Enhancement in Specific CD8+ T Cell Recognition of EphA2+ Tumors In Vitro and In Vivo after Treatment with Ligand Agonists

Amy K. Wesa, Christopher J. Herrem, Maja Mandic, Jennifer L. Taylor, Cecilia Vasquez, Mayumi Kawabe, Tomohide Tatsumi, Michael S. Leibowitz, James H. Finke, Ronald M. Bukowski, Elizabeth Bruckheimer, Michael S. Kinch and Walter J. Storkus

*J Immunol* 2008; 181:7721-7727; doi: 10.4049/jimmunol.181.11.7721

http://www.jimmunol.org/content/181/11/7721

Why The JI?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

References

This article **cites 40 articles**, 15 of which you can access for free at:
http://www.jimmunol.org/content/181/11/7721.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2008 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Enhancement in Specific CD8+ T Cell Recognition of EphA2+ Tumors In Vitro and In Vivo after Treatment with Ligand Agonists

Amy K. Wesa, Christopher J. Herrem, Maja Mandic, Jennifer L. Taylor, Cecilia Vasquez, Mayumi Kawabe, Tomohide Tatsumi, Michael S. Leibowtiz, James H. Finke, Ronald M. Bukowski, Elizabeth Bruckheimer, Michael S. Kinch, and Walter J. Storkus

The EphA2 receptor tyrosine kinase (RTK) plays a pivotal role in disease and development (1). EphA2 is a 130-kDa (type 1) glycoprotein that is expressed at low levels on nontransformed epithelial tissues (2). In these cells, EphA2 localizes to epithelial-cell-to-cell contacts, and it is thought to contribute to the well-known phenomenon of contact inhibition of cell growth and motility (3, 4). In contrast to its role in nontransformed cells, dysregulation in EphA2 expression and function causes EphA2 to support tumor progression and metastasis (4, 5). High levels of EphA2 expression have been observed in a range of malignant cell models and in clinical specimens of many different solid tumors, including metastatic melanoma and carcinomas of the bladder, breast, colon, esophagus, kidney, lung, mesothelium, ovary, prostate, and pancreas, among others (5–14). The highest levels of EphA2 are found on the most aggressive tumors, with tumor cell EphA2 expression levels being predictive of increased metastatic potential and decreased patient survival (7–11, 13–16).

The prevalence of EphA2 overexpression on tumor cells has sparked interest in its use for the development of novel targeted therapeutics. In particular, a class of agonistic EphA2 Abs has been developed that can induce EphA2 internalization and degradation, thereby reducing expression of this powerful oncoprotein. Repeated administration of these reagents has proven successful at inhibiting tumor cell growth in both in vitro and in vivo models, as well as in enhancing the survival of tumor-bearing mice (17, 18). Based on its overexpression on multiple epithelial tumor cell types, EphA2 may represent a pan-tumor-associated Ag for the generalized immune targeting of carcinomas. In this light, we and others (15, 19, 20) have recently identified peptide epitopes derived from human and murine EphA2 that are competent to activate specific CD4+ and CD8+ T cells capable of recognizing tumor cells that constitutively (over)express the EphA2 protein. Notably, dendritic cell-based vaccines incorporating mEphA2 peptides have been reported to promote protective T cell responses in murine melanoma and colon cancer models (21, 22).

However, the clinical expectation would be that vaccines based on EphA2 epitopes would fail to be optimally efficacious in the cancer setting as they would likely elicit only moderate- to low-avidity T cells in patients with EphA2+ cancers, given tolerance mechanisms imposed against the self (nonmutated) EphA2 protein.
as well as immune deviation that is known to occur in these individuals (23). Herein, we investigated whether treatment of EphA2+ human tumor cells with specific agonists would induce proteasome-dependent degradation of EphA2 protein, thereby increasing tumor cell surface expression of MHC class I/EphA2 peptide complexes, resulting in improved recognition of tumor cells by anti-EphA2 CD8+ T cells. We determined that recombinant ligand (i.e., ephrinA1-Fc) and agonist anti-EphA2 mAb208 are both competent to promote the enhanced recognition of EphA2+ tumor cells by specific CD8+ T cells in vitro and in vivo. Such conditional augmentation in immune recognition of EphA2+ tumor cells by recombinant ligand or agonist mAb, in concert with active immunization or adoptive transfer of ex vivo-expanded anti-EphA2 T cells, may serve to define novel and effective combination immunotherapeutic strategies relevant to a large cohort of patients harboring EphA2+ malignancies.

