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Surgical cytoreduction restores the antitumor efficacy of a *Listeria monocytogenes* vaccine in malignant pleural mesothelioma

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Abstract

Recent studies suggest that immunotherapy may offer a promising treatment strategy for early-stage malignant pleural mesothelioma (MPM), but advanced tumor burden may limit the efficacy of immunotherapy. Therefore, we hypothesized that surgical cytoreduction could restore the efficacy of vaccine-based immunotherapy for MPM. We developed a murine model of MPM through transduction of a mesothelioma cell line with mesothelin. We used this model to evaluate the efficacy of a *Listeria monocytogenes* vaccine expressing mesothelin. Tumor growth was significantly inhibited at four weeks in animals vaccinated two weeks prior to tumor cell inoculation as compared to those given an empty vector control ($1371 \pm 420 \text{ mm}^3$ versus $405 \pm 139 \text{ mm}^3$; $p < 0.01$). Mice vaccinated one week prior to tumor challenge also displayed significant reduction in tumor volume ($1227 \pm 406 \text{ mm}^3$ versus $309 \pm 173 \text{ mm}^3$; $p < 0.01$). The vaccine had no effect when administered concurrently with tumor challenge, or after tumors were established. Flow cytometry showed reduced mesothelin expression in large tumors, as well as tumor-associated immunosuppression due to increased myeloid derived suppressor cells (MDSCs). These factors may have limited vaccine efficacy for advanced disease. Surgical cytoreduction of established tumors restored the antitumor potency of the therapeutic vaccine, with significantly reduced tumor burden at post-operative day 18 ($397 \pm 103 \text{ mm}^3$ versus $1047 \pm 258 \text{ mm}^3$; $p < 0.01$). We found that surgery reduced MDSCs to levels comparable to those in tumor-naïve mice. This study demonstrates that cytoreduction surgery restores the efficacy of cancer vaccines for MPM by reducing tumor-related immunosuppression that impairs immunotherapy.

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Keywords

malignant mesothelioma; vaccine immunotherapy; surgical cytoreduction

1. Introduction

Mesothelioma is an insidious neoplasm arising in the pleura, pericardium, peritoneum, or tunica vaginalis, with approximately 80% of cases originating in the thorax. Asbestos exposure is the predominant cause of malignant pleural mesothelioma (MPM), which has a latency period of approximately 30 years.^{1,2} Though modest improvements in the treatment of MPM have been made in the past decade, the disease remains a therapeutic challenge and the median survival of MPM patients is between 12 and 18 months.^{3,4}

A growing body of preclinical research supports the development of immunotherapeutic approaches to the treatment of MPM.^{1,5-9} The genotype and phenotype of MPM has been well characterized, demonstrating surface expression of the 40 kDa glycoprotein mesothelin, a potential tumor antigen amenable to immunotherapeutic targeting.¹⁰⁻¹⁵ Cancer vaccines have been shown to be effective in other neoplasms, and provide an encouraging method of generating an antigen-specific, cell-mediated immune response to mesothelin.¹⁶⁻²⁰

Though cancer vaccines show promise, a number of tumor escape mechanisms have posed persistent challenges to the development of effective vaccines. It has been shown that cancer cells may lose or reduce expression of molecules targeted by effector T-cells, such as tumor-associated antigens (TAA), MHC molecules, and molecules associated with antigen processing.^{21,22} This loss of antigenicity renders vaccines ineffective against the mutated population of neoplastic cells.²³ Furthermore, vaccines may lose their potency as tumor burden increases, due to complex alterations in the tumor microenvironment and systemic immunosuppression that accompanies advanced tumors.²⁴⁻²⁷

Listeria monocytogenes, a Gram-positive intracellular facultative bacteria, is a promising vector for cancer vaccines that may overcome tumor escape via loss of antigenicity. *Listeria* can infect phagocytic and antigen-presenting cells (APCs), where it is able to move from the phagosome into the cytoplasm of the cell, unlike most other intracellular bacteria.^{28,29} This unusual intracellular life cycle allows antigens secreted by *Listeria* to be processed and presented by both MHC class I and II molecules, resulting in strong CD8+ and CD4+ T-cell-mediated immune responses.³⁰ Furthermore, it has been shown that *Listeria*-based vaccines can induce epitope spreading, a phenomenon that may counter tumor cell loss of antigenicity.³¹⁻³³ In epitope spreading, APC processing of tumor cells activates secondary tumor-specific T-cell responses, thereby broadening the host immune response beyond the antigen targeted by the vaccine.³⁴ This process seems critical to the establishment of durable antitumor immune responses, and recombinant strains of *Listeria* expressing TAA have been shown to generate robust *in vivo* antitumor responses in melanoma, breast cancer, and HPV-associated neoplasms.^{33,35-39}

