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TITLE: IL-17-polarizing Vaccination Targeting MUC1 for the Prevention of Lung Cancer

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CONTRACTING ORGANIZATION:
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14. ABSTRACT

Lung cancer disproportionately affects United States Veterans with rates of disease nearly double to those found in the general population. Immunosurveillance, whereby an effective immune response can detect and eliminate abnormal cells before they progress to invasive malignancy, provides rationale for the development of a prophylactic vaccination regimen. Herein, we developed an adenoviral-based vaccination delivering MUC1 tumor-associated antigen and the immunostimulatory molecule ICOS-L to induce a unique subset of effector T cells, IL-17-producing CD4+ T cells, termed Th17s, shown to have superior antitumor reactivity. As peripheral tolerance limits the induction of immunity to self-derived tumor antigens, we have examined the ability to enhance vaccine immunogenicity through the ablation of regulatory T cells (Tregs) prior to immunization. We have illustrated that ICOS-L augmented vaccination significantly enhances Th17, but not Tc17 immune responses against MUC1. We further demonstrate that ablation of Tregs prior to immunization increases both cellular and humoral vaccine-induced immunity against MUC1. Preventative vaccination alters the results in greater accumulation of subsets of T cells within pulmonary tumors, with increased CD8+ T cells and myeloid cells associated with tumor inhibition and progression, respectively. Collectively, these studies represent a novel strategy for the prevention of lung cancer.

15. SUBJECT TERMS
Cancer vaccine, MUC1, T cell, antitumor immunity, lung cancer, ICOS ligand, Th17 cell

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INTRODUCTION: Lung cancer is the leading cause of cancer-related mortality in the United States, resulting in more deaths than colon, prostate, breast, and ovarian cancers combined. Significantly, roughly 70% of patients diagnosed with lung cancer present with locally advanced or metastatic disease at the time of diagnosis. With current medical interventions, ~95% of those who are diagnosed with lung cancer will die from the disease. Recent clinical success of T cell checkpoint inhibitors and adoptive cell transfer of engineered T cells demonstrates that functional tumor-reactive cellular immunity established via immunotherapy may eliminate solid tumors, including in the setting of non-small cell lung cancer (NSCLC). Nevertheless, the majority of patients fail to respond to immunotherapy, indicating additional mechanisms of T cell dysfunction prohibit effective tumor clearance. Our studies seek to induce potent antitumor immunity through vaccination in healthy individuals, prior to the establishment of tumor-derive immune suppression. Herein, we have combined a unique vaccination inducing Th17-mediated cellular immunity against the common tumor-associated antigen MUC1 with depletion of inhibitory regulatory T cells (Tregs) in a novel vaccination strategy for the prevention of lung cancer. Treg ablation directly alleviates mechanisms of host tolerance limiting the induction of tumor-reactive immunity to "self" proteins such as MUC1. These studies evaluate the immunogenicity of vaccination with and without Treg depletion and assess vaccine efficacy to prevent the establishment of lung cancer in a relevant murine model. Study findings characterize the phenotype and function of vaccine-induced T cell-mediated antitumor immunity, employing high-dimensional single-cell protein and transcriptomic analysis. Additionally, profiles of T cells associated with pulmonary tumor inhibition will be identified and characterized, informing future development of novel immunotherapy and identifying potential biomarkers for treatment efficacy. Collectively, study findings will advance our capability to induce effective antitumor immunity through preventative vaccination.

KEYWORDS: Cancer vaccine, Cancer prevention, MUC1, T cell, antitumor immunity, lung cancer, ICOS ligand, Th17 cell.

ACCOMPLISHMENTS: Throughout the time period of the Concept Award and no-cost extension granted, we have successfully met most goals within our proposed study. Regrettably, as I changed institutions during the course of this award, moving my laboratory from the Medical University of South Carolina and Ralph H. Johnson VAMC to the University of Pittsburgh School of Medicine (beginning in May of 2018), some data collection and analysis has been delayed. The following describes our progress completed.

1) What were the major goals of the project: From SOW

Aim1A: Evaluate Preventative Vaccine Efficacy:

- Submit IACUC/SRS protocols: Completed on schedule.
- Hire research technician: Ms. Rebecca Stanton was hired on schedule.
- Purchase adenoviral vectors: Completed on schedule.
- MUC1.Tg mouse breeding: Delayed completion; The colony of MUC1.tg mice previously maintained in my laboratory was expanded upon receipt of ACURO approved protocol. As ACURO approval took several months to obtain after receiving IACUC approval from the Ralph H. Johnson VA Medical Center, expanding our mouse colony to meet requirements of the study was delayed.
- Preventative vaccinations: Completed on schedule; Preventative vaccinations were completed at the RHJ VAMC. Partial findings are presented in the attached publication (Carrell, et. al. Vaccine, 2018) and additional data analysis is ongoing.
- MUC1-expressing Lewis Lung Carcinoma Challenge: Tumor cell challenge of mice following preventative vaccination or vaccination in conjunction with Treg depletion has been completed on schedule and data analysis is ongoing.
- Evaluate lung cancer pathology: Pulmonary histology including H&E and immunohistochemistry has been completed. Slides created and are currently being assessed by a pathologist. Data analysis is ongoing.

- Characterize vaccine-induced cellular immunity: Evaluation of vaccine-induced MUC1-specific T cell responses has been completed and reported in Carrell, et. al. Vaccine, 2018 (appendix). Characterization of the effect of Treg depletion on vaccine-induced immunity is currently ongoing. Computational analysis of flow cytometry and preliminary single-cell RNAseq data is underway. We expect to publish complete findings by Spring of 2020.

Aim1B: Identify Correlates of T cell-mediated Protection From Lung Cancer:

- Repeat preventative vaccinations: Repeat preventative vaccinations have been completed and part of this data set has been reported in Carrell, et. al. Vaccine, 2018 (appendix).
- FACS isolation of tumor-specific T cells following vaccination and tumor challenge: Methodology was established and T cell isolation for analysis or preservation has been performed.
- Isolation and preparation of cDNA from single T cells: Methodology was established at the RHJ VAMC to perform single-cell cDNA preparation using the Fluidigm C1 instrument. As this instrument was not available at the University of Pittsburgh, we have completed analysis using the 10x Chromium platform.
- Single-cell RNAseq analysis of T cells: Behind schedule: Following the establishment of this method, my laboratory moved to the University of Pittsburgh. My current institution uses the 10x Chromium system for single cell isolation and cDNA template preparation for single-cell RNAseq. We have currently collected data and on a subset of the T cells proposed. Complete collection of this data set will occur in the near future.
- Study data analysis and interpretation: Data analysis is ongoing. We have utilized the Cell Ranger program for 10x data analysis, and are currently exploring other computational analysis methods to perform unbiased analysis of transcriptomic data. We are preparing findings on the phenotype of the immune compartment established with or without vaccination regimens for publication in Spring 2020.

Preparation of Findings:

- We have recently published initial findings in Carrell, et. al. Vaccine, 2018 (appendix) including efforts by Dr. LaRue and Dr. Soloff. Dana analysis and manuscript preparation for the remainder of study findings is currently ongoing.
- Partial results and methodologies were presented at the 2018 Annual Meeting of the American Association of Cancer Researchers in an invited talk.
- Generation of proposals advancing study findings: Ahead of schedule. Together with my collaborator, Dr. Rajeev Dhupar (University of Pittsburgh, Pittsburgh VAMC), we have successfully generated three applications for extramural funding based on or building from some part of study findings.

2) What was accomplished under these goals?

1. Major activities: Conducting this investigation allowed me to develop as an early career investigator, gaining experience in management of a laboratory, troubleshooting experiments, development of novel methodologies such as single-cell RNAseq, and strengthen my discipline-specific conceptual knowledge regarding cancer vaccine development and immunologic control of malignancies.

1) Specific Objectives: The Concept Award contains the individual experimental objectives outlined below:

a. *Overcome tolerance to "self" MUC1 antigen in MUC1 transgenic (MUC1.tg) mice*: Induction of effective antitumor immunity is limited by mechanisms restricting the development of immunity against self-derived antigens. Essential to cancer vaccine development is the optimization of immunogens for the induction of immunity against tumor-associated antigens derived from host tissues. Our findings demonstrate that adenoviral based (Ad) vectors containing human MUC1 transgenes are sufficiently immunogenic to overcome tolerance to MUC1 in a transgenic mouse model which recognized human MUC1 as a "self" protein (Fig.1)(Carrell et al.). Herein, wild type C57Bl/6 or MUC1.Tg mice received prime-boost vaccination with Ad vectors containing human MUC1, or MUC1 and murine ICOS-L two weeks apart. Five days after immunization, MUC1-specific reactivity was assessed by intracellular cytokine measurement using flow cytometry. Results indicated that vaccination targeting MUC1, with or without ICOS-L, was sufficient to overcome immunotolerance, with antigen-specific IFNγ production from CD8+ T cells comparable between wild type and MUC1.Tg mice (Fig.1).

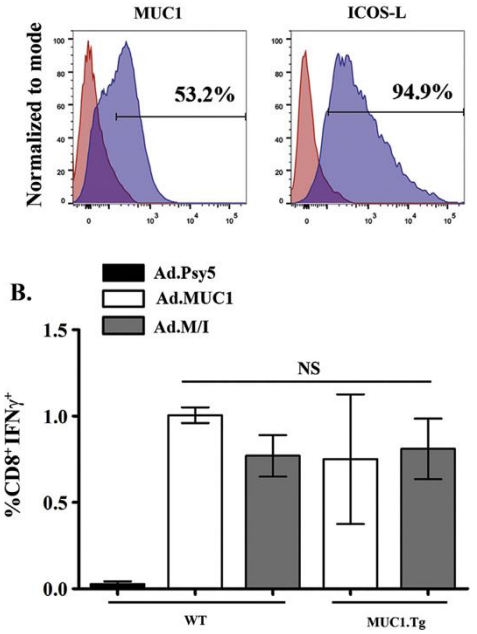

Figure 1: Vaccination induces CD8+ T cell responses in wild type and MUC1.Tg mice. (top) Bone marrow-derived DCs were transduced with Ad.MUC1 or Ad.ICOS.L at an MOI of 100. 24 h later extracellular expression of the MUC1 VNTR region and ICOSL were detected by flow cytometry. (bottom) Wild type or MUC1.Tg mice received primeboost immunizations 2wks apart with either empty vector (Ad.Psy5), Ad.MUC1, or Ad.M/I. 5d after immunization, intracellular IFNc production from CD8+ T cells was measured via flow cytometry from splenocytes cocultured with MUC1 peptidepulsed DCs. NS = non-significant.