Materials and Methods

Cell lines and media

The T2 (HLA-A2+, EphA2+; Refs. 24, 25) cell line (kindly provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN) was used as the peptide-presentation cell in ELISPOT analyses. The EphA2+, HLA-A2-PC-3 prostate carcinoma cell line (5) was used as a positive control for Western blot analyses of EphA2 protein expression and was also used as a negative control target (along with the EphA2 control for Western blot analyses of EphA2 protein expression and was also a positive control target) for improved recognition of tumor cells by anti-EphA2 CD8+ T cells. We determined that recombinant ligand (i.e., ephrinA1-Fc) and agonist anti-EphA2 mAb208 are both competent to promote the enhanced recognition of EphA2+ tumor cells by specific CD8+ T cells in vitro and in vivo. Such conditional augmentation in immune recognition of EphA2+ tumor cells by recombinant ligand or agonist mAb, in concert with active immunization or adoptive transfer of ex vivo-expanded anti-EphA2 T cells, may serve to define novel and effective combination immunotherapeutic strategies relevant to a large cohort of patients harboring EphA2+ malignancies.

Peptides

The HLA-A2-presented EphA2 583–891 (IMNDMIPYTM, EphA2 583–891) (TLADFDPRV) (15), and HIV-nef 180–189 (VLEWRFDSRL) (15) peptides were synthesized using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry by the University of Pittsburgh Cancer Institute’s (UPCI) Peptide Synthesis Facility, as previously described (15). Peptides were >96% pure based on HPLC, with identities validated by mass spectrometric (MS/MS) analyses performed by the UPCI Protein Sequencing Facility.

Mice

Six- to 8-wk-old female C.B-17 scid/scid mice were purchased from Tac-Toxnic and maintained in microisolator cages. Animals were handled under aseptic conditions as per an Institutional Animal Care and Use Committee-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

EphA2 agonists

EphrinA1-Fc (R&D Systems) is a chimeric protein consisting of the ligand binding domain of the EphA2 ligand ephrinA1 fused with the Fc portion of a mouse IgG Ab. mAb208 (kindly provided by MedImmune) is a mouse IgG Ab specific for hEphA2 (16). EphrinB1-Fc (Sigma-Aldrich) and MOPC21 mAb (mouse IgG; Sigma-Aldrich) were also used as specificity controls for ephrinA1-Fc and mAb208, respectively.

Western blot analyses

Tumor cells were grown to 80–90% confluency, then treated with agonists where indicated for up to 48 h before analysis. Additionally, resected SLR24 lesions were obtained before and 24 h after intratumoral injection with ephrinA1-Fc, ephrinB1-Fc, or mAb208, as in text and in the Fig. 5 legend. Tumor samples were analyzed for EphA2 expression via Western blots using the rabbit anti-human EphA2 polyclonal Ab (clone C-20; Santa Cruz Biotechnology). SLR20.A2 and SLR24 cells were also analyzed for expression of the EGFR using rabbit anti-human polyclonal Ab sc-03 (Santa Cruz Biotechnology). Single-tumor cell suspensions isolated from confluent tissue culture flasks or from the enzymatic digestion of resected tumor lesions were lysed using 500 μl lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40; all from Sigma-Aldrich) in PBS containing protease inhibitors (Complete, Roche Diagnostic) for 30 min at 4°C. After centrifugation at 13,500 × g for 20 min, the supernatant was mixed 1:1 with SDS-PAGE running buffer and proteins separated on 7.5% PAGE gels, before electrophoretically onto polyvinylidene difluoride membranes (Millipore). Blots were imaged on Kodak X-OMat Blue XB-1 film (NEN Life Science Products) after using HRP-conjugated goat anti-rabbit Ig (Santa Cruz Biotechnology) and the Western Lightning chemiluminescence detection kit (PerkinElmer). Immunoprecipitations for EphA2 were performed using the anti-EphA2 Ab D7 (Millipore). Anti-phosphotyrosine Ab (clone py99, Santa Cruz Biotechnology) were used to assess pEphA2 content. Mouse anti-β-actin Ab (clone AC-15, Abcam) was used as a loading control.