Surgery may also play a role in bolstering the efficacy of cancer vaccines by overcoming the immunosuppressive effects that accompany advanced tumors. Recent work in our laboratory

indicates that cytoreduction surgery potentiates the effects of immunotherapy in large tumors by reducing systemic myeloid suppressor cell populations.^{25,40,41} Thus, we hypothesized that a *Listeria*-based vaccine coupled with surgical cytoreduction may overcome some of the challenges that have traditionally hampered the development of cancer vaccines, and offer a novel approach to immunotherapy for the treatment of MPM.

Here, we report the development of an *in vivo* model of MPM using a murine mesothelioma cell line transduced with the mesothelin gene. Using this model, we investigated a recombinant *Listeria*-based vaccine for mesothelin, which exhibited robust antitumor effects when delivered prior to tumor cell inoculation. Finally, we demonstrate that cytoreduction surgery in large tumors restores the antitumor efficacy of the *Listeria* vaccine.

2. Materials and Methods

2.1. Animals

Female C57BL/6 (B6, Thy1.2) mice were purchased from Charles River Laboratories (Wilmington, MA). All mice were maintained in pathogen-free conditions and used for experiments at ages 8 week or older. The Animal Use Committees of the Children's Hospital of Philadelphia, The Wistar Institute and the University of Pennsylvania approved all protocols in compliance with the Guide for the Care and Use of Laboratory Animals.

2.2. Cell Lines

Three murine mesothelioma cell lines (AE17, AB12, AB1) that grow in syngeneic mouse strains were transduced with a lentivirus expressing human mesothelin. Flow cytometric sorting was used to purify cells that were successfully transduced with the mesothelin gene. The transduced AE17 cell line was maintained in RPMI media supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin (P/S). The transduced AB12 and AB1 cell lines were maintained in Dulbecco's Modified Eagle Media supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin (P/S). Cells were cultured at 37°C in a humidified incubator containing 5% CO₂.

2.3. Viral Transduction

Using a third-generation self-inactivating lentiviral expression vector encoding human mesothelin driven by the EF-1 α promoter (a generous gift from Dr. Carl June), high-titer replication-defective lentiviral vectors were produced and concentrated as previously described.⁴² 250,000 to 500,000 cells were seeded in 2 ml of their respective serum supplemented growth medium (described above) per well in a 6 well plate. The following day LV-mesothelin was added to the tumor cells at an MOI of 5:1. Transduced cells expressing high levels of mesothelin were collected using a BD FACSAria cell sorter and cultured as described above.

2.4. Immunostaining

Mice were euthanized at which time tumors were harvested and frozen in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA) to be stored at 80 °C, and 5 μ m

sections were cut. The frozen tumor tissue sections were fixed with 4% paraformaldehyde, washed with PBS and incubated with 10% goat serum in PBS + 0.1% Tween-20 for 60 min, followed by labeling with anti-human mesothelin antibody (K1 clone, Cat # SIG-3623, Covance) overnight at 4 °C. After incubation, sections were washed and further incubated with PE conjugated sheep anti-mouse secondary antibody (Cat # P8547, Sigma) for 1 hr at room temperature. Sections were counterstained with DAPI (Invitrogen) and then mounted with slow fade mounting medium (Invitrogen). Sections were visualized under a Nikon E600 microscope.

2.5. *Listeria* Vaccine

A modified *Listeria monocytogenes* vector expressing human mesothelin (CRS-207) was provided by Anza Therapeutics, Inc. (Concord, MA). Mice were vaccinated via intraperitoneal injection with CRS-207 at a dose of 1×10^7 colony forming units (cfu) per mouse two weeks prior to tumor challenge. An empty *Listeria* control vector (ANZ-100) was also provided by Anza Therapeutics. Control mice were vaccinated with ANZ-100 in a similar fashion.

