Possible Role of Arginase-1 in Concomitant Tumor Immunity

Michael J. Korrer1,2,3,4*, John M. Routes1,2,3,4*

1 Department of Pediatrics, Medical College of Wisconsin, Milwaukee, Wisconsin, United States of America, 2 Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin, United States of America, 3 Children’s Research Institute, Milwaukee, Wisconsin, United States of America, 4 Cancer Center, Medical College of Wisconsin, Milwaukee, Wisconsin, United States of America

Abstract

The expression of Adenovirus serotype 2 or serotype 5 (Ad2/5) E1A in tumor cells reduces their tumorigenicity in vivo by enhancing the NK cell mediated and T cell mediated anti-tumor immune response, an activity that correlates with the ability of E1A to bind p300. We determined if E1A could be used as a molecular adjuvant to enhance antigen-specific T cell responses to a model tumor antigen, ovalbumin (OVA). To achieve this goal, we stably expressed a fusion protein of E1A and OVA (MCA-205-E1A-OVA), OVA (MCA-205-OVA) or a mutant version of E1A unable to bind p300 and OVA (E1A-Δp300-OVA) in the B6-derived, highly tumorigenic MCA-205 tumor cell line. MCA-205-E1A-OVA tumor cells were over 10,000 fold less tumorigenic than MCA-205-OVA, MCA-205-E1A-Δp300-OVA, or MCA-205 in B6 mice. However, immunization of B6 mice with live MCA-205-OVA, MCA-205-E1A-Δp300-OVA and MCA-E1A-OVA tumor cells induced nearly equivalent OVA-specific CD4 T cells and CD8 CTL responses. Further studies revealed that mice with primary, enlarging MCA-205-OVA or MCA-205-E1A-Δp300-OVA tumors on one flank exhibited OVA-specific anti-tumor T cell responses that rejected a tumorigenic dose of MCA-205-OVA cells on the contralateral flank (concomitant tumor immunity). Next we found that tumor associated macrophages (TAMs) in progressive MCA-205-OVA tumors, but not MCA-205-E1A-OVA tumors that expressed high levels of arginase-1, which is known to have local immunosuppressive activities. In summary, immunization of mice with MCA-205 cells expressing OVA, E1A-Δp300-OVA or E1A-OVA induced equivalent OVA-specific CD4 and CD8 anti-tumor responses. TAMs found in MCA-205-OVA, but not MCA-205-E1A-OVA, tumors expressed high levels of arginase-1. We hypothesize that the production of arginase-1 by TAMs in MCA-205-OVA or MCA-205-E1A-Δp300-OVA tumor cells leads to an ineffective anti-tumor immune response in the tumor microenvironment, but does not result in inhibition of a systemic anti-tumor immunity.

Introduction

Expression of the Adenovirus E1A oncoprotein in primary cells results in cellular immortalization [1]. Cells stably expressing E1A and the helper protein E1B have been shown to be oncogenic in immunosuppressed rodents [2,3]. Paradoxically, in rodent models the expression of Adenovirus serotype 2 or serotype 5 (Ad2/5) E1A in tumor cell lines significantly reduces tumorigenicity [4] (we now refer to Ad2/5 E1A as simply E1A). The ability of E1A to reduce tumorigenicity is dependent on the induction of a robust NK cell and T cell anti-tumor immune response [5] and correlates with the ability of E1A to bind the transcriptional co-adaptor molecule p300 or CBP [6]. p300 and CBP are highly homologous co-activators of transcription with intrinsic histone-acetyl transferase activity and will hereafter be referred to as simply p300 [7]. The expression of E1A, but not mutant forms of E1A that do not bind p300 (E1A-Δp300), also upregulates NKG2D ligands [8] and sensitizes cells to lysis by macrophages, NK cells and immune effector molecules utilized by these cells [9–13].

Based on these anti-tumorigenic activities of E1A, we sought to determine if E1A could be used to enhance antigen specific, anti-tumor T cell responses to MCA-205 tumor cells that express a model tumor antigen, ovalbumin (OVA). MCA-205 tumor cells that expressed a fusion protein of E1A and OVA elicited an effective anti-tumor T cell response and were rendered non-tumorigenic. Surprisingly, immunization of mice with live MCA-205-OVA or MCA-205-E1A-Δp300-OVA tumor cells elicited a robust anti-tumor immune response, despite forming progressive tumors at the primary site of immunization (concomitant tumor immunity). Further studies examined a possible mechanism whereby immunization of B6 mice with MCA-205-OVA or MCA-205-E1A-Δp300-OVA could induce systemic anti-tumor immunity but fail to clear a local tumor burden.