T cell lines and clones

Bulk CD8+ human T cell lines and clones specific for EphA2 538–66 (i.e., 15/9) and EphA2 583–891 (i.e., E883, 3C1) were generated as previously described (15). The HLA-A2 allotypic CD8+ T clone 2E4 was generated by three rounds of in vitro stimulation of HLA-A2-negative (HLA-DR+) normal donor T cells with irradiated (100 Gy) T2 cells, followed by limiting-dilution cloning. All T cell lines and clones were specifically restimulated every 7–10 days and were maintained in IMDM media (Invitrogen) containing 10% human AB serum (Sigma-Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all from Invitrogen), and rhIL-2 (100 IU/ml; PeproTech). All donor specimens were obtained with written consent under an Institutional Review Board-approved protocol.

ELISA and ELISPOT assays

In vitro T cell responses were evaluated by commercial hIFN-γ ELISA (BD OptEIA; BD Biosciences; limit of detection 4.7 pg/ml) per the manufacturer’s instructions and by IFN-γ ELISPOT assays, as previously described (15).

Preteasome dependency assessment

The impact of preteasome inhibition in Western blot (evaluating agonist-induced EphA2 degradation in tumor cells) and T cell ELISPOT (evaluating enhanced anti-EphA2 T cell recognition of agonist-treated tumor cells) assays was assessed by treatment of tumor cells with either MG-132 (Sigma-Aldrich) or clasto-lactacytin β-lactone (hereafter designated as lactacytin; BIOMOL International), as outlined in relevant text and figure legends.

Cytotoxicity assays

CD8+ T cell clones E883 (anti-EphA2 583–891) and 2E4 (anti-HLA-A2) were evaluated for their capacity to lyse EphA2+ cells. HLA-A2+ SLR24 tumor target cells using standard 4-h 51Cr-release assays, as previously described (26).

Flow cytometry

For phenotypic analyses of control or ligand agonist-treated tumor cells, PE- or FITC-conjugated mAbs against total HLA class I complexes (W6/32; pan-class I specific; Serotec), HLA-A2 complexes (American Type Culture Collection), or empty HLA-A molecules (HC-A2, Ref. 27, the kind gift of Dr. H. Ploegh, Massachusetts Institute of Technology) and appropriate isotype controls (purchased from BD Biosciences) were used, and flow cytometric analyses were performed using a FACSScan (BD Biosciences) flow cytometer. Cell surface expression of EphA2 protein was analyzed using direct immunofluorescence staining monitored by flow cytometry. After treatment for 0–24 h at 37°C with 10 μg/ml ephrinA1-Fc, ephrinB1-Fc, mAb208, or the MOPC21 mAb, tumor cells were stained for 30 min at 4°C with FITC-conjugated anti-EphA2 mAb B2D6 (Millipore; note that this mAb is not sterically inhibited by the binding of ephrinA1-Fc or mAb208 to EphA2, data not shown) before washing using PBS and analysis by flow cytometry. The results of these assays are reported as percentage control (untreated) tumor cell expression based on a comparison of arbitrary mean fluorescence intensity (MFI) units obtained for experimental vs control specimens.