2.6. Animal flank tumor models

C57BL/6 mice were inoculated by subcutaneous injection on the right flank with 1×10^6 AE17M cells. When the tumors reached 250 mm³ the mice were randomized and grouped into cohorts. Tumor dimensions were measured with calipers and tumor size was determined by the equation: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. All experiments had at least five mice per group and were replicated at least once.

2.7. Surgery

Surgery was performed on mice bearing flank tumors using an established partial resection model.⁴³ Partial resection was performed on C57BL/6 mice inoculated subcutaneously with 1×10^6 AE17M cells. Surgery was performed when tumors reached ~300 mm³. Mice were anesthetized with intramuscular ketamine (80 mg/kg) and xylazine (10 mg/kg) and shaved with hair clippers prior to surgery. A 1 to 2 cm incision was made adjacent to the tumor and 90% of the tumor was removed using standard blunt dissection technique. The incision was closed using sterile silk 4-0 sutures. Buprenorphine (0.2 mg/kg) was administered at the time of surgery and 6 hours afterward as postoperative analgesia. Preoperative treatment was unknown to the investigator performing surgery and making tumor measurements.

2.8 In Vivo Depletion of CD8 T-cells

To deplete specific T-cell populations in our model, mice were injected i.p. with monoclonal antibodies purified from the anti-CD8 hybridoma 53-6.7 (American Type Tissue Culture Collection, Manassas, VA). Mice were given 300 µg of purified antibody i.p. dissolved in 200 µL of PBS for CD8+ antibodies. Antibodies against CD8 were administered twice weekly for 3 weeks after tumor resection. Depletions were confirmed by flow cytometry of splenic suspension.

2.9 Detection of Tumor Neutralizing CD8 T-Cells (Winn Assay)

Splenocytes (3 mice/group) or splenic CD8 T cells from tumor bearing mice at various time points or control mice were used. CD8 T-cells were isolated using MACS isolation system (CD8a [Ly-2] mouse MicroBeads -- Miltenyi Biotec, Auburn, Calif). Isolated cells consisted of greater than 90% CD8 T-cells. Fresh AE17 cells were mixed with CD8 T-cells in a ratio of 3 CD8 T-cells to 1 AE17 tumor cell. The resulting mixture (1.5×10^6 CD8 T-cells to 0.5×10^6 AE17 tumor cells) was injected subcutaneously into the flanks of 5 naïve C57Bl/6 mice. A control group of 5 mice were injected with AE17 cells alone (0.5×10^6 cells) in an analogous fashion. Tumor size was assessed over the next 10 days.

2.10. Flow cytometric analysis

For flow cytometric analysis, tumors were removed from euthanized mice and minced into 1–2 mm fine pieces in digestion buffer containing 0.1 mg/mL DNase I and 2.0 mg/mL collagenase type IV (Sigma, St. Louis, MO). Samples were incubated in digestion buffer at 37°C for 30 minutes, filtered through a 70- μ m filter, and washed twice with DMEM supplemented with 10% FBS (vol/vol). Fc receptors were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences PharMingen, San Diego, CA). After 1 wash with staining buffer (PBS supplemented with 2% FBS (vol/vol)), cells were incubated for 30 minutes at 4°C with appropriate antibodies obtained from Abcam (Boston, MA) and BD Biosciences PharMingen (San Diego, CA) and used at the indicated dilutions for flow cytometry. Samples were then washed and re-suspended in staining buffer for immediate flow cytometric analysis. Flow cytometry was completed using a Becton Dickinson FACS Calibur flow cytometer (BD Biosciences), and analyzed using FlowJo software (Ashland, OR).

2.11. Statistical analyses

For flow cytometry, immunohistochemistry, and flank tumor volume studies comparing differences between two groups, we used unpaired Student's t-tests. For studies comparing more than two groups, ANOVA with appropriate post hoc testing was implemented. Differences were considered significant when $p < 0.05$. Data are presented as mean (standard error), unless otherwise noted.