Materials and Methods

Mice

Inbred C57BL/6/J (B6), B6.129S7-Rag1tm1Mom/J (RAG-/-/), B6.SJL-Pepcrca Pepcr/BoyJ (CD43.1), C57BL/6-Tg[TcraTcrb]100Mjb/J (OT-I), and B6.Cg-Tg[TcraTcrb]42Cbn/J (OT-II) mice were purchased from The Jackson laboratories (Bar Harbor, ME). OT-I mice express a
transgene for a T cell receptor that recognizes ovalbumin (OVA) residues 257–264 in the context of H-2K\(\text{b}\) [14]. OT-II mice express a transgene for a T cell receptor that recognizes chicken OVA residues 323–339 in the context of I-A\(\text{b}\) [15]. Male mice six to nine weeks in age were used. All animal work was reviewed and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Reagents

Roswell Park Memorial Institute (RPMI) medium with 5% Fetal Bovine Serum (FBS) (RPMI-5) or 10% FBS (RPMI-10) supplemented with Glutamax (Invitrogen, Carlsbad, CA), glucose and antibiotics was used for all cell culture. FBS (Atlanta Biologicals, Flowery Branch, GA) was heat inactivated for 45 minutes at 56\(\text{°C}\). OVA 257–264 peptide was purchased from Sigma.

Flow cytometry

Flow cytometry was performed with a LSR II (BD biosciences, San Jose, CA) using BD FACSDiva software. Flow cytometry analysis was performed using FlowJo software (Tree Star, Ashland, OR). Antibodies specific to mouse CD3\(\text{e}\) (145-2C11) Alexa Fluor 488 (AF-488); Fluorescein (FITC), CD8a (5H-10) PE; Pacific Orange (PO), CD45.1 (A20) Allophycocyanin (APC), NK1.1 (PK136) PE, and GR-1 (Rb6-8C5) APC were purchased from Biolegend (San Diego, CA). Antibodies specific to mouse CD3\(\text{e}\) (145-2C11) AF-780, CD4 (GK1.5) Efluor 450 (EF-450); Peridinin Chlorophyll (PerCP), CD11b (M170) EF-450, CD11c (N418) PerCP, F4/80 (BM8) APC-Cy7, CD45 (30-F11) PE, H-2K\(\text{b}\) OVA257–264 complex (25-D1.16) APC were purchased from Ebiosciences (San Diego, CA).

Cloning strategy

The wild-type Adenovirus 5 \(E1A\) gene was cloned from Adenovirus 5 (GenBank ID: AY147066.1) bp 44–596,713–1029 Forward primer: 5’-CGT ACT GAA TTC TAA GGT ACC ATG GGC TCC ATC GGT GCA GC-3’, Reverse primer: 5’-GCT GCA CCG ATG ATG GAG CCT GGC CTG TTA CAG CT-3’ by PCR. A mutant version of \(E1A\) unable to bind p300 (\(E1A\)-\(\Delta\)p300) was cloned from the Adenovirus \(E1A\) mutant \(dl1104\) using the same primers as \(E1A\). \(E1A\)-\(\Delta\)p300 has a deletion in amino acids 48–60, which eliminates p300 binding [16]. The \(E1A\) gene was cloned from the pAC-OVA plasmid (GenBank ID: J00895.1), coding sequence (CDS) Forward
fibroblasts (Lonza, Basel, Switzerland) and a Nucleofector II (Lonza, Basel, Switzerland). Cell selection was done in media containing 1 mg/mL G418 (Sigma-Aldrich, St Louis, Missouri). Resistant colonies were screened for E1A expression and OVA expression by western blot analysis.

**Immunoprecipitation/Western blot detection of OVA**

Lysates were generated from tumor lines from $10^3$ cells in 1 mL of radioimmune precipitation buffer (RIPA) consisting of 50 mM Tris-HCl; pH 7.4, 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St Louis, Missouri). OVA was immunoprecipitated with rabbit polyclonal anti-OVA antibody (ab1221) (Abcam, Cambridge, England). The samples were analyzed by Western blot analysis. E1A bands were detected with mouse anti-E1A mAb m73 hybridoma supernatant (produced locally) and OVA bands were detected using mouse anti-OVA mAb (1E7) (Abcam, Cambridge, England). Protein bands were visualized using the Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, Nebraska). Protein bands were quantified using Image Studio software (LI-COR Biosciences, Lincoln, Nebraska).

**Tumor induction studies**

Quantitative tumor induction studies were performed as previously described [8]. Briefly, mice were administered serial log dilutions of tumor cells subcutaneously (s.c.) in the flank and monitored for tumor growth twice weekly using digital calipers. Animals were euthanized by CO$_2$ followed by cervical dislocation when tumors reached 20 mm in diameter, if tumors ulcerated, or at the end of a 12-week monitoring period. Tumor producing dose (TPD$_{50}$) values, which are the log$_{10}$ of the number of cells required to form tumors, were calculated using the Spearman-Karber Formula.