Hu-SCID tumor model

C.B-17 scid/scid mice were injected s.c. in the right flank with 1 × 106 SLR24 (EphA2+, HLA-A2+) renal cell carcinoma cells and tumors allowed to establish to a size of ~30 mm3 (i.e., day 18 postinjection). The
EphA2 agonists induce the phosphorylation and proteasome-dependent degradation of EphA2 in tumor cells in vitro. To determine whether agonist treatment promotes EphA2 phosphorylation, SLR20.A2 (A) and SLR24 (B) renal carcinoma cells (2–4 × 10⁶) were left untreated or were treated for 10 or 30 min with ephrinA1-Fc, ephrinB1-Fc, MOPC21 mAb, or mAb208 (each at 10 μg/ml) at 37°C. Cellular lysates were resolved by SDS-PAGE, and EphA2 protein was immunoprecipitated using the anti-EphA2 Abs D7 in pull-down assays. Western blot analyses were then performed using anti-EphA2 and anti-phosphotyrosine Abs, respectively. To determine whether agonist treatment induces EphA2-specific degradation, SLR20.A2 (C and EphA2 protein was immunoprecipitated using the anti-EphA2 Abs D7 in pull-down assays. Western blot analyses were then performed using H9252 tumor cells were treated as in A and resolved using SDS-PAGE. Western blot analyses were then performed using anti-EphA2 Abs and negative control anti-β-actin Abs. Anti-EGFR Ab was used to image identically prepared lysates as a specificity (negative) control in these experiments. To assess the proteasome dependence of agonist-induced EphA2 degradation, MG-132 (50 μM) or chloroquine (100 μM) were also added to SLR20.A2 cell cultures, where indicated, 30 min before the addition of ephrin-Fc proteins or mAb208 (E). After 24 h, cell lysates were generated and resolved using SDS-PAGE. Western blot analyses were then performed using anti-EphA2 Abs and negative control anti-β-actin Abs. In F, the kinetics of EphA2 down-modulation on the surface of treated (with the indicated agents) SLR20.A2 cells was investigated by flow cytometry. Data are reported as percentage control EphA2 cell surface expression (vs untreated cells) based on MFI values obtained. All data are representative of three independent experiments performed.

**Statistical analyses**

Statistical differences between groups were evaluated using a two-tailed Student’s t test, with p values <0.05 considered significant.

**Results**

**EphrinA1-Fc and mAb208 induce EphA2 phosphorylation and proteasome-dependent degradation in tumor cell lines**

Previous studies have demonstrated that tumor cells exhibit unstable cell-cell contacts and that this impairs the ability of EphA2 to interact with its ephrinA1 ligand on neighboring cells (29–31). Consequently, the EphA2 protein in malignant cells is generally observed to be in a hypophosphorylated state (5). Consistent with these previous reports, our Western blot analyses verified that EphA2 protein expressed in a series of renal cell (SLR20.A2, SLR24) carcinoma cell lines is constitutively nonphosphorylated, but that treatment of these cells with EphA2 agonists (ephrinA1-Fc, anti-EphA2 mAb208) is sufficient to rapidly increase EphA2 phosphotyrosine content (Fig. 1, A and B). As negative controls, treatment of the EphB1+ SLR20.A2 or SLR24 tumor cells with ephrinB1-Fc (a ligand for EphB1, but not EphA2) or control mlgG MOPC21 failed to induce EphA2 phosphorylation (Fig. 1, A and B). Immunoblotting of tumor cell lysates (after 24 h of treatment) verified that ephrinA1-Fc and mAb208, but not ephrinB1-Fc or MOPC21 mAb, induces substantial EphA2 protein degradation (Fig. 1, C and D). These treatments did not alter tumor cell expression of control proteins, including the EGFR and β-actin (Fig. 1, C and D). A reanalysis in the absence or presence of chloroquine or MG-132 suggested that EphA2 degradation in SLR20.A2 cells was predominantly 26S proteasome-dependent (Fig. 1E), consistent with a previous report for the proteasomal dependency of EphA2 destruction in breast and prostate carcinoma cell lines (32). Flow cytometric analysis of agonist-treated SLR20.A2 tumor cells indicated that cell surface EphA2 is rapidly lost (i.e., internalized) within hours after treatment with ephrinA1-Fc and mAb208, but not with ephrinB1-Fc or MOPC21 mAb (Fig. 1F).

**EphrinA1-Fc and mAb208 treatment enhances CD8+ T cell recognition of EphA2+ tumors in vitro**

Since agonistic Abs triggered the proteasomal destruction of EphA2, we hypothesized that this could preferentially increase presentation of EphA2 peptides in tumor cell surface HLA class I.