A second key determinant of an effective preventative cancer vaccine will be to identify the optimal phenotypic and functional character of antitumor cellular immunity for the elimination of malignant cells. Utilizing pre-conditioned T cell subsets in murine adoptive T cell transfer studies for various cancer immunotherapies has demonstrated that Th17 cells possess a less exhausted state akin to "stemness" while retaining potent cytotoxic capabilities and effector cytokine profiles of dual IFNγ/IL-17 expression. Our previous findings suggested that provision of ICOS-L was sufficient to promote Th17-mediated immunity through expansion of pre-existing Th17 memory cells. Our recent findings published in Carrell et. al., demonstrate for the first time, to the best of our knowledge, that provision of ICOS-L is sufficient to induce antigen-specific immunity in vivo through vaccination. These data show that immunization induced MUC1-specific CD4+ T cells producing IFNγ and IL-17 (Fig.2a). Additionally, expression of the transcription factor RORγt, the master

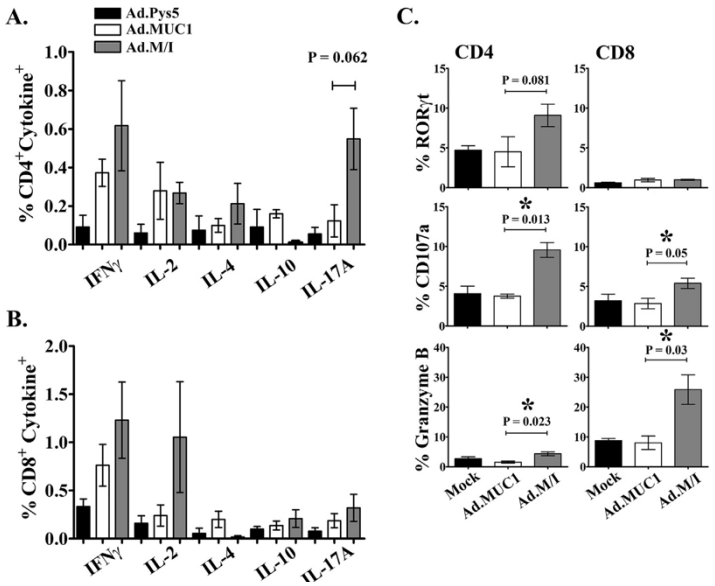

Figure 2: ICOSL promotes a bipolar MUC1-specific Th17/Th1 response to vaccination. MUC1.Tg mice received 3 immunizations with Ad.Psy5, Ad.MUC1, or Ad.M/I at 2wk intervals. 5d after the final immunization, MUC1-specific intracellular cytokine production was determined by flow cytometry in (A) CD4+ or (B) CD8+ T cells from splenocytes cultured with MUC1 peptide-pulsed DCs for 7d. (C) Intracellular expression of the Th17 transcription factor RORct, and cytotoxic effector proteins CD107a and granzyme B were determined 5d after final immunization in splenic CD4+ and CD8+ T cells without DC coculture via flow cytometry. Mean ± SEM; *p < .05.

regulator of Th17 development, was significantly upregulated in the CD4+ T cell populations following vaccination (Fig.2c). Interestingly, although ICOS-L augmented vaccination enhanced production of IFNγ and IL-2 from CD8+ T cells, immunization failed to upregulate their expression of IL-17 or RORγt (Fig.2b,c), suggesting that the pre-existing population of IL-17-polarized precursor memory T cells may be limited in the CD8+ T cell compartment. Findings were further supported through transcriptional profiling of vaccine-induced CD4 and CD8 T cells (Carrell et. al.). Furthermore, results indicate that IL-17-polarized T cell phenotype induced through vaccination was highly durable, persisting for 10 months following immunization. Together, these findings indicate that provision of ICOS-L is capable of promoting antigen-specific IL-17-polarized immunity during vaccination providing a novel method for the induction of highly inflammatory effector responses for the prevention of cancers and infectious diseases.

b. *To characterize the effect of Treg depletion on vaccine-induced immunity*: A major limitation to the induction of preventative antitumor immunity are mechanisms which maintain peripheral tolerance to antigens derived from self proteins. The high molecular weight glycoprotein MUC1 resides at the apical surface of luminal epithelial cells providing a physical barrier from pathogen colonization. During neoplastic transformation, MUC1 becomes overexpressed, loses apical-basal polarity, and importantly, undergoes aberrant glycosylation exposing a highly immunogenic N-terminal region consisting of variable numbers of 20-amino acid tandem repeats. Clinically, naturally occurring MUC1-specific cytotoxic T lymphocyte responses are associated with better prognosis and reduced lifetime risk of developing MUC1-expressing cancers, but are predominantly present in low frequencies and are unable to eradicate tumors. Pre-clinical therapeutic cancer vaccination in mice using MUC1 peptide-pulsed dendritic cells was shown to be strongly inhibited by Tregs. Together, we proposed that ablation of Tregs prior to vaccination would enhance vaccine immunogenicity and increase MUC1-specific immunity. Mice were administered CD25-specific antibody (clone PC-61.5.3) at 0.25mg 4 days prior to Ad-based vaccination as described above. Anti-CD25 administration resulted in near complete depletion of $CD4^+$ $CD25^+$ cells, including $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs, in the spleen and peripheral blood (Fig. 3a). Depletion of Tregs prior to three serial vaccinations with Ad.MUC1, $5x10^9$ vp at monthly intervals, significantly increased levels of MUC1-specific IgG antibody in circulation (Fig. 3b). Similarly, combining Treg ablation prior to vaccination with Ad vectors expressing MUC1 and ICOS-L further enhanced the ability of MUC1-specific CD4 and CD8 T cells to produce IFNγ and IL-17 in response MUC1 peptide re-stimulation (Fig.3c). Although data analysis is currently ongoing, these results demonstrate that transient depletion of Tregs is capable of enhancing vaccine-induced immunity against tumor-associated MUC1.

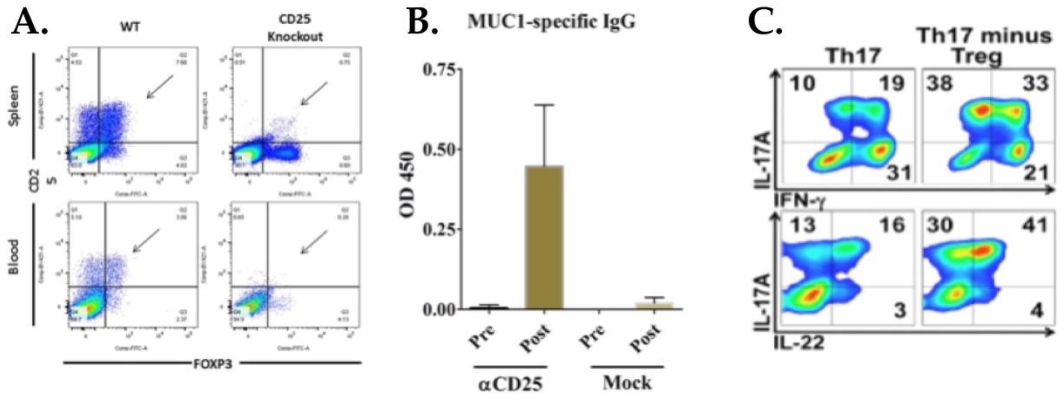

Figure 3: Treg depletion enhances MUC1-specific immunity. A) anti-CD25 Ab depletes $CD25^+$ $FoxP3^+$ and FoxP3- CD4 cells from spleen (top) and blood (bottom) of C57Bl/6 mice. B) MUC1-specific IgG titers are significantly increased in Treg depleted mice following Ad.MUC1. C) Treg depletion increased IFNg and IL-17 production from MUC1-specific CD4 T cells following vaccination with Ad.MUC1/Ad.ICOS-L.

c. *Evaluate the ability of ICOS-L augmented Ad-based vaccination against MUC1 with or without Treg depletion to prevent pulmonary tumor colonization*. We propose that Ad-based vaccination against the tumor-associated antigen (TAA) MUC1, augmented with immunostimulatory ICOS-L, would induce antigen-specific T cell responses capable of inhibiting tumor cell growth upon pulmonary challenge through intravenous administration of MUC1-expressing Lewis lung carcinoma cells (LLC). Further, we propose that depletion of immunosuppressive Treg populations prior to immunization would circumvent peripheral tolerance and increase the magnitude of antitumor immunity induced through vaccination. To test this hypothesis, we engineered LLC cells to express both human MUC1 as well as firefly luciferase allowing for sensitive longitudinal measurement

of tumor burden (Fig.4a). Mice received three immunizations with Ad.MUC1 or Ad.MUC1 and Ad.ICOS-L, termed Ad.M/I, at monthly intervals, with or without Treg depletion. Thereafter, mice were challenged intravenously with LLC-MUC1-Luc and bioluminescence imaging was longitudinally performed from immediately following inoculation (Fig.4b) through 21 days serving as the pre-determined study endpoint. At 21 days post-challenge, significant differences in tumor burden were observed among individual mice between treatment groups as illustrated by luciferase intensity detected between Ad.MUC1 vaccinated and Ad.Psy5 empty vector control treated animals (Fig.4c). Notably, luciferase intensity strongly correlated with pulmonary LLC colonization and disease burden, as illustrated by the significant range of disease detected by gross pathology (Fig.4d). Notably, Ad.M/I vaccination with or without Treg depletion via anti-CD25 administration failed to reduce average pulmonary tumor colonization measured via BLI luminoscore out to 21 days (Fig.5a,b). To date, experiments testing all proposed treatment groups have been conducted and histologic assessment is ongoing. Herein, we propose that failure of vaccination to prevent pulmonary tumor colonization may be due to the high, non-physiologic dose of LLC-MUC1-Luc cells delivered ($5x10^5$ i.v.) and rapid progression of this model, precluding the ability of adaptive immunity to eliminate single cancer cells or clusters as they develop.