**Quantification of OVA specific CD8 and CD4 T cells**

OT-I transgenic CD8 T cells were harvested from the spleens of OT-I RAG$^{-/-}$ CD45.1$^+$ mice and OT-II transgenic CD4 T cells were harvested from the spleens of OT-II RAG$^{-/-}$ CD45.1$^+$ mice. $10^5$ OT-I CD8 T cells or $10^5$ OT-II CD4 T cells were administered i.v. via retro-orbital injection into B6 mice. The following day, mice were administered 10$^5$ live MCA-205-OVA, MCA-205-E1A-OVA or MCA-205-E1A-Dp300-OVA cells s.c. in the hock (the lateral tarsal region just above the ankle). Five days (OT-I) or nine days (OT-II) following tumor injection the popliteal lymph nodes were removed and the CD45.1$^+$ OT-I or OT-II T cells were quantitated by flow cytometry by staining for CD45.1$^+$ CD3$^+$ CD8$^+$ T cells or CD45.1$^+$ CD3$^+$ CD4$^+$ T cells, respectively. The absolute number of cells was determined by multiplying the percentage of the target cell population by the total number of cells in the lymph node.

**In vivo CTL assay**

B6 mice were primed with $10^6$ live tumor cells s.c. in the flank. Seven days later an in vivo CTL assay was performed on the primed mice [18]. B6 splenocytes pulsed with OVA 257-264 peptide were used as targets. OVA pulsed splenocytes were labeled with a low dose (1 mM) of CFSE for one minute in 5% FBS PBS. Untreated splenocytes were labeled with a high dose (10 mM) CFSE for one minute in 5% FBS PBS. The two CFSE labeled target splenocyte groups were mixed equally and injected i.v. into primed mice. $10^6$ total target cells were administered to the
mice. Four hours later, spleens were removed and splenocytes were analyzed by flow cytometry for CFSE expression. The ratio of OVA pulsed splenocytes (low CFSE) to unpulsed splenocytes (high CFSE) was used to determine specific killing. Specific killing was calculated as follows:

\[
\text{Specific lysis} = 1 - \frac{R_{\text{Naive}}}{R_{\text{exp}}} \times 100; \ R = \% \text{OVA pulsed/}\% \text{non-pulsed.}
\]

Concomitant tumor induction studies

Mice were administered three injections of \(1 \times 10^5\) live tumor cells s.c. in the flank five days apart in 100 \(\mu\)L of PBS. Five days after the final injection (day 20) the mice were challenged with serial log dilutions of MCA-205-OVA cells on the contralateral flank and the TPD_{50} was determined. For some studies CD3 T cells were depleted on days 17, 19, 22 and 24 by i.p. injection of 10 \(\mu\)g anti-CD 3 \(\alpha\) mAb 2C11 (BioXcell, West Lebanon, NH) or control Hamster IgG (BioXcell, West Lebanon, NH) into primed mice as previously described [19]. Mice were challenged with \(1 \times 10^4\) MCA-205-OVA cells on the contralateral flank and the percentage of mice tumor free from the MCA-205-OVA challenge was determined.

Immune infiltrate of MCA-205-OVA tumors

B6 mice were administered \(1 \times 10^6\) live MCA-205-OVA cells s.c. in the flank. When the tumor reached a diameter of 10 mm (~ two weeks) tumors were excised. Tumors were minced with scissors and digested with collagenase Type I 5 U/mL (Sigma-Aldrich, St Louis, Missouri), Deoxyribonuclease I 50 U/mL (Sigma-Aldrich, St Louis, Missouri), and Hyaluronidase, Type II 5 U/mL (Sigma-Aldrich, St Louis, Missouri) in 10 mL of RPMI-5 at 37°C for two hours. The digestion was stopped by the addition of 5 mL of 10 mM EDTA and incubated at 37°C for 15 minutes. A single cell suspension was generated by crushing the tissue with glass slides and passing through 40 \(\mu\)m filters. Cells were then characterized by flow cytometry.

Figure 3. Immunization with MCA-205-OVA, MCA-205-E1A-\(\Delta p300\)-OVA and MCA-205-E1A-OVA cells expand similar numbers of OVA-specific T cells in vivo. A, B) B6 mice were injected with \(1 \times 10^5\) CD45.1\(^+\) OT-I cells and then \(1 \times 10^5\) MCA-205, MCA-205-OVA, MCA-205-E1A-\(\Delta p300\)-OVA, or MCA-205-E1A-OVA tumor cells 24 hours later s.c. into the hock. Five days later the draining lymph nodes were removed and the number of OT-I cells was determined by flow cytometry analyzing for CD45.1\(^+\)CD3\(^+\)CD8\(^+\) cells. Data shown is the percent of OT-I cells of the CD8 T cell population (A), and the absolute number of OT-I cells in draining lymph node (B). Data shown in A, B is the mean \(\pm\) SEM, from three experiments with 6–13 mice per group. C, D) Experiments were performed as in A, B, except \(1 \times 10^6\) CD45.1\(^+\) OT-II cells were administered, and the draining lymph nodes were collected on day nine. The number of OT-II CD4 T cells was determined by flow cytometry by analyzing for CD45.1\(^+\)CD3\(^+\)CD4\(^+\) cells. Data shown is mean \(\pm\) SEM, from two experiments with 3–8 mice per group. Data were analyzed by ANOVA followed by Tukey’s HSD post hoc analyses. *, \(p<0.05\); **, \(p<0.01\); ns, no significance.
doi:10.1371/journal.pone.0091370.g003
Tumor associated macrophage arginase expression

Tumors were excised from mice and a single cell suspension was generated as described above. Macrophages were purified using FACS and lysed in 4% Triton-X lysis buffer at a concentration of 1 × 10^5 cells per 100 μL of lysis buffer. Arginase expression was quantified using the QuantiChrom Arginase Assay kit (Bioassay systems, Hayward, CA), following manufacturer's directions.

