB7.2, or isotype controls for 30 min followed by PE-conjugated goat anti-mouse IgG for 30 min. Cells
were analyzed on a Stratedigm S1200Ex flow cytometer and data analysis performed using FlowJo Software (Tree Star, Ashland, OR).

**RL21A peptide specificity T2 assay**

TAP-deficient T2 cells expressing HLA-A*02:01 ($1 \times 10^6$) were pulsed in AIM-V Medium (Gibco) for 4 hours with relevant FLSELTQQL peptide or irrelevant HLA-A2-binding VLQGVLPAL, YLEPGPVTV, YLEEMLWRL, AIMDKNILL, GVLAPLPQV, KIGEGTYGV, TMTRLLQGV, ALMPVLNQV, YELPGPVTV, SVGGVFTSV, NLVPMVATV peptides at 20μM. The pulsed T2-A2 cells were stained with 1 μg/ml RL21A, BB7.2, or isotype control, followed by the PE-conjugated goat anti-mouse IgG. Cells were analyzed on FACSCanto II (BD Bioscience). Data analysis was performed using FlowJo Software (Tree Star, Ashland, OR).

**HLA cross-reactivity screen**

*Assay generation:* Production and purification of 120 different recombinant intact class I sHLA molecules was performed as described previously with minor modifications (31). To immobilize the purified sHLA molecules onto a solid Luminex bead support, they were first tagged with the biotin derivative NHS-PEG12-Biotin (Thermo Fisher Scientific, Rockford, IL) according to manufacturer’s instructions. An empirically determined molar ratio of 20:1 (Biotin-NHS:sHLA protein) was applied so that on average not more than one tag was bound to each sHLA protein. In a total volume of 10 mL, a titrated concentration of individual sHLA probes (to give 95% loading efficiency) was mixed with 2.5 x $10^6$ LumAvidin beads (L100-Lxxx-01; Luminex, Austin, TX) in PBS + 1% BSA and incubated for 60 minutes with light shaking. Beads were washed 4 x in PBS (spins 10 min at 5300 x g) and resuspended in PBS + 1% BSA + 0.05%
sodium azide for storage. To create the multiplex assay, beads were combined into 2 sets: A/C alleles and B alleles. A panel of 25 controls was also created including human immunoglobulins (IgG, IgM, IgA, IgE, IgG1/2/3/4), mouse immunoglobulins (IgG1, IgG2a, IgM), kappa, lambda, β2-microglobulin, BGG, BSA, HSA, as well as HLA-G and E, CD1c and C1q.

**RL21A screen:** The target peptide for RL21A (FLSELTQQL) was incubated with the sHLA-Luminex beads in 6000-fold excess at 53˚C for 15 min, followed by 48 h at 4˚C for complex re-assembly with peptide. (Note that sHLA with incompatible binding motifs for this peptide will not replace the original endogenous peptides.) RL21A (25 μg/ml) was incubated with each set of beads for 60 min at 4˚C, washed four times and then labeled with anti-mouse Fc conjugated to phycoerythrin and incubated for a further 60 min at 4˚C. Fluorescence intensity and bead labels were visualized on a Luminex 100 reader. Data were collected for a minimum of 400 antigen-coated beads. Reported values represent median fluorescence intensity after subtraction of the secondary-only background signal determined for each bead individually and adjusted by applying the internal negative control beads as a lower threshold value.

**Human Tissues**

Cryopreserved human tissue samples were acquired from the University of Oklahoma Stephenson Cancer Center Biospecimen Core (Oklahoma City, OK) following approval from the Scientific Review Committee (#200908-007) and the Institutional Review Board (#3159). Samples were chosen based on a diagnosis of serous ovarian carcinoma, as this is the most common subtype, and HLA-A*02:01+ tissues were selected by high-resolution sequence-based HLA typing of matched blood samples. A total of 84 blood samples were HLA typed, and 39 HLA-A*02:01+ tissues or tissues pairs (tumor and/or fallopian tube) were obtained. H&E
staining was used to verify tissue identities and the presence of evaluable cells. As indicated in Table 1, 27 serous ovarian carcinoma tissues and 24 normal fallopian tube tissues were evaluable, 14 pairs of which were matched tumor and normal. Patient characteristics are summarized in Table 1.

