Adenovirus serotype 5 E1A expressing tumor cells elicit a tumor-specific CD8+ T cell response independent of NKG2D

Michael J. Korrer a,b,c,d,* , John M. Routes a,b,c,d,*

a Department of Pediatrics, Division of Allergy and Clinical Immunology, Medical College of Wisconsin, Milwaukee, WI, USA
b Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI, USA
c Children’s Research Institute, Medical College of Wisconsin, Milwaukee, WI, USA
d Cancer Center, Medical College of Wisconsin, Milwaukee, WI, USA

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The expression of the Adenovirus serotype 2 or serotype 5 (Ad2/5) E1A gene in tumor cells upregulates ligands that are recognized by the NKG2D activating receptor, which is expressed on NK cells and T cells, and reduces their tumorigenicity, a process dependent on NK cells and T cells. In some model systems, the forced overexpression of NKG2D ligands on tumor cells induced antigen-specific CD8 + T cells that mediated anti-tumor immunity. We wanted to determine if the interaction of NKG2D ligands on tumor cells that express E1A with NKG2D on immune cells contributed to the ability of E1A to induce a CD8 + T cell anti-tumor response or reduce tumorigenicity. To address these questions, we used the MCA-205 tumor cell line or MCA-205 cells that expressed Ad5 E1A (MCA-205-E1A cells), a fusion protein of E1A and ovalbumin (MCA-205-E1A-OVA) or OVA (MCA-205-OVA). We found that the expression of E1A or E1A–OVA, but not OVA, upregulated the expression of the NKG2D ligand RAE-1 on the surface of MCA-205 cells. Additionally, MCA-205-E1A cells and MCA-205-E1A-OVA cells were more sensitive to NK cell lysis than MCA-205 or MCA-205-OVA cells in WT B6 mice, but not NK2D deficient B6 mice. Next, we adoptively transferred WT or NK2D deficient OT-1 T cells (CD8 T cells that recognize OVA residues 257–264) into WT B6 mice or B6 mice that were deficient in NKG2D respectively and measured the expansion of OT-1 T cells following immunization with MCA-205-E1A-OVA or MCA-205-OVA cells. We found that the expansion of OT-1 T cells following immunization of either OVA-expressing MCA-205 cell lines was not affected by the presence or absence of NK2D in B6 mice. Finally, we found that the capacity of E1A to reduce the tumorigenicity of MCA-205 cells was impaired in B6-NK2D deficient mice as compared to WT B6 mice. Our results suggest that the ability of E1A to reduce the tumorigenicity of MCA-205 cells, or induce an antigen-specific CD8 + T cell response, is independent of the interaction of NKG2D ligands with the NKG2D receptor.

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1. Introduction

NKG2D is an activating receptor found primarily on NK cells and CD8 + T cells in rodents and humans [1]. The NKG2D ligands are a diverse group of proteins, which are upregulated in response to cellular stress, viral infection, cellular transformation, and DNA damage [2–6]. The engagement of NKG2D on NK cells with NKG2D ligands on the cell surface of target cells, leads to target cell killing and cytokine production by NK cells [1,3]. Ectopic expression of NKG2D ligands on tumor cell surfaces has been shown to significantly reduce the tumorigenicity of tumor cells in an NK cell dependent-manner [3,6–8]. In some tumor models, it appears that the ectopic expression of NKG2D ligands also induces antigen-specific, CD8 + anti-tumor immune responses [8]. How the interaction of NKG2D on CD8 T cells and NKG2D on target cells activates naïve CD8 T cells is unclear, as NKG2D does not appear to be able to directly activate CD8 T cells, but instead provides co-stimulatory signals [4,9–11].

Adenovirus serotype 2 or serotype 5 (Ad2/5) E1A (heretofore referred to as E1A), when stably expressed in tumor cells, reduces their tumorigenicity [12,13]. Both NK cells and T cells [14] mediate the reduction of tumorigenicity by E1A. Tumor cells stably expressing E1A are sensitive to NK mediated killing in vitro and in vivo, in part through the upregulation of NKG2D ligands [7]. In
B6-derived MCA-205 tumor cells, E1A has been shown to only increase the expression of the NKG2D ligands, RAE-1. In B6-RAG<sup>−/−</sup> mice lacking adaptive immune systems, blocking the interaction of NKG2D ligands with the NKG2D receptor, using a specific mAb, inhibited the lysis of MCA-205-E1A tumor cells in vitro and increased their tumorigenicity in vivo [7].