Statistics

Statistical differences between groups were calculated using the ANOVA and Dunnett multiple comparison tests. Mann-Whitney tests were used to compare two sets of data. Survival curves were compared using the log-rank (Mantel Cox) test; p values less than 0.05 were considered significant. Unless otherwise stated, all data are presented as mean ± standard error of mean (SEM). Statistical analysis was done using Prism version 5.0a software (GraphPad Software, La Jolla, CA), following manufacturer’s directions.

Results

Expression of E1A-OVA, OVA or E1A-Δp300-OVA in MCA-205 cells

In an effort to determine if E1A could augment anti-tumor-specific T cell responses to OVA, we first generated MCA-205 tumor lines (see Materials and Methods) that expressed a slightly truncated OVA 3–383 (MCA-205-OVA), a fusion protein of full length E1A and OVA 3–383 (MCA-205-E1A-OVA) or a fusion protein of mutant E1A lacking amino acids 48–60, which results in the inability of E1A to bind p300 (E1A-Δp300) and OVAΔ3–383 (MCA-205-E1A-Δp300-OVA). All MCA-205-OVA, MCA-205-

Figure 4. MCA-205-OVA, MCA-205-E1A-Δp300-OVA and MCA-205-E1A-OVA tumor cells elicited similar in vivo OVA-specific cytotoxic T cell responses. A) Mice were injected with 1 × 10^6 live MCA-205, MCA-205-OVA, MCA-205-E1A-Δp300-OVA, or MCA-205-E1A-OVA tumor cells in the flank s.c. and an in vivo CTL assay against OVA pulsed splenocytes was performed seven days later. Data shown is the mean ± SEM from three experiments, with six mice per group. Data were analyzed by ANOVA followed by Tukey’s HSD post hoc analyses. **, p < .01; ***, p < .001.

doi:10.1371/journal.pone.0091370.g004

Tumor associated macrophage arginase expression

Tumors were excised from mice and a single cell suspension was generated as described above. Macrophages were purified using FACS and lysed in 4% Triton-X lysis buffer at a concentration of 1 × 10^5 cells per 100 μL of lysis buffer. Arginase expression was quantified using the QuantiChrom Arginase Assay kit (Bioassay systems, Hayward, CA), following manufacturer’s directions.

Expression of E1A-OVA, OVA or E1A-Δp300-OVA in MCA-205 cells

In an effort to determine if E1A could augment anti-tumor-specific T cell responses to OVA, we first generated MCA-205 tumor lines (see Materials and Methods) that expressed a slightly truncated OVA 3–383 (MCA-205-OVA), a fusion protein of full length E1A and OVA 3–383 (MCA-205-E1A-OVA) or a fusion protein of mutant E1A lacking amino acids 48–60, which results in the inability of E1A to bind p300 (E1A-Δp300) and OVAΔ3–383 (MCA-205-E1A-Δp300-OVA). All MCA-205-OVA, MCA-205-Δp300-OVA and MCA-205-E1A-Δp300-OVA tumor cells all expressed readily detectable amounts of OVA (Figure 1 A), although the amount of OVA was slightly higher in the MCA-205-E1A-OVA tumor line (Figure 1 B). OVA was detected at the expected molecular weight of approximately 43 kDa, whereas E1A-OVA and E1A-Δp300-OVA bands were detected at significantly higher molecular weights due to the addition of the E1A or E1A-Δp300 proteins.

Next, we confirmed that the OVA protein was processed and presented by the class I molecule H-2K^b by staining the various cell lines with the 25-D1.16 antibody which recognizes OVA 257–264 peptide in the context of H-2K^b. MCA-205-OVA, MCA-205-E1A-Δp300-OVA and MCA-205-E1A-OVA tumor cells expressed large amounts of OVA 257–264 H-2K^b complexes on the cell surface (Figure 1 C). These data demonstrate that MCA-205 lines were generated that stably express E1A and/or OVA and that the OVA protein is processed and presented on the cell surface.