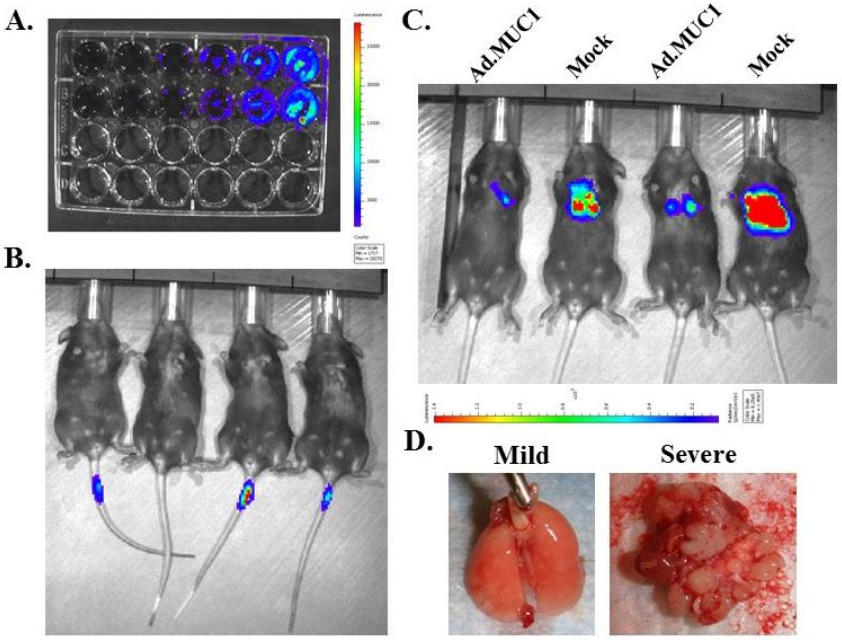

Figure 4: Evaluation of vaccination to prevent pulmonary LLC colonization. A) LLC cells expressing MUC1 (not shown) and firefly luciferase determined via BLI. B) Luciferin measurement by IVIS BLI immediately following I.V. LLC administration. Note, 2nd animal in does not have signal in tail indicating failed injection. C) 21 days post-LLC challenge vaccine treatment group show significant variation in luciferin expression, comparing Ad.MUC1 with mock control. D) Gross pathology of mild and severe LLC take illustrating pulmonary tumor colonization.

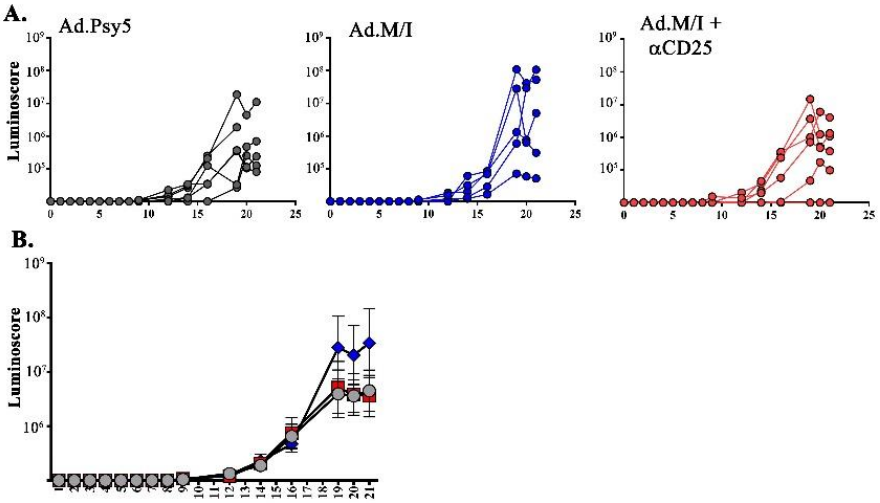

Figure 5: Prophylactic vaccination fails to inhibit pulmonary tumor colonization. MUC1.Tg mice received three vacciations with either mock (Ad.Psy5) or Ad vectors expressing MUC1 and ICOS.L (Ad.M/I) with or without Treg depetion (anti-CD25) then were i.v. inoculated with $5x10^5$ LLC-MUC1-Luc cells. A) Calculated semi-quantitative luminoscore values of luciferase expression in mock, Ad.M/I, or Ad.M/I with Treg depletion animals for 21 days post-challege. B) Grouped averages of above data as mean ± SEM.

d. *Evaluate the immune composition within pulmonary tumor following vaccination.* To further assess the effect of vaccination on tumor formation and progression, pulmonary tumors from mock, vaccinated, or vaccinated/Treg depleted mice were harvested, processed into single-cell suspensions, and polychromatic 24-color spectral cytometry was performed to provide comprehensive immunophenotyping of the tumor-microenvironment. Phenotyping identifies all myeloid and lymphocyte populations, providing information on putative immunosuppressive or tumor-reactive phenotypes. Notably, following mock vaccination, tumors were enriched in myeloid cell population with reduced MHC class II expression, likely representing a immunosuppressive cell subset (Fig. 6E,F). By contrast, vaccinated mice which successfully inhibited tumor progression had tumors enriched in CD8+ T cell subsets. Although further analysis is required and ongoing, these data suggest that establishing anti-tumor immunity prior to challenge is sufficient to alter both myeloid and lymphoid populations of tumor-infiltrating leukocytes, potentially altering the immune balance and resulting in tumor regression. Additionally, such immune signatures may be used for future profiling and predictive analysis of lung cancer patients.

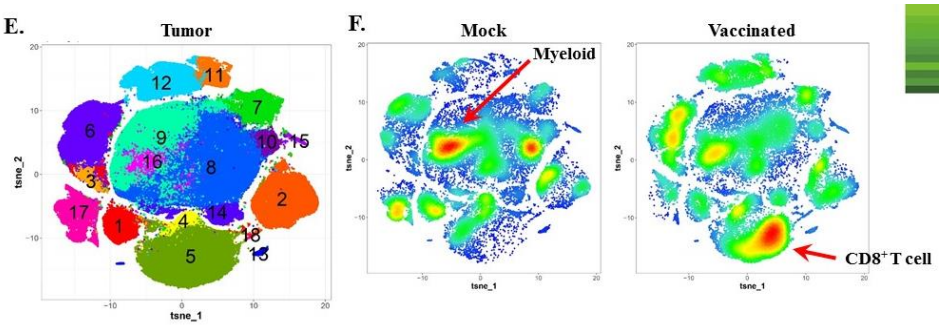

Figure 6: Pulmonary tumors were interrogated with 24-color spectral cytometry. tSNE plots including both mock and vaccinated groups were generated. A) Rphenograph illustrating 18 distinct myeloid and lymphoid cell subsets present in tumors. B) Density maps from tSNE plots highlighting cell subsets which were enriched in either mock treated or vaccinated groups prior to LLC-MUC1-Luc challenge.

e. Characterization of vaccine-induced antitumor T cell response via single-cell RNA sequencing: The final goal of our study is to characterize the transcriptional profile of MUC1-specific T cells isolated from LLC-MUC1-Luc pulmonary tumors using single-cell RNA sequencing (scRNAseq). This aim has not been completed due to my transfer from the Medical University of South Carolina to the University of Pittsburgh. We have requested to complete these studies through subaward with MUSC. Towards our experimental goals, we have established methodology to isolate genomic template and generate cDNA from single immune cells harvested from murine tumors via FACS sorting. To illustrate this technique, other studies in the laboratory have utilized the Fluidigm C1-Biomark system to perform targeted single-cell qPCR on tumor-associated macrophages FACS-sorted from mammary tumors. qPCR analysis of 96 transcripts shows variability in expression levels from individual cells (Fig.7a). FlowSOM (Fig.7b) and tSNE (Fig.7c) analysis depicting heterogeneity in populations highlighting five main subtypes via relationship hierarchy and unbiased high-dimensional clustering, respectively. Collectively, we are well positioned to conduct similar analysis on MUC1-specific T cells employing RNAseq in place of qPCR at the University of Pittsburgh and we are awaiting approval to continue this work as stated below.

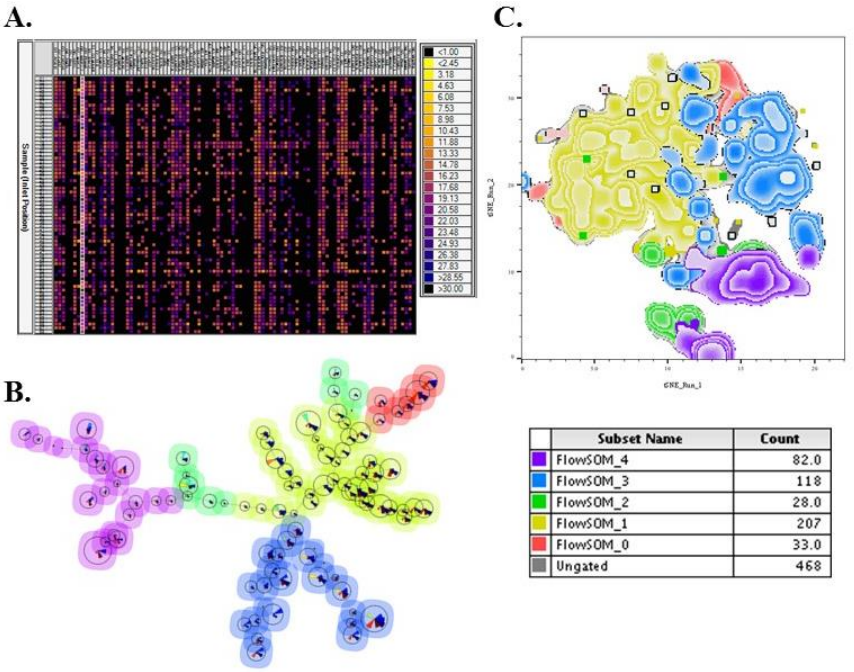

Figure 7: Single-cell genomic analysis. A) Heatmap illustrating raw qPCR transcript expression of 96 genes in single TAMs FACS-sorted from mouse mammary tumors. High-dimensional single-cell data was analyzed depicting lineage hierarchies and associations of 5 major subtypes via B) FlowSOM and C) tSNE analysis.