Immunohistochemistry (IHC)

Cryopreserved tissues were sectioned at 6μm, fixed with 5% methanol in PBS for 12 min, then allowed to air dry for at least 15 min. Tissues were encircled with a PAP pen and blocked for 20 min with normal horse serum (Vector Labs). Primary antibodies were added for 45 min: BB7.2 (pan-HLA-A2) at 1 μg/ml, RL21A (HLA-A2/MIF) at 2 μg/ml, or mouse IgG2a isotype control at 2 μg/ml. An HRP-conjugated anti-mouse Ig secondary was applied for 30 min (ImmPRESS reagent, Vector Labs). Slides were developed for 4 min with DAB, counterstained with hematoxylin, and dehydrated through changes of 80%, 95%, and 100% reagent alcohol, then xylene, and coverslipped with non-aqueous mounting medium. Tissues were scored on a sliding scale from 0-3 for staining intensity and from 0-4 for percentage of cells stained (0, none; 1, 1-10%; 2, 11-30%; 3, 31-60%; 4, 61-100%). These two values were multiplied together to give a composite score of 0-12. The isotype control was used to subtract background staining. Two gynecologic pathologists (authors R.Z. and S.H.) scored the tissues independently and scores were averaged. Scoring was performed from the original slides using OLYMPUS BX43 microscopes, and images were taken with a NIKON NI-U and NIS-Elements software (Nikon).
**Cytotoxicity assays**

Where unconjugated antibody was used, cells were treated with various concentrations of RL21A (0.1 pM – 10 nM) along with 20nM cytotoxic secondary antibody (Fab fragment of anti-mouse IgG Fc conjugated with Duocarmycin DM with cleavable linker, Moradec LLC) for 72 h. Alternatively, RL21A was directly conjugated to the de-antigenized plant toxin deBouganin (32, 33) using a method described previously (34), and cells were treated with various doses of the immunotoxin (0.008 pM – 80 nM) for 72 h. MTS reagent was then added at 20 μl/well and incubated for 3 h. Optical density (OD) was detected at 490 nm by a SpectraMax 190 Microplate Spectrophotometer and viewed using SoftMax Pro 5 Software (Molecular Devices, Sunnyvale, CA). The percent of live cells was calculated by dividing the OD of each well by the average OD of control wells untreated with primary antibody (but treated with secondary toxin) or untreated with conjugated antibody.

**Statistical Analysis**

Graphing and statistical analysis were performed using GraphPad Prism 6 Software version 6.0e.
RESULTS & DISCUSSION

**MIF\textsubscript{19-27} is presented by the HLA-A2 of ovarian cancer cell lines**

The MIF-derived peptide FLSELTQQL (MIF\textsubscript{19-27}) was first identified as a breast cancer biomarker in the context of the common allotype HLA-A*02:01 (25). Here, we investigated whether the same peptide might also mark ovarian cancer. Because the processing and subsequent HLA presentation of antigens relies upon a multifactorial system that is not fully understood, a priori one cannot link protein expression with peptide presentation (35, 36). Thus, despite the established overexpression of MIF protein in ovarian cancer, it is difficult to know which, if any, MIF peptides might be presented. Therefore, we harvested HLA-A*02:01 at high yields from the cancerous human ovarian cell lines A2780, SKOV3, OV90, and FHIOSE to proteomically assess MIF\textsubscript{19-27} peptide presentation. Eluted peptides from each of these lines were compared to a synthetic MIF\textsubscript{19-27} peptide that acted as a reference for LC elution time and MS fragmentation pattern (Fig. 1a-b). For each cell line an eluted peptide peak of the appropriate mass ($m/z$ 539.79), elution time (40 min +/- 2 min), and fragmentation pattern unequivocally confirmed the natural processing and presentation of the MIF\textsubscript{19-27} peptide (Fig. 1a,c). These data demonstrate that the overexpression of the MIF protein across ovarian cancer cell lines and tissues in the literature coincides with consistent HLA-A2 presentation of MIF\textsubscript{19-27} among four different cancerous ovarian cell lines.