3. Results

3.1. Generation of murine mesothelioma cell lines expressing human mesothelin

Three murine mesothelioma cell lines (AE17, AB12, AB1) which grow in syngeneic mouse strains were transduced with a lentivirus expressing human mesothelin. Flow cytometric sorting was used to purify cells that were successfully transduced with the mesothelin gene. [Figure 1A]. Once a purified line was achieved by transduction and flow cytometric sorting, syngeneic mice were inoculated subcutaneously with tumor cells in order to develop a functioning heterotopic murine model. Transduced AE17 mesothelin (AE17M) cell lines that retained expression of the mesothelin antigen grew reproducibly in mice [Figure 1B]. Immunofluorescence staining was performed using harvested AE17M mouse tumors and confirmed expression of the mesothelin antigen [Figure 1C].

3.2. Vaccination with mesothelin-expressing *Listeria* vaccine (CRS-207) slows tumorigenesis

An initial pilot study was performed to determine if pre-vaccination with a *Listeria* vaccine expressing the mesothelin antigen (CRS-207) would protect immunocompetent mice from developing malignant mesothelioma. It was hypothesized that this exposure would lead to development of an immune response against the mesothelin antigen. CRS-207 was tested on the heterotopic murine model of malignant mesothelioma. Five mice were vaccinated intraperitoneally with CRS-207 (1×10^7 cfu per mouse) two weeks prior to tumor challenge. Five control mice received the same dose of empty *Listeria* control vector (ANZ-100). All of the mice were boosted with an additional dose of the appropriate therapy one week later. The following week, 1×10^6 AE17M tumor cells were injected subcutaneously in the flank. The animals were re-vaccinated at one week and two weeks post tumor cell inoculation. Tumor volume, toxicity, and survival were monitored over this time period. Tumor growth was significantly inhibited at four weeks in animals that had been exposed to the therapeutic vaccine (1371 ± 420 mm³ versus 405 ± 139 mm³; $P=0.001$) [Figure 2A]. This study indicates that CRS-207 is effective in prevention of growth of malignant mesothelioma in mice.

To determine the optimal time interval for pre-vaccination, an experiment was performed in which five control and five experimental group mice received a single vaccine dose one week prior to tumor challenge. Additional doses were given at the time of tumor cell inoculation, and then one week, and two weeks after tumor cell inoculation. The details of the remainder of the experiment were identical to the first experiment. Again, CRS-207 demonstrated efficacy in slowing the growth of the tumor. At four weeks, average tumor volume was 1227 ± 406 mm³ in the control group compared to 309 ± 173 mm³ in the treated group ($p=0.001$) [Figure 2B].

To determine whether the vaccine was effective when administered concurrently with tumor challenge, we inoculated five control and five experimental group mice subcutaneously with 1×10^6 AE17M cells in the flank and then vaccinated with ANZ-100 or CRS-207 intraperitoneally one hour prior to tumor cell inoculation. Booster vaccines were given at one and two weeks. Although statistical significance was not achieved, there was some protection provided by concurrent vaccination. Average tumor volume at the conclusion of the experiment was 1370 ± 409 mm³ in the untreated group compared to 967 ± 154 mm³ in the treated group ($p=0.07$) [Figure 2C]. These data provide proof of principle that CRS-207 can generate an immune response to slow tumor growth. Vaccination prior to or concurrent with tumor cell inoculation inhibited tumor cell growth and slowed disease progression.

3.3. CRS-207 does not inhibit growth of established primary tumor

CRS-207 was tested for its ability to treat mice with established malignant mesothelioma tumors. Five mice were inoculated subcutaneously with 1×10^6 AE17M cells in the flank. Once tumors (average volume 200 mm³) had been established, mice received CRS-207 vaccine intraperitoneally. The animals were boosted with additional doses at one week and two weeks. There was no measurable benefit from CRS-207 therapy [Figure 3A].

3.4. Loss of antigenicity may partially explain lack of CRS-207 efficacy in advanced tumor

It has been well established that cancer cells can downregulate tumor-associated antigens to reduce their immunogenicity. We hypothesized that this loss of antigenicity may have played a role in the lack of response of established tumors to CRS-207. To evaluate this theory, we analyzed mesothelin expression levels in small (~100 mm³) and advanced (~500 mm³) AE17M tumors using flow cytometry, finding reductions in mesothelin expression levels in advanced tumors [Figure 3B].