We have continued these analysis using T cells FACS-isolated from tumors harvested from vaccinated or mock treated animals. Using the 10x Chromium platform (Fig.8), we have demonstrated single-cell transcriptional profiling of pulmonary tumors, showing distinct epithelial (blue) and immune (orange) populations. We are currently working with the University of Pittsburgh's bioinformatics core to distinguish differences in immune composition between treatment groups and expect data analysis to be completed in early 2020 and submitted for publication.

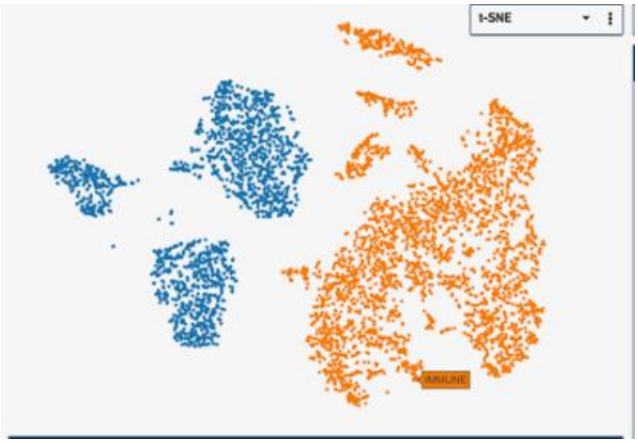

Figure 8: Single-cell genomic analysis using the 10x Chromium platform interrogating pulmonary tumors. Epithelial tumor cells are shown in blue and immune infiltrate is depicted in orange.

3) What opportunities for training and professional development has the project provided?
- Nothing to report.

4) How were the results disseminated to communities of interest?
a. Publications:
Carrell RK, et. al. "ICOSL-augmented adenoviral-based vaccination induces a bipolar Th17/Th1 T cell response against unglycosylated MUC1 antigen." Vaccine. 2018 Oct 8;36(42):6262-6269. doi: 10.1016/j.vaccine .2018.09.010. Epub 2018 Sep 12.

- Second manuscript is being prepared currently.

b. Presentations:
Smits BMG, Rissman AI, Homer-Bouthiette C, Zhao Y, van Peel B, Schulte J, Wilson R, and Soloff AC: Human cancer-associated 8q24 gene desert reduces breast cancer susceptibility through suppression of tissue-resident macrophages throughout mammary gland development. Invited Speaker; Expanding the Definition of the Tumor Microenvironment section, American Association of Cancer Research Annual Meeting April 14th-18th, 2018, Chicago, IL, USA.

5) How were the results disseminated to communities of interest?
- Study findings will be highlighted in a brief report in the upcoming CDMRP Department of Defense Lung Cancer Research Program annual report.

6) What do you plan to do during the next reporting period to accomplish the goals?
- Nothing to report.

IMPACT
1) What was the impact on the development of the principal discipline of the project?
To date, we have published what we believe to be the first report demonstrating that vectored delivery of ICOS-L is capable of inducing Th17-mediated tumor-reactive immunity through vaccination. IL-17-producing T cells may prove to be superior antitumor effector cells compared to conventional Th1 cells. In which case, the induction of such specialized immunity will be a valuable component of combinational therapies which aim to both bolster de novo antitumor immunity as well as target tumor-mediated immunosuppression. In addition, ICOS-L augmented vaccination may be used to prevent or treat infectious diseases as well, potentially providing more potent cellular immune response to pathogens then conventional immunization alone.

2) What was the impact on other disciplines?
- Nothing to report.

3) What was the impact on technology transfer?
- Nothing to report.

4) What was the impact on society beyond science and technology?
- Nothing to report.

CHANGES/PROBLEMS
In early May 2018, I accepted a position as an Assistant Professor at the University of Pittsburgh School of Medicine. Upon accepting this offer, I began to prepare the laboratory for the transition. This required that all experimental work in the laboratory and research involving animals be completed in early to ship reagents and equipment. As such, long-term experiments requiring serial vaccination including repeat LLC-MUC1-Luc challenge experiments and single T cell RNAseq assays could not be completed within the timeframe I was at MUSC, given that these assays require a minimum of three months. We continued this work at the University of Pittsburgh following provision of a subaward and have completed almost all of the proposed experiments. Data analysis is ongoing due to the time required to develop collaborations in bioinformatics.

1) Changes in approach and reason for change:
Due to the transfer from MUSC to the University of Pittsburgh, I no longer have access to the Fluidigm C1 system proposed for single-cell RNAseq. I do have access to the 10x Chromium system and institutional genomics core facility which are fully capable of completion the proposed sequencing experiments. Notably, the 10x system offers greater throughput at a similar cost to that of the Fluidigm system, yielding higher numbers of individual cells analyzed and more robust data.

2) Actual or anticipated problems or delays and actions or plans to resolve them:
The transition from MUSC to the University of Pittsburgh has been a major problem to complete the studies per timeline proposed.

3) Changes that had a significant impact on expenditures:
- Nothing to report.

4) Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
- Nothing to report.

PRODUCTS
1) Publications, conference papers, and presentations:

1. Carrell R, Stanton R, Ethier SP, LaRue AC, and **Soloff AC**: ICOSL-augmented Adenoviral-based Vaccination Induces a Bipolar Th17/Th1 T Cell Response Against Unglycosylated MUC1 Antigen. Vaccine, Oct 8; 36(42):6262-6269, 2018, 2018.

2. Murthy P, Russell KL, Butler SC, Ekeke CN, Wang Y, Luketich JD, **Soloff AC**, Dhupar R, and Lotze MT: Making cold malignant pleural effusions hot: driving novel immunotherapies. In Press, OncoImmunology.

3. Smits BMG, Rissman AI, Homer-Bouthiette C, Zhao Y, van Peel B, Schulte J, Wilson R, and **Soloff AC**: Human cancer-associated 8q24 gene desert reduces breast cancer susceptibility through suppression of tissue-resident macrophages throughout mammary gland development. Invited Speaker; Expanding the Definition of the Tumor Microenvironment section, American Association of Cancer Research Annual Meeting April 14th-18th, 2018, Chicago, IL, USA.

PARTICIPANTS

1) What individuals have worked on the project?

Name: Adam C. Soloff, Ph.D.
Project Role: PI
Researcher Identification: 0000-0002-1467-8168
Contribution to Project: Dr. Soloff performed experiments, analyzed data, prepared findings, and oversaw regulatory and technical aspects of the study.
Funding Support: Susan G. Komen Foundation (CCR15329745)
Name: Rebecca Stanton, B.S.
Project Role: Technician
Researcher Identification: NA
Contribution to Project: Ms. Stanton performed mouse experiments, colony management, and experiments to determine lung cancer burden and immune response to vaccination.
Funding Support: NA

2) Has there been a change in the active other support of the PD/PI since the last reporting period?

- Nothing to report.

3) **What other organizations were involved as partners?**

- Nothing to report.

APPENDICES

Please see attached:

Vaccine 36 (2018) 6262–6269

Contents lists available at ScienceDirect

Vaccine

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ICOSL-augmented adenoviral-based vaccination induces a bipolar Th17/Th1 T cell response against unglycosylated MUC1 antigen

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ABSTRACT

Cellular immunity established via immunotherapy holds the potential to eliminate solid tumors. Yet, cancer vaccines have failed to induce tumor-reactive T cells of sufficient quality to control disease. The inducible T cell costimulator (ICOS) pathway has been implicated in both the selective induction of immunity over tolerance as well as licensing of IL-17-polarized cellular immunity. Herein, we evaluated the ability of ICOS ligand (ICOSL) to augment the immunogenicity of adenoviral-based vaccination targeting the unglycosylated MUC1 peptide antigen. Vaccination disrupted immunotolerance in a transgenic mouse model recognizing human MUC1 as a self-antigen, inducing robust MUC1-specific immunity. Augmenting vaccination with ICOSL induced a bipolar Th17/Th1 effector profile, marked by increased MUC1-specific IL-17A production and RORγt expression in $CD4^+$ but not $CD8^+$ T cells which predominantly expressed IFNγ/IL-2 and T-bet. The polarization and maintenance of Th17 cells established following ICOSL augmented vaccination was highly durable, with elevated IL-17A and RORγt levels detected in $CD4^+$ T cells up to 10 months after initial immunization. Furthermore, provision of ICOSL significantly enhanced MUC1-specific IgG antibody in response to immunization. ICOSL signaling dramatically influenced $CD4^+$ T cell phenotype, altering gene expression of transcription factors and regulators of effector function following immunization. Interestingly, ICOSL augmentation failed to alter the transcriptional profile of $CD8^+$ T cells following immunization, affecting the magnitude, but not distribution, of gene expression. Collectively, ICOSL supports the induction of durable, antigen-specific Th17/Th1-mediated immunity *in vivo*, establishing a vaccination platform to enhance $CD4^+$ T cell-mediated antitumor immunity and providing a crucial component of an effective cancer vaccine.

1. Introduction

The clinical success of T cell checkpoint inhibitors and adoptive cell transfer (ACT) of engineered T cells demonstrates that functional tumor-reactive cellular immunity established via immunotherapy may eliminate solid tumors. Nevertheless, cancer vaccines targeting tumor-associated antigens (TAA) have yet to provide control of disease. In both clinical application of cancer immunotherapy and experimental vaccine development, failure to sustain durable, highly functional effector T cells limits overall response rates and mediates treatment failure [1]. Identifying mechanism to promote long-lived antitumor cellular immunity will be essential to improve the efficacy of cancer immunotherapy regimens.