The purpose of this study was to determine if the interaction of NKG2D ligands on MCA-205 tumor cells that express E1A with NKG2D on immune cells contributed to the ability of E1A to induce an antigen-specific CD8<sup>+</sup> T cell response. Furthermore, we sought to determine if the upregulation of RAE-1 by E1A decreased the tumorigenicity of MCA-205-E1A cells in WT mice. We found that NKG2D–NKG2D ligand interactions were dispensable for the expansion of antigen-specific CD8<sup>+</sup> T cells and for the anti-tumorigenic activity of E1A in WT B6 mice.

2. Results

2.1. Role of interaction of NKG2D ligands and the NKG2D receptor in the NK cell lysis of E1A-expressing MCA-205 tumor cells

We previously demonstrated that the expression of E1A in the B6-derived tumor cell line, MCA-205 cells (MCA-205-E1A), upregulated the NKG2D ligand RAE1 and sensitized MCA-205 cells to NK-mediated killing [7]. First, we generated MCA-205 cell lines that expressed a slightly truncated OVA<sub>3-383</sub> protein (MCA-205-OVA) or a fusion protein of E1A and OVA<sub>3-383</sub> (MCA-205–E1A-OVA) [16]. Next, we determined if expression of E1A–OVA fusion protein induced the NKG2D ligand, RAE1 on the MCA-205 cells as well. We found that expression of E1A–OVA and E1A, but not OVA, induced expression of RAE1 in MCA-205 cells (Fig. 1A). Next, we determined if NKG2D deficient NK cells, compared to normal B6 NK cells, were impaired in their ability to kill MCA-205-E1A or MCA-205–E1A-OVA tumor cells in a 5 h in vitro NK killing assay. These results demonstrated that, in comparison to normal NK cells, NKG2D deficient NK cells were markedly impaired in their capacity to lyse both MCA-205-E1A (Fig. 1B) and MC-205-E1A-OVA cells (C). In fact, the ability of E1A or E1A–OVA to sensitize MCA-205 cells to NK cell killing was nearly lost when using NKG2D deficient NK cells as effector cells (Fig. 1B and C). Collectively, these data showed that the ability of E1A or E1A–OVA to sensitize MCA-205 cells was largely dependent on the interaction between NKG2D on NK cells and RAE-1 on MCA-205 cells.

2.2. Role of the interaction of NKG2D ligands and the NKG2D receptor in the induction of antigen specific CD8<sup>+</sup> T cells

We next asked if the interaction of NKG2D ligands on MCA-205-OVA cells or MCA-205–E1A-OVA cells with NKG2D on immune cells was required for the expansion of OVA-specific CD8<sup>+</sup> T cells. To answer this question, we adoptively transferred NKG2D sufficient OT-1 cells or NKG2D deficient OT-1 CD8<sup>+</sup> T cells into B6 WT or NKG2D deficient B6 mice, respectively. Twenty-four hours after adoptive transfer of OT-1 cells, we injected MCA-205-OVA cells or MCA-205–E1A-OVA cells into the hock of B6 WT or NKG2D deficient B6 mice, respectively. After 5 days, the draining lymph nodes were removed, and OT-1 CD8<sup>+</sup> T cells were enumerated by flow cytometry (Fig. 1A and B). We found that the expansion of OT-1 cells following immunization of E1A–OVA or OVA-expressing MCA-205 cell lines was not affected by the presence or absence of NKG2D in B6 mice. Interestingly, immunization of WT or NKG2D deficient mice with MCA-205-OVA cells, which express little RAE-1, consistently led to a more robust expansion of OT-1 T cells compared to immunization with MCA-205–E1A-OVA (Fig. 2B). The mechanism for this result is under current investigation and is outside the goals of the current manuscript.

2.3. Role of interaction of NKG2D with NKG2D ligands in mediating tumor rejection of MCA-205 cells that express E1A or E1A–OVA

In previous studies using T cell deficient, B6-Rag<sup>−/−</sup> mice, we demonstrated that NK cells contribute to the rejection of MCA-205-E1A cells in an NKG2D–NKG2D ligand dependent manner [7]. Using this finding, we next addressed the role of NKG2D–NKG2D ligand interactions in the rejection of MCA-205 cells that express E1A in WT (T cell sufficient) mice or mice deficient in NKG2D (Fig. 3).