Figure 5. Immunization with MCA-205-OVA, MCA-205-E1A-Δp300-OVA and MCA-205-E1A-OVA tumor cells provided concomitant tumor immunity against MCA-205-OVA challenge on the contralateral flank of B6 mice. Mice were injected with 1 × 10^6 live MCA-205, MCA-205-OVA, MCA-205-E1A-Δp300-OVA, or MCA-205-E1A-OVA tumor cells s.c. in the flank three times, five days apart. Five days after last tumor dose (day 20), mice were challenged with serial log dilutions of MCA-205-OVA tumor cells on the contralateral flank from 1 × 10^1–1 × 10^6 cells. The TPD50 of MCA-205-OVA was calculated six weeks later. Data shown is the mean ± SEM from three experiments, with three mice per dose per experiment. ***, p < .001.

doi:10.1371/journal.pone.0091370.g005

E1A-Δp300-OVA and MCA-205-E1A-OVA tumor cells all expressed readily detectable amounts of OVA (Figure 1 A), although the amount of OVA was slightly higher in the MCA-205-E1A-OVA tumor line (Figure 1 B). OVA was detected at the expected molecular weight of approximately 43 kDa, whereas E1A-OVA and E1A-Δp300-OVA bands were detected at significantly higher molecular weights due to the addition of the E1A or E1A-Δp300 proteins.

Tumorigenicities of MCA-205-OVA, MCA-205-E1A-Δp300-OVA and MCA-205-E1A-OVA cells in B6 mice

We next determined if E1A-OVA expression in MCA-205 tumor cells retained E1A in vivo biological activity by measuring the tumorigenicity of MCA-205, MCA-205-E1A, MCA-205-OVA, MCA-205-E1A-Δp300-OVA and MCA-205-E1A-OVA tumor lines (Figure 2). MCA-205-E1A has been previously shown to have substantially reduced tumorigenicity compared to MCA-205 cells [8] and served as a positive control. Tumorigenicity was measured by determining the tumor producing dose 50 (TPD50), which is the log 10 of the number of tumor cells required to form tumors in half of the B6 mice (Material...
and Methods). MCA-205-E1A and MCA-205-E1A-OVA tumor cells were non-tumorigenic at the highest challenge dose (1 x 10^7 cells) and were at least 10,000 fold less tumorigenic than MCA-205, MCA-205-OVA or MCA-205-E1A-Dp300-OVA tumor lines. MCA-205-OVA and MCA-205-E1A-Dp300-OVA tumor cells were equivalently tumorigenic as MCA-205 tumor cells, indicating that expression of either OVA or E1A-Dp300-OVA in MCA-205 cells does not alter the intrinsic tumorigenicity of the MCA-205 line. In summary, these tumor induction studies showed that the E1A-OVA fusion protein retains the anti-tumorigenic activity of the E1A protein.

Characterization of T cell responses to MCA-205-OVA and MCA-205-E1A-OVA cells

As previously noted, MCA-205-OVA and MCA-205-E1A-Dp300-OVA tumor cells were found to be highly tumorigenic whereas MCA-205-E1A-OVA were non-tumorigenic at the highest challenge dose (Figure 2). One explanation for these results was that, in contrast to MCA-205-E1A-OVA cells, MCA-205-OVA or MCA-205-E1A-Dp300-OVA failed to induce a productive OVA-specific anti-tumor immune response in vivo. As an initial test of this hypothesis, we challenged B6 mice with the various live OVA expressing tumor lines and compared their ability to expand OVA specific CD4 T cells (OT-II cells) or CD8 T cells (OT-I cells) in vivo. 1 x 10^5 CD45.1^+ OT-I CD8 T cells isolated from CD45.1^+OT-I^Rag^-/- mice and were adoptively transferred into CD45.2^+ B6 mice. 24 hours later, MCA-205, MCA-205-OVA, MCA-205-E1A-OVA or MCA-205-E1A-Dp300-OVA cells were injected into the hock. Five days after tumor injection, the popliteal lymph nodes were removed and the number of OT-I cells was determined by enumerating the number of CD45.1^+ CD8 T cells by flow cytometry. In contrast to MCA-205 cells, challenge of mice with MCA-205-OVA, MCA-205-E1A-Dp300-OVA or MCA-205-E1A-OVA tumor cells resulted in an equivalent expansion of OT-II cells present in the draining lymph node (Figure 3 B, C). Collectively these data indicate that challenge of mice with MCA-205-OVA, MCA-205-E1A-Dp300-OVA or MCA-205-E1A-OVA tumor cells induced an OVA-specific CD4 and CD8 T cell response.

Characterization of the functional T cell response to MCA-205-OVA and MCA-205-E1A-OVA cells

Challenge with tumorigenic MCA-205-OVA or MCA-205-E1A-Dp300-OVA cells expanded OVA-specific CD8 T cells slightly better than challenge with MCA-205-E1A-OVA cells. Therefore, we determined if there was a functional difference between the CD8^+ T cell responses in mice challenged with MCA-205-E1A-OVA or MCA-205-OVA tumor cells, which could account for differences in tumorigenicity. To test the functional response of OVA-specific CD8 T cells in mice to challenge with live MCA-205-OVA, MCA-205-E1A-Dp300-OVA or MCA-205-E1A-OVA tumor cells, we performed an in vivo CTL killing assay (Figure 4). As expected, mice challenged with MCA-205 cells in the absence of OVA did not induce OVA-specific CTLs. In contrast, mice primed with MCA-205-OVA, MCA-205-E1A-Dp300-OVA or MCA-205-E1A-OVA cells induced equivalent OVA-specific CTL killing (Figure 4). These results suggest that injection of live MCA-205-OVA, MCA-205-E1A-Dp300-OVA or
MCA-205-E1A-OVA cells elicits OVA-specific CTL capable of killing OVA expressing targets.