T cell costimulation provides requisite signaling during peptide/MHC-TCR-mediated activation to support the development of phenotypically and functionally distinct T cell subsets. Identification of multiple stimulatory receptors with non-redundant signaling pathways including the inducible T cell costimulator (ICOS/CD278), CD40, OX40, CD137, and GITR has expanded our understanding of how T cell plasticity is regulated. Upon activation, T cells upregulate ICOS, which following binding to its ligand (ICOSL/B7-H2/CD275) expressed by antigen presenting cells, augments cytokine production, proliferation, and survival [2]. ICOS signaling results in biased augmentation of effector T cell (Teff) responses over those mediated by regulatory T cells (Tregs) during the early inflammatory response [3]. Furthermore, signaling via the ICOS pathway drives the expansion of IL-17-expressing $CD4^+$ T cells (Th17) via

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upregulation of c-Maf, IL-21, and IL-23R [4]. Notably, Th17 cells possess heightened cytotoxicity, durability, and mediate superior tumor clearance following ACT therapy compared to conventional Th1 counterparts [5–7]. The ability to promote a superior Teff response while mitigating immunosuppressive Treg function indicate ICOS may be uniquely suited for the induction of tumor-reactive immunity during cancer immunotherapy.

Adenoviral (Ad)-based vectors have progressed clinically as potent transgene delivery systems, armed with cytokines to modulate tumor microenvironments or TAA to target cancer cells [8]. Owing to the inherent pathogen associated molecular patterns (PAMP) of adenoviruses, infection results in recognition of unmethylated CpG viral sequences, activation of the ERK1/2, MAPK, and Jak/Stat pathways, nuclear translocation of NF-κB, and subsequent pro-inflammatory interferon response. Ad vectors do not integrate into the host genome, circumventing safety concerns of lentiviral vectors activating proto-oncogenes through integration into transcriptionally-active sites [9]. Ad vectors transduce large numbers of terminally differentiated and non-dividing host cell types including antigen presenting myeloid and plasmacytoid dendritic cells (DC) [10]. In addition, Ad-transduced DCs are capable of continual synthesis of antigen and were found to be superior immunogens compared to adjuvanted DNA or peptide-pulsed DCs where antigen presentation is limited by dissociation of the MHC class I peptide complex [11]. Ad-based vaccine regimens have successfully increased the magnitude of antitumor immunity, yet the ability to augment vector immunogenicity to elicit IL-17-mediated immunity has yet to be examined.

Herein, we evaluate the ability of ICOSL costimulation to selectively promote immunity over tolerance following vaccination against unglycosylated MUC1 in a murine model which recognizes MUC1 as a self-antigen. We propose that ICOSL may additionally induce IL-17-polarized cellular immunity *in vivo*, establishing highly inflammatory antitumor Teff. Our studies compared the immunogenicity of Ad-based vaccination against MUC1, with or without ICOSL augmentation, on the phenotype, function, and durability of the cellular immune response, antibody response, and transcriptional profile of antitumor immunity. Our findings demonstrate that ICOSL selectively promotes bipolar Th17/Th1-mediated cellular immunity, but has limited direct effect on $CD8^+$ T cell phenotype or function.

2. Methods

2.1. Animals

MUC1.Tg mice (C57Bl/6 background) expressing the human MUC1 gene under the control of the endogenous murine MUC1 promoter were kindly provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) [12]. Wild type C57BL/6 animals were purchased from the Jackson Laboratory. Mice were maintained in a specific pathogen-free environment at the Animal Research Facility of the Ralph H. Johnson Veterans Affairs Medical Center in accordance with guidelines of the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee.

2.2. Ad-based vectors and vaccination

cDNA was generated encoding a 100mer peptide consisting of 5 tandem repeats of the 20 amino acid sequence HGVTSAPDTRPAPGSTAPPA from the variable number of tandem repeats (VNTR) region of human MUC1 or the murine ICOSL (GenBank accession NM_015790.3) (GenScript) and subcloned into pAd-lox plasmid for vector assembly. Individual E1/E3-deleted, replication-deficient Ad serotype 5-based vectors expressing MUC1 100mer, ICOSL, or lacking transgene (Ad.Psy5) under direction of the cytomegalovirus promoter were constructed through Cre-lox recombination by Dr. Andrea Gambotto at the University of Pittsburgh's Viral Vector Core Facility [13]. Mice (N = 6–12/group) received subcutaneous immunizations of 5×10^9 Ad particles in sterile saline at indicated timepoints.

2.3. Dendritic cell (DC) generation and T cell stimulation

Bone marrow cells from the femurs and tibiae of C57BL/6 mice were cultured in complete DMEM supplemented with 20 ng/ml GM-CSF and 10 ng/ml IL-4. Cells were cultured for 5–7 days and fed with fresh media every other day, yielding immature DCs. At day 5–7, DCs were harvested and loaded overnight with 20 μg/ml MUC1 100mer peptide representing 5 replicates of the VNTR as above (a kind gift from Dr. Olivera Finn, University of Pittsburgh). Prior to T cell co-culture, DCs were exposed to 1 μg/ml LPS (4–6 h) to induce maturation and cultured at 1:5 ratio with spleen cells in the presence of 20U/ml IL-2 for 7 days. Additional MUC1 peptide was added at day 3 and 6 [14,15]. Cultures were treated with Brefeldin A and Monensin overnight prior to analysis.

2.4. Flow cytometry

Phenotypic characterization and intracellular cytokine detection were performed as previously described [13,16]. Reagents were purchased from BioLegend unless otherwise noted. Surface Fc receptors were blocked by addition of FcBlock (anti-CD16/CD32) prior to extracellular staining. $1–5x10^6$ cells were labeled via amine-reactive viability dye UV-LiveDead (Molecular Probes) then stained using combinations of mAbs specific for: MUC1 VNTR(SM3;eBioscience), ICOSL(HK5.3), CD3e(145-2C11), CD4 (GK1.5), CD8(53–6.7). For detection of intracellular proteins, cells were subsequently fixed then labeled with mAbs to IFNγ (XMG1.2), IL-2(JES6-5H4), IL-4(11B11), IL-10(JES5-16E3), and IL-17A(TC11-18H10.1) or CD107a(1D4B) and granzymeB(GB11) in the presence of permeabilizing staining buffer. Transcript factors RORγt(B2D), T-bet(4B10), Gata3(TWAJ), and FoxP3(FJK-16 s) were measured using True-Nuclear transcription factor buffer set per manufacturer's instructions. Data was collected on a LSRFortessa X-20 cytometer (Becton Dickinson) or isolated using a MoFlo AstriosEQ cell sorter (Beckman Coulter). Data was analyzed using FlowJo V10 software (TreeStar).

2.5. MUC1 IgG ELISA

Mice were bled via cardiac puncture and plasma separated by centrifugation. MUC1-specific ELISA was performed as previously described using 10 μg/ml MUC1 peptide as capture antigen [15]. Data represent the average of triplicate wells.

2.6. Cytometric bead array

T cells were FACS-sorted from cryopreserved single-cell suspensions from spleens. 2×10^5 $CD4^+$ or $CD8^+$ T cells were cultured in 100 μl complete media with PMA (40.5 μM) and ionomycin (669.3 μM) (BioLegend) for 24 h. The levels of IFNγ, TNFα, IL-2, IL-4, IL-6, IL-10, and IL-17A in cell culture supernatants were determined via cytometric bead array Th1/Th2/Th17 cytokine kit (BD Biosciences). Data was collected using a BD FACS Canto and cytokines quantified using FCAP Array Software version 3.0 (BD Biosciences).

2.7. Quantification of gene expression

Transcript expression was determined using the Mouse Immunology Panel on the nCounter GX system (NanoString Technologies). 2×10^5 FACS-isolated $CD4^+$ or $CD8^+$ T cells from homogenates of fresh spleens from immunized MUC1.Tg mice (N = 3/group) were stimulated for 18 h with PMA/ionomycin, harvested, and RNA isolated using an RNeasy Mini kit (Qiagen). 150 ng of RNA per sample was hybridized with fluorophore barcoded probes for 16 h, loaded into the nCounter prep station, and quantified using the nCounter Digital Analyzer. Transcript expression of 547 immunology-related genes were measured. Data were normalized in relation to both positive spiked-in controls and according to the geometric means of 14 internal reference controls (*Alas1*, *Eef1g*, *G6pdx*, *Gapdh*, *Gusb*, *Hprt*, *Oaz1*, *Polr1b*, *Polr2a*, *Ppia*, *Rpl19*, *Sdha*, *Tbp*, and *Tubb5*). Samples with either positive control normalization factors outside the recommended range of 0.3–3 or background greater than 3 standard deviations from the mean were excluded from analysis. Data was analyzed using nSolver Analysis Software version 3.0 and heat maps generated with Heatmapper web-enabled analysis [17].

2.8. Statistical analysis

All results were expressed as means ± standard error of the mean (SEM). Data were analyzed with a two-tailed Student's *t*-test performed using GraphPad Prism5 (Graphpad Software). For all hypothesis tests, a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ad-based vaccination overcomes immunotolerance inducing MUC1-specific CTL response in a MUC1.Tg host

Without alteration, Ad vectors induce robust Th1-mediated immunity due to the presence of PAMPs [13]. We sought to harness the potency of Ad vectors while providing costimulation via ICOSL to enhance targeted immunity against the MUC1 TAA. To this end, we generated individual vectors containing either the human *MUC1 VNTR* domain or the mouse *ICOSL* transgene, termed Ad.MUC1 and Ad.ICOS.L, respectively. To confirm transgene production, we analyzed MUC1 and ICOSL protein expression in bone marrow-derived DCs following 24 h transduction. At a multiplicity of infection (MOI) of 100, ~53% of DCs transduced with Ad.MUC1 and ~95% of DCs transduced with Ad.ICOS.L expressed MUC1 or ICOSL on the cell surface, respectively, indicating efficient transduction and protein production from antigen presenting cells (Fig. 1A).