Tumorigenicity was measured by determining the tumor-producing dose 50 (TPD<sub>50</sub>), which is the log<sub>10</sub> of the number of tumor cells required to form tumors in half of the B6 mice (Material and methods). In previous studies, we demonstrated that the TPD<sub>50</sub> of MCA-205–E1A cells and MCA-205–E1A-OVA cells were equivalent.
that the reduction of tumorigenicity by E1A is dependent on NK cells and T cells [14]. Expression of E1A induces NK2G2 ligands on the surface of cells, and NK2G2 is expressed on NK cells and CD8+ T cells. The NK cell mediated killing of tumor cells that express E1A is dependent, at least in part, on the interaction between NK2G2–NK2G2 ligands [7]. Therefore, it seemed possible that the interaction of NK2G2 ligands with NK2G2 could link the innate and CD8+ T cell response to E1A expressing tumor cells in mice.

We found that the expression of E1A, or a fusion protein of E1A and OVA, induced the expression of RAE-1 on MCA-205 cells when the number of CD8+ T cell responses were equal to MCA-205-E1A-OVA cells by NKG2D deficient NK cells was significantly decreased compared to NK2G2 sufficient NK cells (Fig. 1B). These results are consistent with our prior studies and the large literature on the importance of the interaction of NK2G2 ligands and NK2G2 in the NK cell mediated lysis of specific target cells that express high levels of NK2G2 ligands.

In contrast to NK cell mediated killing of tumor cells that express E1A, we found that the interaction of NK2G2 ligands with NK2G2 was dispensable for the expression of OVA-specific CD8+ T cells following challenge with MCA-205-OVA cells or MCA-205-E1A-OVA cells (Fig. 2). Prior studies on the role of NK2G2–NK2G2 interactions in the induction of CD8+ antigen-specific antitumor responses are mixed. Diefenbach et al. [8] found that the ectopic expression of NK2G2 ligands on tumor cells reduced tumorigenicity and led to the induction of antigen-specific, CD8+ T cell antitumor immunity. Similar to our results, Cerwenka et al. found that the forced expression of NK2G2 ligands on tumor cells reduced tumorigenicity, but did not enhance the generation of tumor-specific CD8+ T cell responses [3].

We hypothesize that MCA-205-OVA tumor cells induce a greater OVA-specific CD8+ T cell response than MCA-205-E1A-OVA cells due to increased tumor antigen load. The number of MCA-205-OVA cells used for these studies is well above the TPD50, which would result in persistent and growing MCA-205-OVA tumor cells in vivo. Conversely, MCA-205-E1A-OVA cells are non-tumor forming and rapidly eliminated in vivo, which likely leads to reduced antigen load over the course of the experiment. OT-1 cell expansion similar to that of MCA-205-OVA cell immunization were observed with MCA-205-E1A-OVA cells when the number of cells inoculated was increased 10-fold, (data not shown), suggesting the amount of tumor antigen load may be responsible for the differences observed between MCA-205-OVA and MCA-205-E1A-OVA cells.

The reasons for the disparity in the role of NK2G2–NK2G2 interactions in the elicitation of tumor-specific CD8+ T cell responses is unclear. It is possible that NK2G2 signaling was dispensable in our model due to the presence of a strong foreign antigen, OVA. Natural tumor antigens may provide weaker activation signals than OVA, and therefore may require additional co-stimulation which could be provided by NK2G2 signaling. MCA-205 is mildly immunogenic and therefore expresses an endogenous tumor antigen [23]. However, the tumorigenicity of either MCA-205 or MCA-205-E1A tumor cells was not increased in NK2G2 sufficient mice compared to wild type mice. Collectively, these data suggest that in the MCA-205 model, the interaction of NK2G2 with NK2G2 ligands is dispensable for the anti-tumor immune response.