Presence of Protective Systemic Tumor Immunity in Mice with Progressive MCA-205-OVA and MCA-205-E1A-Δp300-OVA Tumors

The observation that MCA-205-OVA and MCA-205-E1A-Δp300-OVA cells elicited an OVA-specific T cell response was puzzling. One possible explanation for these results was that MCA-205-OVA and MCA-205-E1A-Δp300-OVA tumors suppressed OVA-specific T cells in the tumor microenvironment, but not at sites distal to the primary tumor. If this hypothesis was correct, mice with primary MCA-205-OVA tumors in one flank should be resistant to a subsequent challenge with tumors in the contralateral flank. Therefore, we injected B6 mice with either PBS, MCA-205-OVA, MCA-205-E1A-Δp300-OVA or MCA-205-E1A-OVA tumor cells into the flank of mice, on three occasions, and then determined the TPD50 of MCA-205-OVA tumor cells injected on the contralateral flank (Figure 5). As predicted, mice that received injections of PBS did not exhibit measurable anti-tumor immunity to a subsequent challenge with MCA-205-OVA cells. In contrast, immunization of B6 mice with tumorogenic MCA-205-OVA cells and MCA-205-E1A-Δp300-OVA cells, as well as non-tumorogenic MCA-205-E1A-OVA cells, all induced significant protective immunity to a subsequent challenge with tumorogenic MCA-205-OVA tumor cells (Figure 5). Following injection of MCA-205-OVA, MCA-205-E1A-Δp300-OVA, or MCA-205-E1A-OVA tumor cells, there was an approximately 1,000 fold increase in the numbers of MCA-205-OVA cells required to form tumors on the contralateral flank compared to PBS primed mice (Figure 5). Concomitant tumor immunity occurred even in the presence of growing tumors from the primary tumor.

Figure 7. Composition of immune infiltrate of MCA-205-OVA tumors. A) Representative gating strategy to determine immune infiltrate of MCA-205-OVA tumors. B6 mice were administered 1x10^6 MCA-205-OVA cells, and tumors were excised and digested when the tumor reached 15 mm. The immune cells which infiltrated the tumor were determined by flow cytometry. CD45^+, live cells were first gated. Macrophages (CD45^+CD11b^+ CD11c^lo F4/80^+Gr-1^-), myeloid-derived suppressor cells ([MDSC) CD45^+CD11b^+ CD11c^lo GR-1^-], CD4 T cells (CD45^+CD11b^-CD3^-CD4^+), CD8 T cells (CD45^-CD11b^-CD3^-CD8^-), and NK cells (CD45^+CD11b^-CD3^-CD8^-CD4^-NK1.1^-) were quantified in B as the percentage of CD45^+ cells. Tregs were <1%, not shown in figure. Data shown is the mean ± SEM from three mice.

doi:10.1371/journal.pone.0091370.g007
arginase activity compared to TAMs from MCA-205-OVA tumors. TAMs from MCA-205-E1A-OVA tumors have significantly more arginase activity compared to TAMs from MCA-205-E1A-OVA tumors. TAMs from MCA-205-E1A-OVA tumors express arginase-1 activity at a concentration of 1 x 10^6 TAMs per 100 μL of lysis buffer using a colorimetric assay. Data shown is the mean ± SEM from four-six mice per group. Data were analyzed by ANOVA followed by Tukey's HSD post hoc analyses. *, p < .05; ***, p < .001.

doi:10.1371/journal.pone.0091370.g008

Discussion

In this study we used OVA as a model tumor antigen to evaluate the ability of E1A expression to augment antigen-specific anti-tumor T cell responses in mice. We found that the expression of an E1A-OVA fusion protein rendered MCA-205 cells essentially non-tumorigenic in normal B6 mice. In contrast, the tumorigenicity of MCA-205 cells was not substantially changed by the expression of OVA or E1A-Dp300-OVA in normal B6 mice. Immunization with either MCA-205-OVA, MCA-205-E1A-Dp300-OVA, or MCA-205-E1A-OVA tumor cells induced a robust OVA-specific anti-tumor T cell response. For example, following injection of either MCA-205-OVA,
MCA-205-E1A-D\textsubscript{p300}-OVA, or MCA-205-E1A-OVA tumor cells, approximately 1,000 fold more MCA-205-OVA cells were required to form tumors in comparison to tumor challenge with naïve mice (Figure 5). These results indicated that while MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.