To model vaccination against a self-derived TAA, we utilized MUC1.Tg mice which display analogous levels and similar tissue-specific distribution of MUC1 compared to humans, possess an intact immune system, and are tolerant to human MUC1 as a "self" antigen [12,18–20]. Wild type or MUC1.Tg mice received prime-boost immunizations two weeks apart with either Ad.MUC1, Ad.MUC1 with Ad.ICOS.L (herein called Ad.M/I), or Ad.Psy5 (empty vector control). Five days after the final immunization, IFNγ production from splenic $CD8^+$ T cells was determined following stimulation with MUC1 peptide-pulsed DCs. Immunization against MUC1, with or without ICOSL, induced MUC1-specific IFNγ production in 0.8–1% of $CD8^+$ T cells from wild type mice (Fig. 1B). Notably, administration of either Ad.MUC1 or Ad.M/I in tolerant MUC1.Tg mice induced MUC1-specific IFNγ responses of similar magnitude to those observed in wild type mice (Fig. 1B). Immunization with empty vector failed to induce an antigen-specific response (Fig. 1B). These data indicate that Ad-based vaccination is sufficiently immunogenic to generate MUC1-specific cellular immunity in transgenic mice tolerant to MUC1.

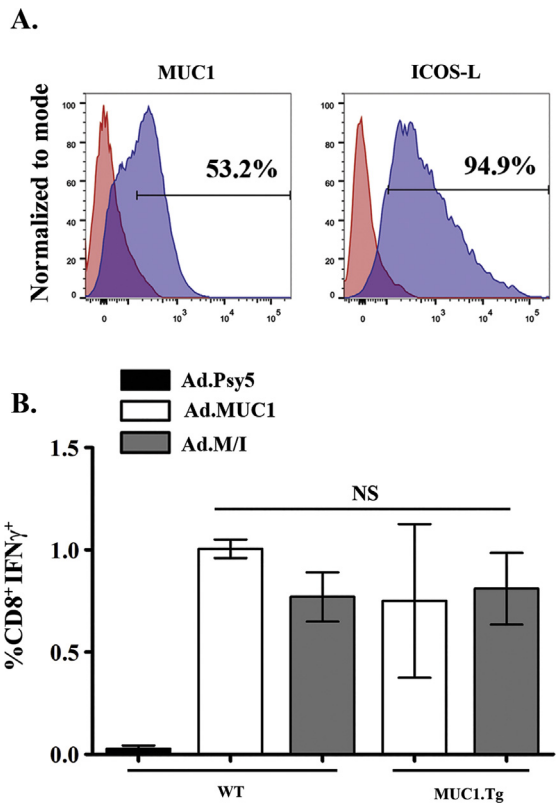

Fig. 1. Vaccination induces $CD8^+$ T cell responses in wild type and MUC1.Tg mice. (A) Bone marrow-derived DCs were transduced with Ad.MUC1 or Ad.ICOS.L at an MOI of 100. 24 h later extracellular expression of the MUC1 VNTR region and ICOSL were detected by flow cytometry. (B) Wild type or MUC1.Tg mice received prime-boost immunizations 2wks apart with either empty vector (Ad.Psy5), Ad.MUC1, or Ad.M/I. 5d after immunization, intracellular IFNγ production from $CD8^+$ T cells was measured via flow cytometry from splenocytes cocultured with MUC1 peptide-pulsed DCs. NS = non-significant.

3.2. ICOSL augmented vaccination promotes bipolar Th17/Th1 cellular immunity against MUC1

We next determined the impact of ICOSL on the breadth of T cell cytokine production in response to vaccination. MUC1.Tg mice received three immunizations at bi-weekly intervals with Ad.MUC1, Ad.M/I, or Ad.Psy5. Five days after the final immunization, MUC1-specific cytokine production from $CD4^+$ and $CD8^+$ T cells was determined by flow cytometry following stimulation with MUC1 peptide-pulsed DCs. Cytokines were selected as representative effector molecules of specific T cell subsets: IFNγ/IL-2 (Th1), IL-4 (Th2), IL-10 (Treg), and IL-17A (Th17). Administration of Ad.MUC1 induced modest production of MUC1-specific IFNγ and IL-2 from $CD4^+$ T cells. Notably, co-administration of Ad.ICOS.L with Ad.MUC1 significantly enhanced MUC1-specific IL-17A production and slightly increased IFNγ levels from $CD4^+$ T cells (Fig. 2A). As expected, Ad.MUC1-induced MUC1-specific cytokine responses in $CD8^+$ T cells consisted predominantly of IFNγ (Fig. 2B). Interestingly, Ad.M/I immunization enhanced the IL-2 response and to a lesser extent IFNγ production from $CD8^+$ T cells compared to levels induced by Ad.MUC1 alone, but failed to promote IL-17A (Fig. 2B).

We next examined levels of the transcription factors RORγt (Th17), T-bet (Th1), Gata3 (Th2), and FoxP3 with CD25 (Treg) as the master regulators of T cell subset development. Although Ad.

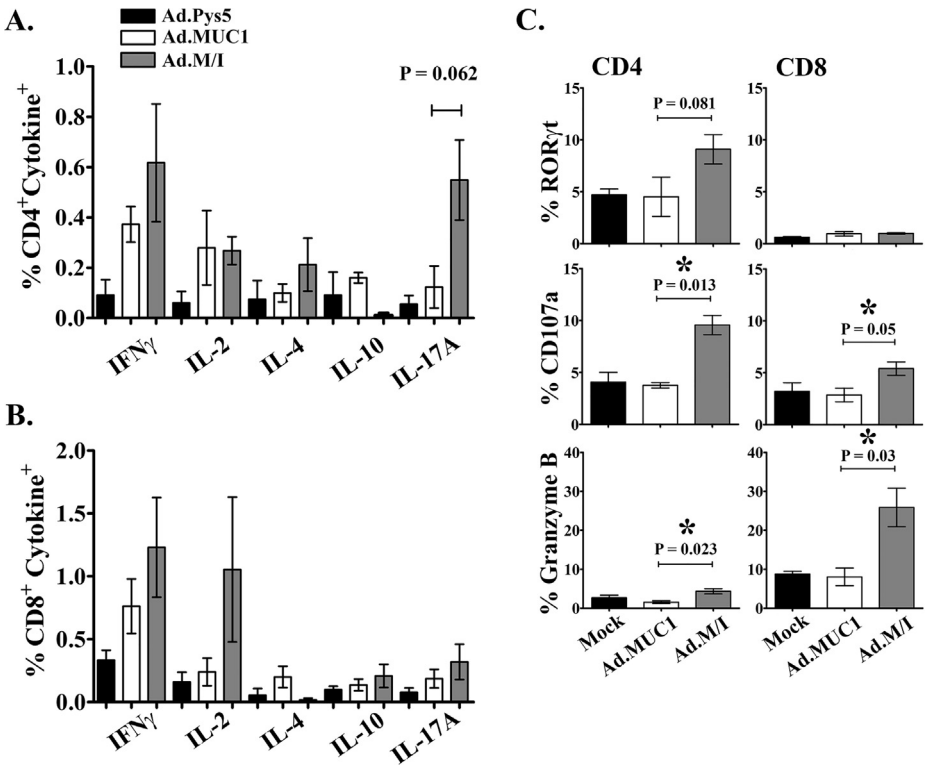

Fig. 2. ICOSL promotes a bipolar MUC1-specific Th17/Th1 response to vaccination. MUC1.Tg mice received 3 immunizations with Ad.Psy5, Ad.MUC1, or Ad.M/I at 2wk intervals. 5d after the final immunization, MUC1-specific intracellular cytokine production was determined by flow cytometry in (A) $CD4^+$ or (B) $CD8^+$ T cells from splenocytes cultured with MUC1 peptide-pulsed DCs for 7d. (C) Intracellular expression of the Th17 transcription factor RORγt, and cytotoxic effector proteins CD107a and granzyme B were determined 5d after final immunization in splenic $CD4^+$ and $CD8^+$ T cells without DC coculture via flow cytometry. Mean ± SEM; $^*p < .05$.

MUC1 did not increase RORγt levels above those identified after administration of empty vector, Ad.M/I resulted in dramatic upregulation of nuclear RORγt in $CD4^+$, but not $CD8^+$ T cells (Fig. 2C). No differences in expression were observed for T-bet, Gata3, or FoxP3 between immunization groups in either $CD4^+$ or $CD8^+$ T cell populations (data not shown). As surrogate markers for cytotoxic potential, we determined expression of CD107a and granzyme B, involved in vesicle degranulation and apoptotic induction, respectively. Although vaccination enhanced T cell-mediated IFNγ production (Fig. 2A and B), Ad.MUC1 immunization failed to increase CD107a or granzyme B in either $CD4^+$ or $CD8^+$ T cells (Fig. 2C). By contrast, ICOSL augmentation induced robust expression of CD107a and granzyme B in both $CD4^+$ and $CD8^+$ T cell subsets, which was significantly greater than levels detected in animals administered Ad.MUC1 or Ad.Psy5 (Fig. 2C). Together, these findings indicate that ICOSL promotes distinct Teff responses within $CD4^+$ and $CD8^+$ T cell subsets in MUC1.Tg mice.

3.3. Ad.ICOS.L co-administration enhances MUC1-specific antibody response to vaccination

Next, we evaluated the effect of ICOSL augmentation on vaccine-mediated humoral immunity. Plasma MUC1-specific IgG levels were measured from wild type or MUC1.Tg mice receiving three immunizations as described above. Consistent with our initial observations, immunization against MUC1 in wild type animals, which recognize human MUC1 as foreign, resulted in robust MUC1-specific IgG responses by ELISA. Ad.MUC1 immunization of MUC1.Tg mice overcame immunotolerance, resulting in increased, albeit minimal, MUC1-specific antibody titers compared to mock immunized controls. Notably, co-administration of Ad.ICOS.L significantly enhanced MUC1-specific antibody titers above those attained with Ad.MUC1 immunization alone, animals reached roughly 50% of those induced in wild type mice (Fig. 3).