#### FIGURE 1.

EphrinA1-Fc and mAb208 induce EphA2 phosphorylation and proteasome-dependent degradation in tumor cell lines

(a) EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control

(b) EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control

(c) EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control

(d) EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control

(e) EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control

(f) EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control

<table>
<thead>
<tr>
<th>Time Post-Treatment (h)</th>
<th>% Control Cell Surface EphA2 Expression</th>
<th>Grapevine State EphrinA1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</td>
</tr>
<tr>
<td>25</td>
<td>100</td>
<td>EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control EphrinA1-Fc EphrinB1-Fc MOPC21 mAb mAb208 Control</td>
</tr>
</tbody>
</table>
complexes. If correct, it would then logically follow that EphA2 agonists could selectively enhance tumor cell recognition by EphA2-specific CD8$^+$ T cells. To address this question, EphA2$^+$ tumor cell lines were incubated with mAb208 for 24 or 48 h before evaluating the ability of these target cells to be recognized by HLA-A2-restricted CD8$^+$ T cell lines and clones specific for the EphA2$_{883–891}$ (19) (located in the EphA2 extracellular domain) or EphA2$_{883–891}$ (15) (located in the EphA2 intracellular domain) peptide epitopes. Rather than initially assessing differential tumor sensitivity to T cell killing (which could involve changes in both T cell and tumor cell functions induced by agonists), we instead chose to more directly interrogate changes in T cell functional recognition of treated tumor cells using the IFN-γ ELISPOT assay as a readout of effector T cell reactivity.

Pretreatment of SLR20.A2 (EphA2$^+$, HLA-A2$^+$) tumor cells with mAb208 significantly enhanced their recognition by both anti-EphA2 CD8$^+$ T cell lines (Fig. 2, A and B) and moderate avidity (Fig. 2, C and D) CD8$^+$ T cell clones 15/9 and 3C1 (Fig. 2, E and F, respectively). Notably, improved T cell recognition of treated tumor cells was sustained for a period of at least 48 h (Fig. 2, A, B, E, and F). SLR20 (EphA2$^+$, HLA-A2$^+$) tumor cells failed to be recognized by any of these T cell populations, even after treatment with mAb208 (that promotes EphA2 degradation, data not shown). Furthermore, treatment of SLR20.A2 cells with control IgG (MOPC21 mAb) failed to enhance tumor cell recognition by anti-EphA2 CD8$^+$ T cells (Figs. 2, A, B, E, and F).

While unlikely, we considered the trivial explanation that increased tumor cell recognition by anti-EphA2 CD8$^+$ T cells could be the result of a general up-regulation in tumor cell expression of HLA-A2 class I molecules (and hence a compensatory increase in the cohort of HLA-A2 complexes containing EphA2-derived peptides). To address this possibility, the SLR20.A2 and SLR24 cell lines were treated with ephrinA1-Fc or mAb208 and analyzed by flow cytometry for cell surface expression levels of total HLA class I molecules (using the W6/32 mAb), peptide-loaded (monitored using the BB7.2 mAb) complexes, and empty (monitored using the HC-A2 mAb) HLA-A2 complexes. We noted no significant changes in the MFI of any of these parameters as a consequence of tumor treatment with these EphA2 agonists (data not shown).

To demonstrate whether enhanced CD8$^+$ T cell recognition of tumor cells was due to processing through the proteasome, SLR20.A2 tumor cells were pretreated with MG-132 or β-lactone to block proteasomal function, and then cells were cultured with EphA2 agonists before use as targets for CD8$^+$ T cell recognition. Due to concerns for the toxicity of proteasome inhibitors using a prolonged exposure, we chose a short 3-h period for tumor pretreatment, with no impact on tumor cell morphology or viability noted (data not shown). As a confirmation and extension of data depicted in Fig. 1E, application of MG-132 or lactacystin prevented agonist-induced EphA2 degradation (Fig. 3A). These proteasome inhibitors also completely abrogated any enhancement in recognition of SLR20.A2 cells by anti-EphA2 T cells resulting from treatment with the EphA2 agonists, mAb208 and ephrinA1-Fc (Fig. 3B).