Based on the observation of concomitant tumor immunity in the presence of primary tumor formation, we investigated the tumor microenvironment. We found that TAMs were the predominant inflammatory cell in progressive MCA-205-OVA tumors in WT B6 mice. TAMs isolated from MCA-205-OVA, MCA-205-E1A-D\textsubscript{p300}-OVA induced systemic immunity, local tumor immunity was hampered as progressive tumors formed at the site of the primary challenge site.
activity in the tumor microenvironment also provide a possible explanation for concomitant tumor immunity induced by MCA-205-OVA tumors in the presence of a progressive primary MCA-205-OVA tumor.

Further studies need to directly examine the role of arginase-1 production by TAMs, concomitant tumor immunity and the anti-tumorigenic effect of E1A. Definitive studies examining the role of arginase-1 production by TAMs and anti-tumor immunity are currently hampered by a relative lack of arginase-1 specific reagents. The most commonly used in inhibitor of arginase is N[α]-hydroxy-nor-L-arginine (nor-NOHA). Nor-NOHA inhibits both arginase-1 and arginase-2 and in conventionally used doses the inhibition of arginase-1 in vivo is less than 50% [28]. Mice genetically deficient in arginase-1 [29] or conditionally-induced to be deficient in arginase-1 [30], succumb to an illness that mimics human arginase deficiency far too rapidly to permit tumor induction experiments examining concomitant tumor immunity. The development of mice conditionally deficient of arginase-1 in macrophages or better arginase-1 inhibitors will facilitate progress in this important area.

The mechanism whereby tumor cells that express E1A lead to the decreased expression of arginase-1 by TAMs is unknown and also needs to be explored. Arginase-1 activity has been postulated as one of the mechanisms for the failure of adoptive cellular immune therapy to be effective against solid tumors. Determining how E1A is able to inhibit tumor cells from inducing arginase-1 in TAMs could have important implications in augmenting local anti-tumor immune responses in the setting of progressive tumor enlargement. Additionally, novel uses of E1A could be considered to augment local anti-tumor immune responses. For example, studies could be performed to determine if administration of liposomes with E1A protein into a tumor could augment local tumor anti-immune responses and lead to tumor rejection.

Finally, the use of the MCA-205-OVA, MCA-205-E1AΔp300-OVA, or MCA-205-E1A-OVA tumor lines in the B6 mouse is a new model of concomitant tumor immunity that also allows for the quantitation of antigen-specific T cell responses. In the B16 melanoma concomitant tumor immunity model developed by Turk et al. [31], concomitant tumor immunity was observed only after manipulating B16 melanoma cells to express GM-CSF, or by depleting/inhibiting regulatory T cells. In our MCA-205-OVA model, concomitant tumor immunity occurred by day 20, and required no further experimental manipulation of the tumor cells or B6 mice. To our knowledge, this model of concomitant tumor immunity is unique and may more accurately replicate ongoing systemic anti-tumor immune responses in a human in the face of enlarging primary or secondary tumors.

**Author Contributions**

Conceived and designed the experiments: MJK JMR. Performed the experiments: MJK. Analyzed the data: MJK JMR. Contributed reagents/materials/analysis tools: JMR. Wrote the paper: MJK JMR.