3.4. ICOSL signaling promotes long-lived Th17 compartment

We previously observed that ICOSL enhanced the vaccine-induced response from Th17 cells, a subset shown to have unique long-lived potential [21]. To determine if T cell phenotypes initially induced through ICOSL augmentation were sustained, MUC1.Tg mice were immunized at day 0, 14, and 270, being allowed to rest for roughly 9 months. At day 300, cytokine production and transcription factor expression were determined in $CD4^+$ and $CD8^+$ T cells. LiveDead^{neg}CD3$^+$CD4$^+$ and LiveDead^{neg}CD3$^+$CD8$^+$ T cells were FACS-sorted, polyclonally stimulated with PMA/ionomycin for

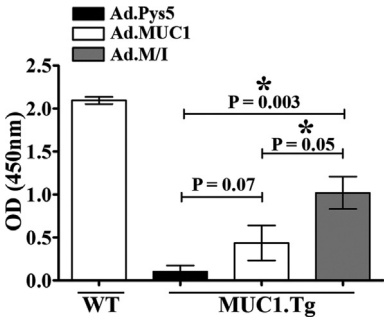

Fig. 3. Co-administration of Ad.ICOS.L enhances IgG antibody response against MUC1 in MUC1.Tg mice. Wild type or MUC1.Tg mice received 3 immunizations at 2wk intervals. 5d after the final immunization, plasma was isolated from cardiac blood and levels of MUC1-specific IgG were determined by ELISA using MUC1 100mer peptide as capture antigen. Mean ± SEM; $^*p < .05$.

24 h, and cytometric bead array assays were performed on T cell culture supernatant. Following stimulation, $CD4^+$ T cells isolated from Ad.M/I immunized MUC1.Tg mice produced significantly greater amounts of IL-17A then Ad.MUC1 or mock immunized controls (Fig. 4A). In addition, co-administration of Ad.ICOS.L substantially reduced the production of IL-10 by $CD4^+$ T cells. By contrast, $CD8^+$ T cells failed to generate IL-17A or IL-10, with cytokine production among all vaccine groups consisting of IFNγ, TNFα, and IL-2 (Fig. 4A). No significant amount of IL-4 or IL-6 was detected among cell types or vaccine groups (data not shown). Consistent with cytokine production profiles, $CD4^+$ T cells from Ad.M/I immunized MUC1.Tg mice contained an increased proportion $ROR\gamma t^+$ cells and cells expressing both $ROR\gamma t^+$ and T-bet^+ (Fig. 4B). Inclusion of ICOSL during vaccination resulted in a significant decrease in $FoxP3^+CD25^+$ expressing $CD4^+$ T cells at the conclusion of the 10-month immunization regimen (Fig. 4B). By contrast, $CD8^+$ T cell subsets predominantly expressed T-bet^+, with minimal levels of RORγt, Gata3, or FoxP3 detected (Fig. 4B). Although these experiments cannot distinguish between Teff responses induced following initial or booster immunizations, the findings suggest that provision of ICOSL during Ad-based immunization sustains a population of memory Th17 cells while inhibiting the Treg subset.

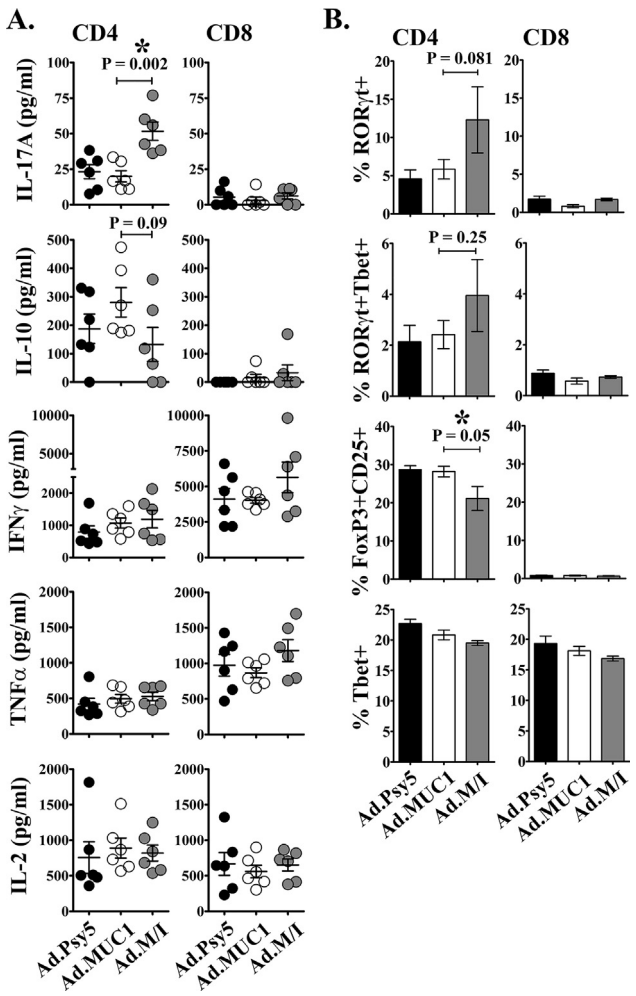

Fig. 4. *Ad.ICOS.L supports long-lived Th17 expansion in vivo.* MUC1.Tg mice were immunized at day 0, 14, and 270 and tissues harvested on day 300. (A) cytokine production was determined by cytometric bead array following 24 h PMA/ionomycin simulation of FACS-sorted splenic $CD4^+$ and $CD8^+$ T cells. (B) Flow cytometric measurement of nuclear transcription factors RORγt, T-bet, and FoxP3 with CD25 for $CD4^+$ and $CD8^+$ T cell subset identification. Mean ± SEM; $^*p < .05$.

3.5. ICOSL alters the transcriptional profile of $CD4^+$ but not $CD8^+$ T cells in response to vaccination

The above findings suggest that ICOSL augmentation influenced the polarization of MUC1-specific $CD4^+$ T cell-mediated immunity, but had limited effect on $CD8^+$ T cell responses beyond Ad.MUC1 vaccine alone. To further characterize the discordant effects of ICOSL, we evaluated transcript expression in T cell subsets isolated from MUC1.Tg mice following three bi-weekly immunizations against MUC1 with or without ICOSL. FACS-purified $CD4^+$ and $CD8^+$ T cells were stimulated with PMA/ionomycin, and mRNA expression of 547 immune-related genes was quantified [22]. ICOSL profoundly altered the activated $CD4^+$ T cell phenotype, with N = 57 genes upregulated and N = 74 downregulated at least 25% in $CD4^+$ T cells from Ad.M/I compared to Ad.MUC1 immunized mice (Fig. 5A). Transcript expression of *RORγt*, *IL-23R*, *IL-17A*, *IL-17F*, and *CCL20* was increased following Ad.ICOS.L co-administration, supporting our previous findings that ICOSL promotes Th17-mediated immunity *in vivo*. Additionally, ICOSL upregulated genes associated with cytotoxicity and inflammation such as *granzyme A*, *perforin*, and *IL-6* in $CD4^+$ T cells. By contrast, Ad.ICOS.L co-administration failed to alter the phenotype of the $CD8^+$ T cell response, but instead increased expression of genes identified following Ad.MUC1 immunization including transcription factors, costimulatory receptors, cytokines, and markers of effector function (Fig. 5B). The differential effect of ICOSL signaling among T cell populations is highlighted when comparing transcriptional profiles from the $CD4^+$ or $CD8^+$ T cell compartments following ICOSL augmented immunization yielding an $R^2 = 0.84746$ (Fig. 5C). Collectively, findings indicate that administration of ICOSL during vaccination dramatically alters $CD4^+$ T cell phenotype, including expression of Th17-associated factors, but does not effect the polarization of the $CD8^+$ T cell compartment.

4. Discussion

Peripheral tolerance to self-derived TAAs limits the induction of tumor-reactive immunity. Mechanisms which impart immunogenicity over tolerance are thus necessary to establish a therapeutic or preventative effector T cell response from a cancer vaccine. Studies have shown that ICOS, unique from all other T cell costimulatory receptors, is preferentially induced in the setting of immune reactivity compared to establishment of tolerance [3]. Furthermore, ICOS signaling in the context of immunologic danger signals (PAMPs/DAMPs) results in selective and massive expansion of $CD4^+$ Teff while leaving the Treg compartment largely undisturbed [2,3,23]. Our findings indicate that provision of ICOSL in the context of Ad-derived PAMPs enhances effector responses from both $CD4^+$ and $CD8^+$ T cells against MUC1 antigen in a tolerogenic model, arming cytotoxic potential and cytokine production. Concomitantly, we observed reductions in both IL-10 production and the percentage of $FoxP3^+CD25^+$ Tregs in Ad.M/I immunized animals, indicating that although ICOS is highly expressed on Tregs, vectored delivery of ICOSL does not contribute to their activation. These findings suggest that activating the ICOS pathway during vaccination, through provision of ICOSL, receptor agonists, or stimulating antibodies, may circumvent immunotolerance to self-derived TAAs, further supporting the development of cancer vaccines.