3.5 Adaptive immune responses are responsible for vaccine efficacy

Due to the inability of CRS-207 to effectively treat established tumors, we investigated whether cytoreduction surgery would enhance the antitumor efficacy of the vaccine in established tumors. This notion was based upon past work in our laboratory indicating that advanced tumor burden is characterized by an immunosuppressive environment, and that surgical cytoreduction may lessen tumor-associated immunosuppression.

In order to test this hypothesis, mice were injected subcutaneously with 1×10^6 AE17M and at the same time point were given either CRS-207 or ANZ-100. Mice were boosted with appropriate vaccines after one week. When tumor reached ~300 mm³ in mice, 90% of the tumor was resected and mice were given an additional dose of CRS-207 or ANZ-100. Recurrent tumor growth was monitored after surgery and it was observed that CRS-207 significantly inhibited tumor growth (397 ± 103 mm³) compared to ANZ-100 at post-operative day 18 (1047 ± 258 mm³) ($p = 0.002$) [Figure 4].

Previous reports have described that immune responses are a primary mechanism of the effects of adaptive immune response. To more precisely define this role in our model, mice with recurrent AE17 flank tumors after surgery were randomized to four groups: (1) ANZ-100 (control), (2) ANZ-100 with CD8 T-cell depletion, (3) CRS-207, or (4) CRS-207 with CD8 T-cell depletion. Depletion of the T-cell population was confirmed weekly by analysis of spleen cells by flow cytometry (data not shown). Although mice receiving CRS-207 had smaller nodules after 14 days of tumor growth, effects were negated in the presence of CD8 T-cell depletion (Fig. 5A). These results confirm the role of CD8 T-cells as an essential element in anti-tumor effects of Listeria vaccination protocols in our recurrent tumor model.

To further confirm that CD8 T-cells were responsible for this antitumor effect, we isolated CD8 T-cells from mice 3 to 5 days following the first dose of Listeria vaccination. These CD8 T-cells were injected along with fresh AE17 tumor cells (in a ratio of 3 CD8 T-cells: 1 AE17 cell) into the flank of tumor naïve C57bl/6 mice. The growth of these nodules was then observed for 7 days. We found that CD8 T-cells obtained from mice receiving CRS-207 were better able to neutralize AE17 cells as compared to mice receiving ANZ-100 ($p=0.02$) (Fig. 5B). These results suggest that CRS-207 decreases tumor burden primarily by augmentation of anti-tumor CD8 T-cell function.

3.6. Surgical cytoreduction restores antitumor efficacy of CRS-207 by systemic reduction in myeloid derived suppressor cells

Past work in our laboratory has shown that surgery reduces systemic myeloid suppressor cell (MDSC) populations to restore the efficacy of T-cell mediated immunotherapy in a mouse model of non-small cell lung cancer.²⁵ To determine whether a similar phenomenon was responsible for the restoration of the antitumor effects of CRS-207 in our model of mesothelioma, we evaluated the number of MDSCs (CD11b+Gr1+) present in mice without tumors, mice bearing established tumors (~300 mm³), and at postoperative day 3. Mice were sacrificed and spleens were evaluated for MDSC populations using flow cytometry. We found a significant increase in MDSCs as a fraction of CD45 splenocytes in mice bearing tumors compared to tumor-naïve mice (3.2% to 57.5%; $p < 0.01$). Three days after cytoreduction surgery, the percentage of MDSCs decreased to levels comparable to those in tumor-naïve mice (4.7%; $p < 0.01$) [Figure 6]. These data indicate that surgery has the ability to restore the efficacy of cancer vaccines by reducing the tumor-related immunosuppression networks that impair CD8 T-cells.

4. Discussion

Cancer vaccines represent a promising immunotherapy strategy that has produced some striking responses in animal models for a variety of malignant diseases.¹⁸ However, these results have translated poorly to human trials, and a number of challenges have hampered the development of clinically effective vaccines, especially for advanced tumors.⁴⁴ It has been demonstrated that cancer cells are able to lose or reduce their immunogenicity through a number of mechanisms, including downregulation of TAA and MHC molecules.^{21,22} Here, we show a significant reduction in expression of mesothelin in large tumors, further demonstrating the loss of antigenicity that may significantly limit the efficacy of vaccines for advanced disease. Further work needs to be completed in order to determine if this phenomenon is truly due to selection pressure against mesothelin in tumor cells compared with simply loss of mesothelin expression over time.