**EphrinA1-Fc and mAb208 treatment enhances the therapeutic efficacy of adoptively transferred anti-EphA2 CD8$^+$ T cells in a Hu-SCID tumor model**

To determine whether the conditional (agonist-induced) enhancement of EphA2$^+$ tumor cell recognition by anti-EphA2 CD8$^+$ T cells could be of potential clinical significance, we established a Hu-SCID tumor model system for the analysis of combinational adoptive cellular immunotherapy. Human SLR24 (EphA2$^+$, HLA-A2$^+$) renal carcinoma cells were injected s.c. into the right flanks of C.B-17 scid/scid mice and allowed to progress to a size of ~30 mm$^3$, at which time animals were either left untreated or were pretreated with no Ab, control IgG (MOPC21 mAb), or mAb208 (10 µg/ml each) for 24 or 48 h, as indicated, before ELISPOT analyses. Data are reported as mean IFN-γ ELISPOT spots/10$^5$ CD8$^+$ T cells ± SD from triplicate determinations. All data are representative of three independent experiments.
treated with intratumoral injection of ephrinA1-Fc, ephrinB1-Fc, or mAb208 and/or i.v. delivery of an HLA-A2-restricted, anti-EphA2\textsubscript{883–891} CD8\textsuperscript{+} T cell clone (i.e., clone E883 was used vs clone 3C1 due to its ability to be expanded to the high numbers of T cells required for these experiments). As depicted in Fig. 4A, clone E883 mediates the lysis of SLR24 tumor cells in vitro in an HLA-A2-restricted manner, with cytolytic dramatically increased if the tumor cell line is pretreated with either ephrinA1-Fc or mAb208, but not with ephrinB1-Fc. Treatment-induced sensitization of SLR24 to T cell-mediated cytolysis is specific to anti-EphA2 CD8\textsuperscript{+} T cells, since alloreactive (anti-HLA-A2) CD8\textsuperscript{+} T cell clone 2E4 lysed SLR24 tumor cells to a comparable degree regardless of in vitro pretreatment conditions applied to the tumor cells (Fig. 4B).

In the Hu-SCID tumor model, intratumoral injection of 50 μg ephrinA1-Fc, ephrinB1-Fc, or mAb208 (on day 18 posttumor inoculation) had minimal effect on the continued progressive growth of SLR24 lesions (Fig. 5A), despite specific, acute reduction in situ EphA2 expression 24 h after injection with ephrinA1-Fc or mAb208, but not with ephrinB1-Fc (Fig. 5B). The adoptive transfer of 5 × 10\textsuperscript{6} CD8\textsuperscript{+} T cells (either the anti-EphA2 clone E883 or the anti-HLA-A2 allospecific clone 2E4 on day 19 posttumor inoculation) also failed to significantly alter consequent SLR24 lesional growth in vivo (Fig. 5, C and D). However, combined application of ephrinA1-Fc or mAb208 (on day 18) along with the adoptive transfer of E883 T cells (on day 19) resulted in complete tumor eradication to enhanced SLR24 to T cell-mediated cytolysis is specific to anti-EphA2 CD8\textsuperscript{+} T cells, since alloreactive (anti-HLA-A2) CD8\textsuperscript{+} T cell clone 2E4 10\textsuperscript{6} human SLR24 (HLA-A2\textsuperscript{+}) RCC tumors s.c. in the right flank and allowed to establish to a size of ~30 mm\textsuperscript{3} (i.e., day 18). Animals were then randomized into four cohorts (six animals each) receiving no treatment (control) or intratumoral injections of ephrinA1-Fc (50 μg), ephrinB1-Fc (50 μg), or mAb208 (50 μg) on day 18. Tumor size was evaluated every 3–4 days, with results reported in mean mm\textsuperscript{3} ± SD. In B, tumors were resected from one mouse per cohort on day 19 (i.e., 24 h after treatment and Western blots performed to validate EphA2 degradation in situ). In C and D, 5 × 10\textsuperscript{6} CD8\textsuperscript{+} T cells (either anti-EphA2\textsubscript{883–891}, clone E883 or anti-HLA-A2 allospecific clone 2E4, respectively) were adoptively transferred by tail vein injection on day 19 posttumor inoculation alone or in combination with prior day 18 intratumoral injections of ephrinA1-Fc, ephrinB1-Fc, or mAb208 (50 μg each). Tumor size was evaluated every 3–4 days, with results reported in mean mm\textsuperscript{3} ± SD. Data are representative of three independent experiments performed.