**RL21A recognizes HLA-A2+ ovarian cancer cells**

Having confirmed that MIF\textsubscript{19-27} is naturally prepared for HLA-A2 presentation in ovarian cancer cells, we proceeded to test surface staining of the same cell lines with the monoclonal antibody RL21A. RL21A is specific to the HLA-A2/MIF\textsubscript{19-27} complex and does not bind HLA-A2
presenting various irrelevant peptides ((26) and Supplementary Fig. 1). First, FHIOSE cells that naturally express HLA-A*02:01 were stained with the pan-HLA-A2 antibody BB7.2 confirming robust HLA-A2 surface expression (Fig. 2a,f). These cells then also demonstrated substantial RL21A staining, suggesting that a considerable fraction of their HLA-A2 molecules are loaded with the MIF_{19-27} peptide and that HLA-A2/MIF_{19-27} is a naturally occurring and high abundance surface biomarker on these cells (Fig. 2b,f). As A2780 and SKOV3 do not naturally express HLA-A2, these cell lines were transfected to express a full-length HLA-A*02:01 surface molecule (Fig. 2a). These A2780-A2 and SKOV3-A2 cells also stained efficiently with RL21A (Fig. 2c-d,f). Thus, various ovarian cancer cell lines all appear to make MIF_{19-27} available in HLA-A2 at the cell surface.

**RL21A antigen cross-recognition benefits population coverage**

Receptors of the adaptive immune system are empowered by fine specificity, yet both monoclonal antibodies and T cell receptors can at times be promiscuous in their recognition of antigen. An example is seen in Fig. 2f where the pan-HLA-A2 antibody exhibits a known cross-reactivity with a close serologic relative, A*68:01, which is endogenous to the untransfected SKOV3 cells. In therapeutic development, immune cross-recognition of similar antigens must be investigated to avoid off-target effects, and this is addressed in the next section. However, limited cross-reactivity may be beneficial if the same cancer-associated peptide can be recognized in the context of multiple HLA molecules, widening the relevant patient population. The OV90 cells that do not express HLA-A*02:01 are a case in point as they encode the closely related HLA-A*02:02 and A*02:05 alleles and demonstrated cross-reactivity with both BB7.2 and RL21A (Fig. 2a,e-f). This OV90 reactivity suggests that MIF_{19-27} is able to bind one or both
of the alternate HLA-A2 alleles for recognition by RL21A. To more fully characterize which HLA allotypes might be relevant to MIF_{19-27} and RL21A, microspheres were coated with a panel of 120 class I HLA molecules and subjected to MIF_{19-27} peptide loading and staining with RL21A. A degree of cross-reactivity was observed among all of the HLA-A*02:0x isoforms tested with no reactivity toward any of the other HLA-A, -B, or -C molecules (Supplementary Fig. S2 and data not shown). In the event RL21A demonstrates promise as a cancer immunotherapy, recognition of MIF_{19-27} in the context of multiple HLA-A2 subtypes provides wider HLA class I population coverage.

**RL21A selectively stains ovarian cancer tissues**

Ovarian cancer cell lines represent a valuable model of disease, and the panel of cell lines tested here demonstrated consistent HLA-A2 presentation of MIF_{19-27} by proteomic analysis as well as by RL21A surface staining. Next, in order to test the natural tumor environment while at the same time testing for off-target reactivity, RL21A was used to directly stain ex vivo normal and tumor tissue samples from patients with serous ovarian cancer (Table 1). Fallopian tube (FT) epithelium is increasingly considered the primary site from which the majority of serous ovarian cancers develop (37-39), and thus this cell type was evaluated as the primary normal comparison for RL21A staining. The comparison revealed dramatically increased RL21A staining intensities in the ovarian cancer cells with an intensity score \( \geq 1+ \) observed in 67\% (18/27) of tumors compared to 5\% (1/19) of the normal epithelial tissues, and a score of \( \geq 2+ \) was seen in 41\% (11/27) of tumor tissues compared to none of the normal epithelia (Fig. 3a-b). Incorporating intensity and percentage of cells stained into overall staining scores for each tissue, a significant increase in RL21A reactivity was found in ovarian tumors compared to normal epithelial tissues.
(median 0.25 vs. 3.50, p < 0.0001) (Fig. 4a). In patients where both tumor and normal FT epithelial cells were evaluable (see Table 1) an upward slope between matched data points demonstrates an increase in staining upon cancerous transformation (Fig. 4b). The relatively high percentage of tumor tissues positive for this complex corresponds to the consistent MIF_{19-27} sequencing and RL21A staining of cell lines (Fig. 1-2) as well as the prevalent overexpression of the MIF protein observed across ovarian tumors (17, 18). This dramatic transformation-associated increase in RL21A epithelial staining demonstrates that the HLA-A2/MIF_{19-27} complex represents an ovarian tumor-specific biomarker on patient specimens.