Finally, studies reported herein suggest that the reduction of tumorigenicity by E1A is largely independent of NK2G2–NK2G2 ligand interactions in WT B6 mice. Previous work by our laboratory has shown that the rejection of tumor cells that express E1A in WT B6 mice is partially dependent on T cells. In RAG−/− B6 mice, blocking the interaction of NK2G2 with NK2G2 ligands increased the tumorigenicity of MCA-205-E1A cells [7]. Because the expansion of antigen-specific CD8+ T cells occurs in an NK2G2–

3. Discussion

In this study, we investigated the role of the interaction of NK2G2 ligands on tumor cells that express E1A and NK2G2 on immune cells in elicitation of antigen-specific CD8+ T cells, and the anti-tumorigenic properties of E1A. Prior studies have shown

in B6 mice [16]. Furthermore, the ability of E1A and E1A–OVA to upregulate RAE-1 expression or sensitize MCA-205 cells to NK cell lysis was also equivalent (Fig. 1A–C). Therefore, to decrease the numbers of mice, only MCA-205 cells and MCA-205-E1A cells were used in these tumor induction studies. We found that the TPD50 of MCA-205-E1A cells were equivalent in WT B6 mice compared to mice that lacked NK2G2. Collectively, these studies suggest that NK2G2–NK2G2 ligand interactions play a relatively minor role in the ability of E1A to reduce tumorigenicity in WT (T cell sufficient) mice.

Fig. 2. NK2G2 deficiency does not alter the expansion of OT-1 CD8 T cells in response to injection of MCA-205-E1A-OVA cells in vivo. B6 or NK2G2 deficient mice were adoptively transferred with 1 x 106 CD45.1+ OT-1 cells that were NK2G2 sufficient or NK2G2 deficient respectively and 24 h later administered MCA-205, MCA-205-OVA or MCA-205-E1A-OVA tumor cells s.c. in the flank. Five days later the draining lymph node was examined by flow cytometry for the percentage of OT-1 cells (A) and the total number of OT-1 cells (B). OT-1 cells were identified as CD45.1+ CD3+ CD8+ cells. Data shown is the mean ± SEM from two experiments, with three to six mice per group. Data were analyzed by ANOVA followed by Tukey’s HSD post hoc analyses.

Fig. 3. NK2G2 deficiency does not alter the tumorigenicity of MCA-205-E1A cells. Serial log dilutions of MCA-205 cells from 1 x 102–1 x 106 cells or MCA-205-E1A cells from 1 x 104–1 x 106 cells (3 mice/dose tumor cells) were injected s.c. into the flank of either WT B6 or NK2G2 deficient mice. The TPD50 was calculated 12 weeks later. Data shown is the mean ± SEM from two experiments. Data were analyzed by a Student’s t-test. * p < 0.01.
NKG2D ligand independent manner (Fig. 2), these results suggest that there is a redundant role for NK cells in WT B6 mice that is fulfilled by T cells in an NKG2D-independent manner.

Technical issues intrinsic to the MCA-205 model may contribute to the inability to detect a change in tumorigenicity in a WT B6 mice compared to B6 mice lacking NKG2D. Injection of MCA-205-E1A cells or MCA-205-E1A-OVA cells fail to cause tumor formation in a WT animal at the highest challenge dose (10⁷ tumor cells), which is the highest inoculum we can give in a small volume, due to technical limitations. Thus, with a higher tumor cell burden, NKG2D dependent and independent mechanisms may be required for rejection of MCA-205 tumor cells that express E1A in WT B6 mice. This hypothesis is consistent with results demonstrating that that ectopic expression of NKG2D ligands on tumor cell lines reduces tumorigenicity [3,6–8]. Further investigations on the role of NKG2D–NKG2D ligand interactions in the reduction of tumorigenicity by E1A are needed to clarify this issue.

We found the ability of E1A to sensitize cells to NK cell mediated killing is largely NKG2D–NKG2D ligand dependent. In contrast, the interaction of NKG2D ligands with NKG2D appears to be dispensable for the ability of E1A to generate antigen specific CD8+ anti-tumor immune responses, and for the reduction of tumorigenicity in WT B6 mice. These findings are consistent with our previous work that showed that E1A may reduce tumorigenicity by modulation of tumor microenvironment through the inhibition of arginase 1 produced by tumor associated macrophages [16]. Arginase 1 produced in the tumor microenvironment results in a depletion of l-arginine, which is essential for T cell effector function [18]. Additional potential mechanisms whereby E1A may decrease tumorigenicity include sensitizing cells to immune effector molecules such as TNF-α, TRAIL, nitric oxide, perforin and fas-dependent killing [19–22].

In summary, we found that the ability of E1A to reduce the tumorigenicity of MCA-205 cells and elicit an antigen-specific CD8 T cell response is independent of the interaction of NKG2D ligands with NKG2D.