Advances in molecular immunology, coupled with increased knowledge of pathogen physiology, have facilitated the use of attenuated bacteria as vaccine vectors, a strategy that may counter loss of antigenicity through epitope spreading. Epitope spreading is a process by which the T-cell response is expanded beyond the antigen targeting the vaccine, thereby broadening and enhancing vaccine efficacy.⁴⁵ *Listeria monocytogenes* seems to be especially promising in this regard, and several studies have demonstrated its propensity to induce epitope spreading in a number of malignant neoplasms.³¹⁻³³

In this study, we add to the relatively abundant literature demonstrating the potency of *Listeria*-based vaccines targeting model tumor antigens. We report the establishment of a mouse model of MPM by the successful transduction of a murine mesothelioma cell line with mesothelin, a surface protein overexpressed in MPM. Using this model, we show robust antitumor activity elicited in a response to a recombinant *Listeria* vector expressing mesothelin. Tumorigenesis was significantly inhibited when mice were pre-vaccinated at two time points over a two-week period prior to tumor cell inoculation. Inhibition of tumor

growth was also observed in mice vaccinated with a single dose either one week prior to or concurrently with tumor cell inoculation.

Despite its effectiveness in slowing tumorigenesis when administered prior to or concurrently with cancer cell inoculation, the mesothelin-expressing *Listeria* vaccine did not exhibit antitumor activity against established primary tumors, a result that is consistent with previous studies of other cancer vaccines, and one that points to another persistent challenge in the realization of the potential of vaccine-based immunotherapy.⁴⁰ Past work in our laboratory and elsewhere has demonstrated that local and systemic host immunosuppression accompanies advanced tumor burden and may limit vaccine effectiveness. This immunosuppression appears to be mediated by a complex array of factors, including extensive systemic suppressive cell populations, poor T-cell trafficking, and increased levels of immunosuppressive cytokines.^{24,25,27}

Recently, a number of studies have suggested that surgery may play a role in restoring immunotherapy for advanced tumors. Surgery may enhance immunotherapy not only through simple mechanical cytoreduction, but also by reducing systemic tumor-related immunosuppression.^{25,41,46} Here, we demonstrate that surgery reduces systemic myeloid derived suppressor populations, which are known to inhibit CD8 T-cell responses. This approach is not without limitations, as surgery has been shown to generate a transiently immunosuppressive tumor microenvironment that allows increased tumor growth.^{47,48} However, the results of this study provide support for pairing cytoreductive surgery with neoadjuvant immunotherapy in advanced disease to re-establish the effectiveness of immunotherapy. We demonstrate that surgical debulking of established tumors restores the antitumor efficacy of the mesothelin-expressing *Listeria* vaccine.

5. Conclusions

In summary, the results of this study add to the growing body of literature demonstrating the effectiveness of *Listeria*-based cancer vaccines, and suggest a role for a mesothelin-targeted vaccine in the treatment of MPM, a cancer that remains a significant therapeutic challenge. A recent clinical trial of a similar *Listeria* vaccine expressing mesothelin found that the immunotherapy was well-tolerated and induced immune activation in patients with various advanced cancers.⁴⁹ This result underscores the potential clinical application of our findings. Finally, we expand upon the concept of a synergy between surgery and immunotherapy, demonstrating that debulking of tumor restores the antitumor efficacy of vaccine-based immunotherapy for MPM by reducing systemic MDSC populations. Ultimately, the combination of a mesothelin-expressing *Listeria* vaccine and surgical cytoreduction warrants further study and may one day offer an attractive therapeutic strategy for patients with advanced MPM refractory to conventional therapy.

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6. Vitae

Gregory T. Kennedy is a medical student at the Perelman School of Medicine at the University of Pennsylvania. He graduated *summa cum laude* from Princeton University, where he was elected to Phi Beta Kappa, and received a graduate degree from the University of Cambridge, where he was a Rotary Ambassadorial Scholar. He plans to pursue a career in academic surgery.