In the Hu-SCID tumor model, intratumoral injection of 50 μg ephrinA1-Fc, ephrinB1-Fc, or mAb208 (on day 18 posttumor inoculation) had minimal effect on the continued progressive growth of SLR24 lesions (Fig. 5A), despite specific, acute reduction in situ EphA2 expression 24 h after injection with ephrinA1-Fc or mAb208, but not with ephrinB1-Fc (Fig. 5B). The adoptive transfer of 5 × 10\textsuperscript{6} CD8\textsuperscript{+} T cells (either the anti-EphA2 clone E883 or the anti-HLA-A2 clone 2E4 on day 19 posttumor inoculation) also failed to significantly alter consequent SLR24 lesional growth in vivo (Fig. 5, C and D). However, combined application of ephrinA1-Fc or mAb208 (on day 18) along with the adoptive transfer of E883 T cells (on day 19) resulted in complete tumor eradication in all treated animals (Fig. 5C, Table I). In contrast, combined use of ephrinB1-Fc and E883 T cells yielded a tumor growth curve that was indistinguishable from single agent controls, with no animals rejecting their tumors (Fig. 5C). Furthermore, even though the allospecific 2E4 CD8\textsuperscript{+} T cell clone efficiently kills SLR24 tumor
cells in vitro (Fig. 4B), improved efficacy was not observed in combination approaches using EphA2 agonists and 2E4 T cells (Fig. 5D). This result is consistent with the failure of SLR24 pretreatment with ephrin-A1-Fc or mAb208 to augment tumor sensitivity to 2E4 T cell-mediated lysis in vitro (Fig. 4B).

Discussion

In the current age of cancer therapy, some of the most promising new therapies include Abs and small molecule modulators of RTKs (over)expressed by tumor cells (32). The mechanisms of action associated with therapeutic efficacy are varied (i.e., silencing of receptor signaling, promotion of receptor down-regulation, induction of tumor cell death mediated via Ab-dependent cellular cytotoxicity, among others) (32). The results of our studies suggest that additional immune-based mechanisms may be cooperative in the setting of such therapies, and that if optimized, such combination treatments may yield enhanced clinical benefits to patients with many forms of (EphA2-+) cancer.

The major finding of the present study is that the treatment of tumor cells with agonists that promote EphA2 autophosphorylation and proteasomal degradation/processing also result in improved recognition by EphA2-specific CD8+ T cells both in vitro and in vivo. As a consequence, moderate-to-low functional avidity (i.e., ~1 μM ED50 for peptide recognition on T2 cells) EphA2-reactive CD8+ T cells are rendered more effective in reacting against, and mediating the regression of, EphA2+ tumor lesions in vivo. Notably, EphA2 was capable of yielding epitopes (EphA258–66 and EphA2883–891, located in the extracellular domain and intracellular domain of the target protein, respectively), which could consequently be presented by MHCI class I molecules for extended periods of time. However, the intermediate steps involved in this mechanism remain unclear, but likely depend on alternate, nonclassical mechanisms of Ag processing (33). Cytosolic proteins generally are thought to be the primary substrates for the proteasome and are fed into the endoplasmic reticulum via TAP1/TAP2 transporters (33, 34). In contrast, EphA2 is a transmembrane protein, generally poised for degradation by lysosomes after ligand-induced internalization (35). It remains unknown whether the cohort of EphA2 protein associated with agonist-enhanced T cell recognition derives from EphA2 molecules that are retrotranslocated into the endoplasmic reticulum or deposited into the cytoplasm via a loss of endocytic vesicle integrity or a “ratcheting” mechanism applied to ubiquitinated transmembrane protein substrates (33, 36–38). We are currently undertaking pharmacologic studies to begin to delineate such intermediate steps. A better understanding of the mechanism(s) involved in agonist-induced EphA2 molecule processing may allow for the accentuation of relevant pathways, allowing for even greater enhancement in therapeutic immune recognition of EphA2+ tumor cells.

