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Early expression of stem cell-associated genes within the CD8 compartment after treatment with a tumor vaccine

M. Eric Kohler [a,1], William H.D. Hallett [a,1], Qing-Rong Chen [b], Javed Khan [b], Bryon D. Johnson [a], Rimas J. Orentas [c,*]

[a] Dept. Pediatrics, Section of Hematology Oncology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226, United States
[b] Oncogenomics Section, Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Advanced Technology Center, 8717 Grovemont Circle, Gaithersburg, MD 20877, United States
[c] Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, 10 Center Dr., 1W3840, Bethesda, MD 20892, United States

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ABSTRACT

Using a mouse neuroblastoma cell line, we have demonstrated that vaccination of tumor-free mice with a cell-based vaccine leads to productive immunity and resistance to tumor challenge, while vaccination of tumor-bearing mice does not. The T cell immunity induced by this vaccine, as measured by in vitro assays, is amplified by the depletion of Treg. Our goal is to understand this barrier to the development of protective cellular immunity. mRNA microarray analyses of $CD8^+$ T cells from naïve or tumor-bearing mice undergoing vaccination were carried out with or without administering anti-CD25 antibody. Gene-expression pathway analysis revealed the presence of $CD8^+$ T cells expressing stem cell-associated genes early after induction of productive anti-tumor immunity in tumor-free mice, prior to any phenotypic changes, but not in tumor-bearing mice. These data demonstrate that early after the induction of productive immune response, cells within the $CD8^+$ T cell compartment adopt a stem cell-related genetic phenotype that correlates with increased anti-tumor function.

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1. Introduction

Neuroblastoma is the most common extracranial solid cancer in childhood, accounting for 15% of childhood cancer deaths [1]. Patients with high-risk disease have poor prognoses (<40% long-term survival) despite the use of intensive therapies [2,3]. Therefore, new therapeutic strategies are needed to improve the survival of these patients. Immune therapies to cancer potentially offer specificity with minimal toxicity. These therapies include the administration of monoclonal antibodies, adoptive transfer of immune cells, and tumor vaccines in the form of whole tumor cells, purified proteins or peptides, or gene vectors [4,5]. However, immune therapies for neuroblastoma have proven difficult due to lack of known tumor antigens and inability to identify tumor-responsive $CD8^+$ T cells.

The development of long-term immunity to a particular tumor antigen is dependent on generation of long-lived memory T cells. $CD8^+$ memory T cells have unique properties that make them well suited to respond quickly and effectively during immunotherapy for neuroblastoma. Memory $CD8^+$ cells remain primed for rapid proliferation in response to antigen reemergence, and they maintain mRNA expression of several cytotoxic proteins as well as pro-inflammatory cytokines [6,7]. The factors involved in the development of $CD8^+$ T cell memory responses remain poorly understood. Following recognition of a cognate ligand and appropriate co-stimulatory signals, $CD8^+$ T cells expand into armed effector cells as well as memory cells, though the factors involved and timing of memory development remain elusive. Multiple models have been developed to explain the development of $CD8^+$ T cell memory [8]. It has been reported that the induction of memory T cells is programmed and/or initiated as early as the first day of antigen exposure [9–11].

We have previously reported that a cell-based vaccine for neuroblastoma that transiently expressed CD80, CD86, CD54, and CD137L generated protective immunity in resting mice [12] and following HSCT [13]. The protective immunity was based on both $CD8^+$ and $CD4^+$ T cells and generated tumor-reactive, IFN-γ producing T cells in the spleens of vaccinated mice. Anti-CD25 therapy is known to deplete and functionally inactivate $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) [14–16]. The combination of anti-CD25 to the cell-based vaccine therapy enhanced vaccine efficacy compared to vaccine treatment alone [16]. These data demonstrated that anti-neuroblastoma immunity could be generated by the use of a cell-based vaccine and that the strength of the vaccine was enhanced by anti-CD25 therapy.

* Corresponding author. Fax: +1 414 456 7010.
E-mail address: rimas.orentas@nih.gov (R.J. Orentas).
[1] These authors contributed equally to this work.

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We report that a productive vaccine-induced immune response in mice results in $CD8^+$ T cells with a genetic signature that is shared with stem cells of hematopoietic, neuronal, and embryonic origin. Interestingly, an opposing effect of vaccination is observed in tumor-bearing mice, where $CD8^+$ T cells are driven away from the stem cell-related genetic signature and attenuated immune responses are observed. Therefore, we believe the "stemness" signature of $CD8^+$ T cells represents an early signature of developing functional immune responses and may represent incipient $CD8^+$ memory cells.

2. Materials and methods

2.1. Animals and cell lines

A/J mice were purchased from The Jackson Laboratory and used at 6–8 weeks of age. All experiments were performed under approved protocols in a specific pathogen-free environment at the Medical College of Wisconsin, which contains an American Association for the Accreditation of Laboratory Animal Care-certified facility. Generation of AGN2a, a subclone of Neuro-2a (obtained from American Type Culture Collection), was described previously [17]. Cryopreserved stocks of wild-type AGN2a cells were generated and used for all tumor challenge experiments to minimize experimental variability. For tumor vaccine generation (AGN2a-4P), AGN2a cells in log growth were electrotransfected with plasmids encoding CD80, CD86, CD54, and CD137-L using the AMAXA nucleofection system (Gaithersburg, MD) as previously described [12].