**References**

1. Kelekis A, Cole MD (1987) Immortalization by η-csvc, H-ras, and E1a oncogenes induces differential cellular gene expression and growth factor responses. Mol Cell Biol 7: 3899–3907.
2. Gallimore PH, McDougall JK, Chen LB (1973) In vitro traits of adenovirus-transformed cell lines and their relevance to tumourigenicity in nude mice. Cell 10: 669–678.
3. Cook JL, Lewis AM Jr, Kirkpatrick CH (1979) Age-related and thymus-dependent rejection of adenovirus 2-transformed cell tumors in the Syrian hamster. Cancer Res 39: 3335–3340.
4. Walker TA, Wilson BA, Lewis AM Jr, Cook JL (1991) E1A oncogene induction of cytolytic susceptibility eliminates sarcoma cell tumorigenicity. Proc Natl Acad Sci U S A 88: 6941–6945.
5. Routes JM, Ryan S, Li H, Steinke J, Cook JL, et al. (2000) Disimilar immunogenicities of human papillomavirus E7 and adenovirus E1A proteins influence primary tumor development. Virology 277: 48–57.
6. Cook JL, Krantz CK, Routes BA (1996) Role of p300-family proteins in E1A oncogene induction of cytolytic susceptibility and tumor cell rejection. Proc Natl Acad Sci U S A 93: 13985–13990.
7. Wang F, Marshall CB, Ikura M (2013) Transcriptional/epigenetic regulator CBP/p300 in tumorigenetic structural and functional versatility in target recognition. Cell Mol Life Sci 70: 3889–4000.
8. Routes JM, Ryan S, Morris K, Takaki R, Cerwenka A, et al. (2005) Adenovirus serotype 5 E1A sensitizes tumor cells to NKG2D-dependent NK cell lysis and tumor rejection. J Exp Med 202: 1477–1482.
9. Cook JL, Lewis AM Jr (1984) Differential NK cell and macrophage killing of hamster cells infected with nononcogenic or oncogenic adenoviruses. Science 224: 612–615.
10. Cook JL, Walker TA, Lewis AM Jr, Rudaie HE, Graham FL, et al. (1986) Expression of the adenovirus E1A oncogene during cell transformation is sufficient to induce susceptibility to lysis by host inflammatory cells. Proc Natl Acad Sci U S A 83: 6965–6969.
11. Cook JL, May DL, Wilson BA, Holkin B, Chen MJ, et al. (1989) Role of tumor necrosis factor-alpha in E1A oncogene-induced susceptibility of noncellular cells to lysis by natural killer cell killers and activated macrophages. J Immunol 142: 4527–4534.
12. Miura TA, Morris K, Ryan S, Cook JL, Routes JM (2003) Adenovirus E1A, not human papillomavirus E7, sensitizes tumor cells to lysis by macrophages through nitric oxide- and TNAα-specific mechanisms despite up-regulation of 70kDa heat shock protein. J Immunol 170: 4119–4126.
13. Routes JM, Ryan S, Clase A, Miura T, Khali A, et al. (2000) Adenovirus E1A oncogene expression in tumor cells enhances killing by TNAα-related apoptosis-inducing ligand (TRAIL). J Immunol 165: 4525–4527.
14. Hoggquist KA, Jameson SC, Heath WR, Howard JL, Bevans MJ, et al. (1994) T cell receptor antagonist peptides induce positive selection. Cell 76: 17–27.
15. Barnede MJ, Allison J, Heath WR, Carbone FR (1998) Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol 76: 34–40.
16. Egan C, Jelma TN, Howe JA, Bayley ST, Ferguson B, et al. (1988) Mapping of cellular protein-binding sites on the products of early-1 region 1A of human adenovirus type 5. Mol Cell Biol 8: 3953–3959.
17. Spiess PJ, Yang JC, Rosenberg SA (1987) In vivo antitumor activity of tumor-infiltrating lymphocytes expanded in recombiant interleukin-2. J Natl Cancer Inst 79: 1067–1073.
18. Marzo AL, Kianer BF, Lake RA, Frelinger J, Collins EJ, et al. (2000) Tumor-specific CD4+ T cells have a major “post-licensing” role in CTL mediated anti-tumor immunity. J Immunol 165: 6047–6053.
19. Loubaki L, Tremblay T, Bazin R (2013) In vivo depletion of leukocytes and platelets following injection of T cell-specific antibodies into mice. J Immunol Methods 393: 38–44.
20. Lin EY, Li JF, Gnaikovitch L, Deng Y, Zhu L, et al. (2006) Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 66: 11238–11246.
21. Torisu H, Ono M, Kiryu H, Furuse M, Ohmoto Y, et al. (2000) Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFα and IL-1α. Int J Cancer 85: 102–106.
22. Qian B, Deng Y, Im JH, Musch RJ, Zou Y, et al. (2009) A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PLoS One 4: e6562.
23. Rodriguez PC, Zoa AH, Colotta KS, Zabala J, Ochoa JB, et al. (2002) Regulation of T cell receptor CD3ζα chain expression by L-arginine. J Biol Chem 277: 21123–21129.
24. Rodriguez PC, Quiceno DG, Ochoa AC (2007) L-arginine availability regulates T lymphocyte cell-cycle progression. Blood 109: 1560–1573.
25. Talma R, Ochoa JB, Faghri N, Collotta K, Park HJ, et al. (2001) L-Arginine regulates the expression of the T-cell receptor zeta chain (CD3ζα) in Jurkat cells. Clin Cancer Res 7: 9536–9545.
26. Stein M, Kesek S, Harris N, Gordon S (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 176: 207–292.
27. Rodriguez PC, Hernandez CP, Morrow K, Sierra R, Zabaleta J, et al. (2010) L-arginine deprivation regulates cyclin D3 mRNA stability in human T cells by controlling H2R expression. J Immunol 184: 5308–5304.
28. Prat C, Berthelot A, Cantelip B, Wendling D, Demougeot C (2012) Treatment with the arginase inhibitor Nω-hydroxy-nor-L-arginine restores endothelial function in rat adjuvant-induced arthritis. Arthritis Res Ther 14: R130.
29. Iyer RK, Yoo PK, Kern RM, Rozengurt N, Tsea R, et al. (2002) Mouse model for human arginase deficiency. Mol Cell Biol 22: 4491–4498.

30. Kasten J, Hu C, Bhargava R, Park H, Tai D, et al. (2013) Lethal phenotype in conditional late-onset arginase 1 deficiency in the mouse. Mol Genet Metab 110: 222–230.

31. Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, et al. (2004) Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. J Exp Med 200: 771–782.