Prevalent HLA upregulation was observed in ovarian tumors

HLA downregulation has been reported as a means of immune escape in several cancers, but the extent to which this occurs is controversial. As a control along with RL21A staining, tissue sections in this study were stained with BB7.2 to confirm expression of HLA-A2 (Fig. 3a, Supplementary Fig. S3). Unexpectedly, an overall increase in total HLA-A2 expression was detected in the tumor cells compared to the normal FT epithelium (median 5.00 vs. 7.50, p < 0.05) (Fig. 4a). Ten of the eleven matched cases displayed an increase in HLA-A2 staining score between normal and cancerous epithelial cells, while only one displayed HLA-A2 downregulation (Fig. 4b). The literature for ovarian cancer reports HLA downregulation in as few as ~20% of cases and in as many as ~70% (10, 40-43). This variation could be attributed to differing antibodies, tissue preservation and fixation methods, cohort characteristics, and various technical definitions of ‘downregulation’. In comparison, this is the first report to our knowledge where ovarian cancer HLA class I expression was studied using cryopreserved tissues. A delicate fixation buffer (5% methanol) was used to avoid HLA denaturation as BB7.2 and RL21A
antibodies require intact HLA/peptide complexes, an approach that contrasts with traditional IHC methods using strongly fixed, paraffin-embedded tissues and antibodies that recognize only a denatured HLA conformation (44). The strong correlation between cytotoxic T cell tumor infiltration and survival in ovarian cancer also suggests that the class I HLA system is largely operative and that immune escape hinges predominantly on effector cell inhibition rather than on HLA downregulation (10-12). Our observations suggest a low incidence of HLA downregulation and reveal an increase in HLA-A2 expression in >90% of ovarian tumor tissues (Fig. 4b). These findings enhance the prospect of therapies for ovarian cancer that directly target HLA/peptide complexes on the tumor, including monoclonal antibodies or CAR T cells directed against HLA-A2/MIF_{19-27}.

**RL21A recognizes the tumor stroma**

In addition to the epithelial comparison, stromal tissue and blood vessel endothelial cells were separately evaluated for RL21A and BB7.2 reactivity. RL21A staining increased significantly in the tumor stroma (median 0.50 vs. 2.75, p < 0.0001), and among the different cell types the tumor stroma exhibited the biggest difference in total HLA-A2 expression as compared to its normal counterpart (median 2.50 vs. 5.88, p < 0.001) (Fig. 4c). Tumor stroma is increasingly recognized as a prominent player in ovarian cancer progression, and multiple therapeutic strategies are now targeting this compartment (45). Furthermore, increased MIF expression is reported in the ovarian tumor stroma as contributing to tumor promotion (46). A therapeutic that can target both tumor epithelium and tumor stroma would be of great value, and the IHC data presented here suggest that both cell types process and present the MIF_{19-27} peptide for recognition by RL21A. In contrast, blood vessel endothelium yielded high HLA-A2 but low
RL21A staining in both normal and tumor tissues (Fig. 4d). Thus, RL21A can efficiently and specifically react with both ovarian tumor cells and the associated cancer stroma.

**Potential for a therapeutic window**

As in the example of HER2, successful targeting of cell surface tumor antigens requires a significant increase in antigen availability on the cancerous cells in comparison to normal tissues. Just as tumor availability of HLA-A2/MIF19-27 dramatically increases in comparison to normal epithelium (Fig. 4a), it is also significantly higher in comparison to the stromal and endothelial tissues of the normal FT samples (p < 0.001, p < 0.0001, respectively) (Fig. 4e). Thus, all of the normal tissue regions observed here remained significantly below tumor RL21A staining levels. These findings correspond with previous RL21A cadaveric studies in which no significant off-target staining was observed across multiple normal human organs (26). In the same study, fresh peripheral blood samples were also tested from healthy volunteers with little to no RL21A staining observed across 18 donors (26). A clear visual representation of RL21A tumor specificity is seen in Fig. 4f, an example of when metastatic lesions were found embedded within the normal FT tissue of ovarian cancer patients. RL21A strongly reacts with the tumor focus yet shows no reactivity with directly adjacent normal epithelial cells. These data demonstrate that a significant therapeutic window may exist for RL21A targeting of ovarian cancer.