4. Materials and methods

4.1. Mice

WT C57BL/6J (B6), B6.129S7-Rag1tm1Mom/J (RAG−/−), B6.129S7-PpcrePepcbBoyJ (CD45.1) and C57BL/6-Tg[TcraTrcb]1100Mjb/J (OT-1) mice were purchased from the Jackson Laboratories. OT-1 mice express a transgene for a T cell receptor that recognizes ovalbumin (OVA) residues 257–264 in the context of H-2Kb. B6- Klrk1tm1Dhr (NKG2D−/−) mice were kindly provided by David H. Raulet [15] (University of California, Berkeley, Berkeley, CA). NKG2D−/− mice were bred with CD45.1+ OT-1+ RAG−/− mice to generate NKG2D−/−CD45.1+ OT-1+ RAG−/− mice. All animal work was reviewed and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

4.2. Reagents

Roswell Park Memorial Institute (RPMI) medium with 5% Fetal Bovine Serum (FBS) (RPMI-5) or 10% FBS (RPMI-10) supplemented with Glutamax, glucose and antibiotics was used for all cell culture. FBS (Atlanta Biologicals) was heat inactivated for 45 min at 56 °C.

4.3. Flow cytometry

Flow cytometry was performed with an LSR II (BD biosciences) using BD FACSDiva software. Flow cytometry analysis was performed using FlowJo software (Tree Star). Antibodies specific to mouse CD3 (145-2C11) Alexa Fluor 488 (AF-488); Fluorescein (FITC), CD8+ (5H-10) PE: Pacific Orange (PO), and CD45.1 (A20) Allophycocyanin (APC) were purchased from Biologend. Pan-RAE1 (CX1) PE antibody was purchased from BD Biosciences.

4.4. Cell lines

MCA-205, a C57BL/6 derived fibrosarcoma cell line, was kindly provided by N. Restifo (National Cancer Institute, National Institutes of Health, Bethesda, MD). MCA-205 lines that stably expressed E1A−/−OVA (MCA-205-E1A-OVA), or OVA (MCA-205-OVA) were generated as previously described [16].

4.5. NK cell cytolysis assays

Mouse NK cells were enriched from either B6 or NKG2D deficient splenocytes by nylon wool enrichment as previously described [17] and used in a standard 5 h ⁵¹Cr release assay [7]. NK purity was confirmed by flow cytometry, and only NK cell preparations with greater than 90% NK cells were used.

4.6. Quantification of OVA specific CD8+ T cells

OT-1 transgenic CD8+ T cells were harvested from the spleens of either NKG2D+/−CD45.1+ OT-1+ RAG−/− mice or NKG2D−/−CD45.1+ OT-1+ RAG−/− mice. 1 × 10⁵ NKG2D+/−CD45.1+ OT-1+ cells or NKG2D−/−CD45.1+ OT-1+ cells were adoptively transferred via retro-orbital injection into WT or NKG2D−/− B6 mice respectively. The following day, mice were immunized with 1 × 10⁵ live MCA-205-OVA or MCA-205-E1A-OVA cells subcutaneously (s.c.) in the hock (the lateral tarsoal region just above the ankle). Five days after tumor cell injection, the popliteal lymph nodes were removed from WT or NKG2D−/− B6 mice and the CD45.1+ OT-1+ cells were quantitated by flow cytometry by staining for CD45.1+ CD3+ CD8+ T cells. The absolute number of CD8+ T cells was determined by multiplying the percentage of the target cell population by the total number of cells in the lymph node.

4.7. Tumor induction studies

Quantitative tumor inductions studies were performed as previously described [7]. Briefly, B6 or NKG2D deficient mice were administered serial log dilutions of MCA-205 or MCA-205-E1A tumor cells s.c. in a total volume of 0.2 cc in the flank of WT B6 or NKG2D and then monitored for tumor growth. Animals were euthanized when tumors reached 20 mm in diameter, or at the end of a 12-week monitoring period. Tumor producing dose 50 (TPD₅₀) values, which are the log₁₀ of the number of cells required to form tumors, were calculated using the Spearman–Karber Formula. Due to technical limitations, 10² was the highest number of tumor cells that could be given in 0.2 cc volume. Therefore, if tumors did not occur at 10⁷ tumor cells, the TPD₅₀ was estimated 0.5 logs higher than the highest attainable dose (e.g. TPD₅₀ = 7.5).

4.8. 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. Any 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) (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
Authors’ contributions

MK performed the studies and prepared the manuscript. JR conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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