The ability of agonistic reagents to conditionally trigger the proteasome-dependent degradation of overexpressed EphA2 molecules on tumor cells in vivo may provide opportunities for the development of new combinational therapeutic strategies for the treatment of patients with EphA2+ cancers. In particular, our present results suggest the potential therapeutic benefits of using “off-the-shelf” agonists to sensitize EphA2+ tumor cells to anti-EphA2 CD8+ T cells that could be preactivated via specific immunization (15, 19) or provided by the adoptive transfer of Ag-specific ex vivo-expanded, autologous CD8+ T cell populations. At present, however, many questions remain unanswered with regard to the optimal implementation of such a treatment strategy, including: 1) Must a tumor grossly overexpress EphA2 protein (relative to normal epithelia and such) in order for agonists to enable modest- to low-avidity anti-EphA2 CD8+ T cells to mediate improved therapeutic benefit? 2) Can agonist-enhanced CD8+ T cell recognition of tumor cells be further enhanced by the co-application of IFN-α or IFN-γ (i.e., cytokines that up-regulate the MHC class I Ag processing machinery) (39) without destroying tumor-presented EphA2 epitopes due to the concomitant activation of the immunoproteasome?; and 3) Will this strategy sensitize normal EphA2+ tissues to the spectre of autoimmune pathology? Based on preliminary data, we can suggest that even tumor cells exhibiting only modestly overexpressed levels of (hypophosphorylated) EphA2 protein appear capable of being sensitized by agonists to specific CD8+ T cells (Fig. 4 and data not shown) and that the EphA2883–891 peptide (i.e., identical sequence occurs in both human and murine EphA2) elicits potent antitumor CD8+ T cell responses in the absence of autoimmune pathology in HLA-A2 Tg mice that constitutively express EphA2 protein in normal lung, liver, and kidney cells (20). Furthermore, based on analysis using a web-based algorithm (www.imtech.res.in/raghava/propred1/index.html), at least the EphA2883–891 peptide epitope is not predicted to be destroyed by the immunoproteasome. Hence, we think that combinational immunotherapies targeting EphA2 will prove both safe and effective.

One surprising aspect in our work is that the combinational therapy works or fails in vivo, presumably based on an ~2–4-fold increase in tumor cell recognition by anti-EphA2 CD8+ T cells after agonist treatment in vitro. This may suggest that additional mechanisms of action are in play in vivo, only some of which relate to tumor presentation of EphA2 epitopes. Clearly, one might envision that tumor EphA2 processing in vivo could be more efficient than that observed in vitro for a given tumor cell line. We are currently attempting to address this possibility by performing mass spectrometry analyses for the EphA258–66 and EphA2883–891 peptide epitopes extricated from HLA-A2 complexes of SLR24 tumor cells grown in vitro vs in vivo with and without agonist treatment for 24 h (via addition to culture or intratumoral injection). Beyond this mechanism, we have not observed any effects of EphA2 agonists directly on T cells, and, indeed, our T cell lines/ clones fail to express discernable levels of EphA2 (data not shown). However, it is possible that EphA2 agonists could trigger alterations in production of inflammatory chemokines that would serve to enhance T cell recruitment or survival within the tumor microenvironment. Given the speciation of many chemokines, relevant alterations in our Hu-SCID model would likely be tumor cell (rather than stroma)-dependent. Hence, we are currently investigating whether EphA2 agonists promote alterations in expression of chemokines, such as CXC chemokines IFN-induced protein of
10 kDa (IP-10), monokine induced by IFN-γ (Mig), and IFN-inducible T-cell α-chemoattractant (I-TAC), by SLR24 tumor cells that may facilitate CTL recruitment in vivo. Alternatively or additionally, the enhanced efficacy of the combinatorial therapy in vivo could relate to the direct targeting of the EphA2 tumor-associated vasculature (40) by anti-EphA2 T cells after agonist administration. Indeed, we have recently shown that the vaccination of mice with peptides representing CD8+ T cell epitopes derived from mEphA2 protein inhibits the growth of EphA2-negative tumor cells in vivo and limits the neoangiogenesis of Matrigel implants containing vascular endothelial growth factor (22). This suggests that combinatorial treatments using EphA2 agonists and T cell-based immunotherapy will likely have multiple strategic EphA2+ cellular targets within the tumor microenvironment, potentially opening patient accrual to individuals harboring any form of vascularized cancer.

Acknowledgments

We thank Drs. Hassane Zarour, William Chambers, and Russ Salter for careful review and helpful comments provided during the preparation and revision of this manuscript.

Disclosures

Elizabeth Bruckheimer and Michael S. Kinch were employees of MedImmune, Inc. while studies were being conducted.

References