**Toxin conjugated RL21A mediates killing of ovarian cancer cells**

Toxin conjugation is a promising avenue for converting tumor-specific antibodies into potent anticancer therapeutics (47). For instance, a toxin-conjugated version of trastuzumab significantly improves progression free survival in breast cancer trials, including a decrease in
adverse events (48). To begin assessing therapeutic potential of RL21A, A2780-A2 cells were treated with increasing RL21A concentrations alongside the HLA-A2 negative A2780 parent cells in the presence of a constant concentration of secondary Fc-binding duocarmycin-based cytotoxin. Toxin-associated RL21A killed the A2780-A2 cells in a concentration-dependent manner with an IC$_{50}$ of 0.048 nM ($R^2 = 0.945$) while cells lacking HLA-A2 were unaffected (Fig. 5a). Next, RL21A was directly conjugated to deBouganin, a de-immunized plant toxin derived from Bougainvillea spectabilis (32, 33). This conjugate (RL21A-deB) exhibited dose-dependent cytotoxicity against A2780-A2 with an IC$_{50}$ of 0.324 nM ($R^2 = 0.970$) (Fig. 5b). To also test effectiveness against cells that naturally express HLA-A*02:01, FHIOSE cells were exposed to the same concentrations of RL21A-deB and a similar cytotoxicity pattern was observed with an IC$_{50}$ of 9.376 nM ($R^2 = 0.925$) (Fig. 5b). These data correlate well with the relative levels of RL21A staining observed for each cell line by flow cytometry (Fig. 2f). Thus, HLA-A2 presents MIF$_{19-27}$ at a level whereby toxin-bound RL21A kills cancerous ovarian cells without harming cells that lack the antigen. RL21A-deB immunotoxin represents one of the potential avenues toward therapeutic targeting of HLA-A2/MIF$_{19-27}$ complexes on ovarian cancer.

**Summary**

Immune therapies for cancer are enjoying a renaissance. Monoclonal antibodies continue to evolve into potent anti-cancer therapies via drug conjugation, through bi-specific technologies, and using antibody variable regions to engineer highly active CAR T cells against cell surface tumor markers. In addition, HLA-presented cancer vaccines are stimulating endogenous T cells against tumor antigens, while immune checkpoint inhibitors can further unleash both
manipulated and natural effector T cells (7). As immunologists and cancer biologists join forces to harness the specificity and power of immunotherapies, the need for a robust source of cell surface cancer markers becomes increasingly apparent; T cells and antibodies are only as powerful as their targets. Cell surface HLA class I molecules are a natural means of revealing intracellular indicators of neoplasticity to the immune system, and given the observation that tumor infiltrating CD8+ lymphocytes correspond to better clinical outcomes, we have embarked upon targeting ovarian cancer via class I HLA-presented peptides.

Here we explored the availability of the MIF-derived peptide FLSELTQQL in the context of ovarian cancer HLA-A*02:01 molecules. We found that tumorigenic ovarian cell lines expressing HLA-A2 display this precise MIF\textsubscript{19-27} peptide and are reactive with the RL21A antibody specific for this complex. When cryopreserved tumor samples from ovarian cancer patients were tested for reactivity, strong staining for HLA-A2/MIF\textsubscript{19-27} complexes was observed which was significantly increased from normal FT epithelium, stroma, and endothelium. An unexpected increase in HLA-A2 expression was noted in the tumors, and alternate HLA-A2 variants were recognized as capable of presenting the MIF peptide and reacting with this antibody. As monoclonal reagents are enabling a number of immunotherapies for cancer, we toxin-conjugated RL21A and demonstrated its potential for specific killing of ovarian cancer lines \textit{in vitro}. These data merit the future testing of a humanized version of RL21A to confirm continued cytotoxicity \textit{in vitro}, to investigate efficacy \textit{in vivo} using animal models of human ovarian cancer and, if results continue to be promising, to work toward a transition to clinical trials for ovarian cancer immunotherapy.
ACKNOWLEDGEMENTS

We thank the Stephenson Cancer Center at the University of Oklahoma Health Sciences Center, Oklahoma City, OK for the use of the Biospecimen Acquisition Core and Bank, which provided the cryopreserved tissues, and the Cancer Tissue Pathology Core, which provided cryosectioning and H&E staining services (special thanks to Dr. David Brown and Louisa Williams, respectively).
REFERENCES