Herein, we assessed if augmenting an Ad-based vaccination regimen with ICOSL effected IL-17-producing T cells *in vivo*. Although ICOS signaling is not required for Th17 cell differentiation, this pathway is crucial for IL-23R upregulation and subsequent IL-21 expression in naïve $CD4^+$ Th0 cells, facilitating the survival and expansion of resident Th17 cells [4]. Our data indicate that

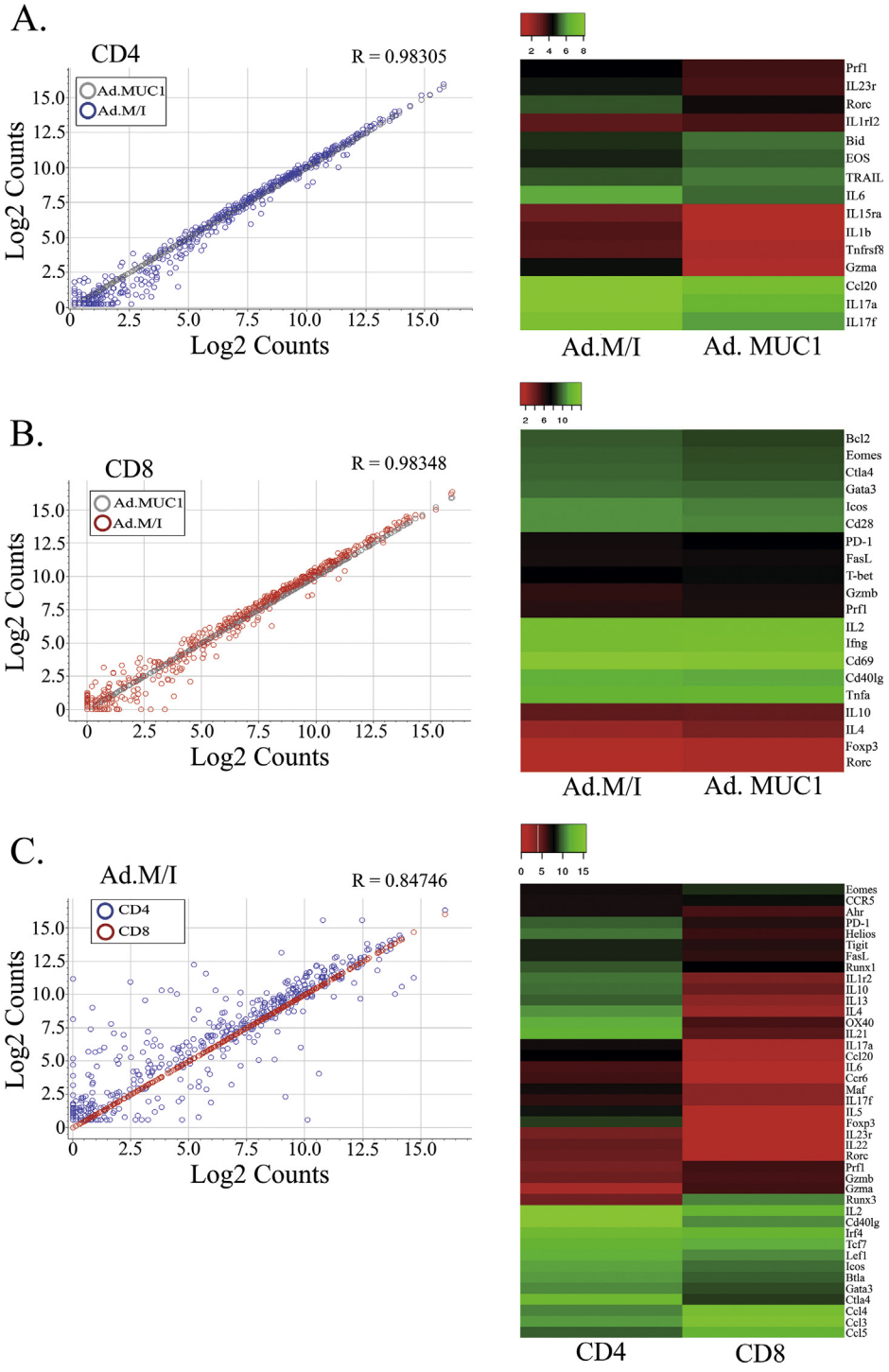

Fig. 5. *Ad.ICOS.L alters the gene expression profile of CD4⁺ but not CD8⁺ T cells in response to immunization.* FACS-sorted splenic CD4⁺ or CD8⁺ T cells from immunized MUC1.Tg mice were stimulated for 18 h with PMA/ionomycin. Expression of 547 endogenous genes were analyzed using the Nanostring nCounter Mouse Immunology Panel. Scatter plots (left) and heat maps (right) illustrating Log2-fold differential gene expression and selected expression of highlighted immune genes, respectively. Gene expression comparing stimulated (A) CD4⁺ T cells or (B) CD8⁺ T cells from MUC1.Tg mice receiving Ad.MUC1 (grey) or Ad.M/I (blue/red, respectively) immunizations. (C) Comparison of transcript levels from CD4⁺ (blue) or CD8⁺ (red) T cells from Ad.M/I immunized groups. N = 3 immunized mice per group.

co-administration of ICOSL was sufficient to modify the Th1-mediated cellular immune response induced by Ad vectors towards a bipolar Th17/Th1 response, as demonstrated by enhanced MUC1-specific IL-17A production, markers of cytotoxicity, and transcriptional signature. Findings further suggest that augmenting vaccination with ICOSL may stabilize the Th17 population, enhancing persistence of antigen-specific cellular immunity demonstrated by detection of enhanced Th17 markers in long-term immunization studies. Although absolute numbers of T cells were not determined, the elevated proportion of RORγt⁺ and RORγt⁺T-bet⁺ CD4⁺ T cells observed in Ad.M/I treated mice at 10 months suggests the persistence of vaccine-induced Th17/Th1

cells possessing increased self-renewal, replicative capacity, and/or half-life [21,24]. A similar effector profile was identified in pre-polarized Th17 cells engineered to express a mesothelin-specific chimeric antigen receptor containing a ICOS intracellular domain [25]. Interestingly, we found that ICOSL augmentation failed to promote IL-17-mediated immunity within the $CD8^+$ T cell compartment. Although IL-17-producing $CD8^+$ T cells (Tc17) are responsive to ICOS signaling following *in vitro* polarization, it is possible that limited numbers of Tc17 precursors in MUC1.Tg mice prohibit effective vaccine-mediated expansion [26]. Collectively, these findings support the use of ICOSL costimulation to promote antigen-specific Th17-based immunity through vaccination for the treatment or prevention of cancers and infectious diseases [27].

Tumor-binding antibodies capable of mediating antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity, or direct tumor cell death provide non-redundant mechanisms of tumor clearance [28]. In accordance with our previous findings, we demonstrate that Ad-based immunization induced detectable, albeit minimal, transgene-specific humoral immunity in an immunotolerant mouse model [29]. ICOSL significantly increased MUC1-specific IgG responses above those detected following Ad. MUC1 immunization alone. It is possible that enhanced antibody production results from the direct effects of ICOSL on $CD4^+$ T cells, as ICOS signaling supports the development of T follicular helper (Tfh) cells and germinal center function, which provide subsequent T cell help for B cells [4,30]. Accordingly, ICOS deficiency induces defects in antibody production inhibiting antibody affinity maturation and class switching [30–32]. As such, augmenting Tfh function via ICOS signaling modalities during immunization may further optimize strategies to induce humoral immunity.

During neoplastic transformation, the MUC1 glycoprotein becomes overexpressed and loses apical-basal polarity resulting in basolateral distribution and presentation to sentinel immunity. Premature termination of O-glycosylation on tumor-associated MUC1 results in the accumulation of short carbohydrate precursors (monosaccharide Tn, disaccharide T, TF, and sialylated sTN and sT antigens) within the immunogenic N-terminal subunit consisting of variable numbers of 20-amino acid tandem repeats [33,34]. Vaccine trials targeting MUC1 have demonstrated safety and immunogenicity, improving patient outcome over chemotherapy alone [35–37]. Yet, conventional vaccination strategies have failed to eliminate established MUC1-expressing tumors. Although our efforts utilized unglycosylated MUC1 antigen to examine disruption of immunotolerance through vaccination, future vaccine trials will benefit from targeting unique MUC1 glycoforms specific to the cancerous state. MUC1 glycosylation patterns are maintained throughout antigen processing by dendritic cells, effect peptide structure and presentation by major histocompatibility molecules, and are recognized by T cells [38–40]. In addition, immunization regimens utilizing chemoenzymatically synthesized MUC1 glycopeptide antigens induce antibodies with dramatically increased specificity against tumor-associated MUC1 compared to MUC1 present on healthy tissue, demonstrating enhanced diagnostic capability to distinguish human mammary and pancreatic cancers [41–45]. As atypical MUC1 expression has been identified in over 80% of all cancers including lung, breast, and pancreatic cancers, development of an effective MUC1-targeting immunotherapy possesses immense clinical value [33,46].

Vaccination holds the potential to generate long-lived antitumor immunity capable of eliminating established tumors and importantly, prevent metastasis through the establishment of persistent immunologic memory. Vaccination is associated with low toxicity and high specificity. We believe the cancer vaccine described herein supports two key features which may improve antitumor responses: the induction of a potent antigen-specific $CD4^+$ T cell response and the expansion of a Th17 response. Harboring robust cytotoxicity as well as classical helper functions, durable antitumor $CD4^+$ T cell-mediated immunity is increasingly recognized as a critical component to sustained and effective cancer immunotherapy [15,47–49]. As a unique Th subset endowed with specialized, non-redundant mechanisms to promote inflammation at the cell and tissue levels, Th17 cells displayed unparalleled cytotoxicity and durability resulting in superior tumor clearance in preclinical models over that of Th1 counterparts [5–7]. Incorporating ICOS signaling into cancer immunotherapy may dramatically improve patient outcomes and, as such, multiple ICOS agonists are being evaluated in clinical trials alone or in combination with T cell checkpoint inhibitors [50]. Rational design of next generation immunotherapies incorporating cancer vaccines and immune modulators must address mechanism to establish persistent, highly functional cellular immunity to potentiate tumor clearance.

5. Data availability statement

All relevant data from this study are available from the authors.

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Competing interests

The authors declare no conflict of interest.

Contributions

R.K.C., R.S., A.C.S performed the experimental manipulations including immunizations, monitoring immune responses, and data analysis. S.P.E., A.C.L., A.C.S. provided experimental designed and interpretation of results. A.C.S. supervised all aspects of these experiments. All authors contributed to data presentation and writing of the manuscript.

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