2.2. Tumor inoculation, vaccination and anti-CD25 administration

Tumor-bearing mice were inoculated with 10^5 or 10^6 AGN2a cells by subcutaneous (s.c.) injection of the right hind flank (day 0). Mice receiving vaccination were given 2×10^6 AGN2a-4P cells by s.c. injection in the right hind flank on day 6 or on days 6 and 13. Mice treated with anti-CD25 monoclonal antibody (clone PC61) received 250 μg of PC61 by intraperitoneal injection on day 3. Control mice not receiving tumor, vaccine or anti-CD25 were injected with PBS.

2.3. Antibodies

The following monoclonal antibodies (mAbs), with or without a fluorescent label, were used in this study. Anti-CD80 (16-10A1), anti-CD4 (OX-35), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD25 (7D4) and anti-CD137L (TKS-1) were purchased from BD Biosciences Pharmingen (San Jose, CA). Anti-CD8 (53-6.7), anti-CD86 (GL1) and anti-CD54 (YN1) was purchased from eBioscience (San Diego, CA). Anti-CD25 monoclonal antibody (PC61) was generated from a hybridoma obtained from the American Type Culture Collection. PC61 was produced using Integra CL 1000 bioreactors (Chur, Switzerland).

2.4. $CD8^+$ cell enrichment

Spleens from A/J mice were collected, processed into single cell suspensions, and incubated with anti-CD8a conjugated microbeads (Miltenyi Biotec, Auburn, CA). $CD8^+$ T cells were isolated by automated immunomagnetic cell sorting (AutoMACS, Miltenyi Biotec). $CD8^+$ T cell enrichment was confirmed by flow cytometric analysis, and the cells were typically >95% $CD8^+$. Samples used in gene expression studies were processed and sorted in ice-cold buffers to minimize changes in gene expression due to the experimental procedure.

2.5. Analysis of cells by flow cytometry

Surface expression of T cell activation markers and immune co-stimulatory molecules on modified AGN2a tumor cells was verified as described previously [12]. Antibody-labeled cells were analyzed on a Becton–Dickinson FACSCalibur flow cytometer and data analyzed using Flow-Jo software (Tree Star, Ashland, OR).

2.6. IFN-γ ELISPOT assays

ELISPOT assays to assess numbers of tumor-reactive IFN-γ-secreting $CD8^+$ T cells were performed using a kit from BD Biosciences Pharmingen as previously described [16]. Briefly, 96-well hydrophobic polyvinylidene difluoride membranes were coated with IFN-γ capture monoclonal antibody. Two-fold dilutions of purified $CD8^+$ T cells were added to the plates (concentrations varying from 10^5 to 2×10^3 per well) in triplicate along with 5×10^4 irradiated ANG2a target cells expressing CD80 and CD86 (AGN2a-80/86) or CD80 and CD137L (AGN2a-80/137L), which increased sensitivity of the response while maintaining specificity. Controls consisted of $CD8^+$ T cells cultured alone and irradiated tumor cells cultured alone. The plates were incubated overnight at 37°C. After incubation, the plates were washed to remove the cells and biotinylated anti-IFN-γ detection antibody was added for 2 h at room temperature. Extravidin alkaline phosphatase (Sigma–Aldrich Corp, St. Louis, MO) was added, and the spots developed by adding BCIP/NBT substrate (Sigma–Aldrich). Numbers of spots, corresponding to numbers of IFN-γ-secreting cells, were quantified with an ImmunoSpot Analyzer using included acquisition and analysis software (CTL Analyzers, LLC, Cleveland, OH).

2.7. RNA isolation and microarrays

To minimize processing-related changes in gene expression and RNA degradation, cells were collected, processed and isolated on ice. Splenic $CD8^+$ T cells were isolated from individual mice as described above. RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and further purified with the RNeasy Micro Kit (Qiagen, Valencia, CA). The purified RNA was quantified with a Nanodrop 1000 UV/Vis-spectrophotometer (Thermo Scientific, Wilmington, DE) and equal amounts of RNA from five to six mice were pooled. Pooled samples were sent to Expression Analysis Inc. (Durham, NC), where labeled cRNA was generated and hybridized to Affymetrix GeneChip® Mouse Genome 430.2 microarrays (Santa Clara, CA). Microarray data were exported from Affymetrix GCOS software as CEL files and normalized in dCHIP to the median intensity using the PM-only model [18]. The data were Log2 transformed and filtered to exclude probe sets with an "Absent" call in every sample.

2.8. Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) (http://www.broad.mit.edu/gsea/) was used to interrogate the filtered data for the enrichment of gene sets in the C2 collection of the Molecular Signature Database (MSigDB) [19]. Expression data were collapsed to the median probe set signal for each gene and genes were ranked based on the "Difference of Classes" metric by GSEA. Ranked gene lists were generated by comparing microarray data of tumor-bearing and tumor-free mice given the same treatments. Gene Set Enrichment Analysis was performed on ranked gene lists using 1000 permutations of the gene sets to normalize the enrichment scores. Gene sets were considered to be significantly enriched with a p value >0.05 and a false discovery rate (FDR) <0.05. Results were generated from averaging and normalizing two independent experiments. In each experiment, five to six mice from each group