Table 1. Donor tissue characteristics

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FIGO: International Federation of Gynecology and Obstetrics
Gray boxed: Corresponding to tissue numbers in Fig. 4
FIGURE LEGENDS

Figure 1: The MIF peptide FLSELTQQL is detected in HLA-A2 from all four cancerous ovarian cell lines. (A) Overlaid extracted ion chromatograms of the 539.79 m/z ion from the synthetic MIF_{19-27} LC-MS run and that of the corresponding fraction from each cell line. (B) MS/MS spectrum showing the fragmentation pattern of the synthetic MIF_{19-27} peptide. B and Y ions are labeled along with some prominent internal fragment ions. (C) The identical fragmentation pattern to the synthesized MIF_{19-27} peptide in (B) was observed in all ovarian cancer cell lines at the corresponding m/z.

Figure 2: RL21A stains ovarian cancer cells in an HLA-A2 dependent manner. (A) Overlaid histograms comparing total HLA-A2 staining for each cell line. (B) Flow cytometry dot plots showing RL21A staining of FHIOSE compared to isotype control. (C,D) RL21A staining of HLA-A2-transfected A2780-A2 (C, left) or SKOV3-A2 (D, left) compared to the untransfected parent cells (right). (E) RL21A staining of OV90 compared to isotype control. (F) Summarized RL21A and pan-HLA-A2 mean fluorescence intensities for all lines from three independent experiments. Data are mean + SD.

Figure 3: RL21A tissue staining intensities. (A) Four different stains were performed on each patient tissue as indicated along the top. Three tissues are shown, each representing the indicated RL21A intensity range where x = intensity. Original magnification, x 200 (including zoomed insets). (B) Percentages of tumor and normal epithelial tissues falling within each RL21A intensity range. Actual number of tissues is indicated above each bar.
**Figure 4:** RL21A specifically targets ovarian tumor tissues. Normal FT or tumor tissues from ovarian cancer patients were stained by IHC for total HLA-A2 using BB7.2 or for the specific HLA-A2/MIF complex using RL21A. Scores integrate intensity and percent cells stained. Individual data points and median are plotted for (A) total epithelial scores, (B) matched-only epithelial scores with normal and tumor pairs connected, (C) stromal scores, and (D) blood vessel endothelial scores. Mann-Whitney U tests were performed for all but (B), which represents a Wilcoxon matched-pairs signed rank test. (E) RL21A scores only, comparing normal FT stromal and endothelial cells with the epithelial ovarian cancer cells using a Kruskal-Wallis one-way analysis of variance with Dunn’s multiple comparisons. (F) Normal FT tissue with tumor focus boxed and further magnified. Black arrows, epithelial ovarian cancer metastasis. White arrows, normal epithelium. Original magnification, x 40 (top), x 200 (bottom).

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Figure 5:** Toxin-bound RL21A kills HLA-A2+ ovarian cancer cells. Cells were treated with dilutions of either bare RL21A in the presence of a constant concentration of cytotoxic secondary (A), or RL21A-deB (B) for 72 h. Live cells were detected by MTS assay. Data are mean ± SD from at least three independent experiments with at least 3 replicate wells per experiment. Data are fit with sigmoidal 4-parameter logistics curves (A2780-A2 and FHIOSE) or best-fit lines (A2780).
Figure 1
Figure 2

A

B

C

D

E

F

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Figure 3

A

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Normal FT Tissue

- **RL21A Staining Intensity (x)**
  - $x < 1$
    - 18/19 Normal
    - 9/27 Tumor
  - $1 \leq x < 2$
    - 1/19 Normal
    - 7/27 Tumor
  - $x \geq 2$
    - 11/27 Tumor

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Figure 4

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Figure 5

A

% Live Cells

RL21A (log[nM])

-4 -3 -2 -1 0 1 2

0 50 100

A2780

A2780-A2

B

% Live Cells

RL21A-deB (log[nM])

-5 -4 -3 -2 -1 0 1 2

0 50 100

A2780

A2780-A2

FHIOSE
Molecular Cancer Therapeutics

Human Leukocyte Antigen-Presented Macrophage Migration Inhibitory Factor is a Surface Biomarker and Potential Therapeutic Target for Ovarian Cancer

Andrea M Patterson, Saghar Kaabinejadian, Curtis P McMurtrey, et al.

Mol Cancer Ther Published OnlineFirst December 30, 2015.

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