Brendan F. Judy is a medical student at Jefferson Medical College. He graduated from the University of Richmond in 2010 with a degree in political science. He subsequently worked in the Thoracic Surgery Research Laboratory before beginning medical school in 2013. He is interested in a career in surgery.

Pratik Bhojnagarwala graduated from the University of Illinois at Urbana-Champaign with his B.S. in Molecular Biology. He also received his MS in Biotechnology from the University of Pennsylvania. Before joining Dr. Singhal's lab, Pratik worked in the Department of Pharmacology at UPenn. Currently, his primary focus involves developing a better understanding of the function of myeloid cells in lung cancer. This will hopefully lead to design of better immunotherapies in the future. Future plans involve getting a Ph.D. in immunology and eventually working to bring new and novel therapies to the market.

Edmund K. Moon is a junior faculty member in Pulmonary, Allergy, and Critical Care at the University of Pennsylvania. Two and half years of his training and two years of his initial faculty career have been spent doing basic science research under the mentorship of Dr. Steven Albelda in the Thoracic Oncology Research Group. Dr. Moon's research interest is in adoptive T cell immunotherapy for thoracic and pleural malignancies with the overarching goal of becoming established as a successful physician-scientist in academic medicine.

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Sunil Singhal is the Director of the Thoracic Surgery Research Laboratory and Assistant Professor of Surgery at the University of Pennsylvania School of Medicine. He obtained his Doctor of Medicine degree from the University of Pennsylvania and received post-graduate training at Johns Hopkins and the University of Pennsylvania. His research interests include nanotechnology, intraoperative immune suppression, muscle physiology, patient outcomes, myeloid derived suppressor cells, and gene-expression and profiling.

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Highlights

- A murine model of malignant pleural mesothelioma was developed.
- A *Listeria* vaccine expressing mesothelin was tested against an empty vector control.
- Mice vaccinated prior to tumor inoculation had significant reduction in tumor volume.
- There was no effect on established tumors due to tumor-related immunosuppression.
- Surgery restored vaccine efficacy by reducing tumor-related immunosuppression.

Figure 1. Establishment of a murine model of malignant mesothelioma

A. Flow cytometry indicates that transduced cell lines (shown in blue) express higher levels of mesothelin than parental cell lines (shown in red). **B.** Tumor growth curve demonstrating tumorigenesis of AE17M in mice after transduction of mesothelin as described in Materials and Methods. **C.** Fluorescent staining of flank tumors harvested from female C57/BL/6 mice 3 weeks after tumor cell inoculation demonstrating overexpression of mesothelin in AE17M.

Figure 2. Vaccination with CRS-207 inhibits tumorigenesis

A. Tumor growth curve demonstrating significantly reduced tumor growth in mice (n=5) prevaccinated with CRS-207 two weeks and one week prior to tumor challenge. Booster vaccines were given one and two weeks after tumor challenge. **B.** Tumor growth curve demonstrating significantly reduced tumor growth in mice (n=5) pre-vaccinated with CRS-207 one week prior to tumor challenge. Booster vaccines were given one and two weeks after tumor challenge. **C.** Tumor growth curve demonstrating reduced tumor growth in mice (n=5) vaccinated with CRS-207 concurrent with tumor challenge. Booster vaccines were given one and two weeks after tumor challenge. Arrow denotes vaccination with either CRS-207 or the empty vector control ANZ-100. * Signifies $p < 0.05$ and ** signifies $p < 0.01$.

Figure 3. CRS-207 is not effective against established tumors

A. Tumor growth curve demonstrating no alteration in tumor growth in mice (n=5) vaccinated with CRS-207 after tumors had grown to 200 mm³. Booster vaccines were given one and two weeks after initial vaccination. Arrow denotes vaccination with either CRS-207 or the empty vector control ANZ-100. * Signifies $p < 0.05$ and ** signifies $p < 0.01$. **B.** Flow cytometry data indicating reduction in expression of mesothelin in large tumors harvested four weeks after tumor cell inoculation as compared to small tumors harvested at one week. Arrow denotes vaccination with CRS-207.

Figure 4. Cyto-reduction surgery restores antitumor efficacy of CRS-207

Tumor growth curve demonstrating synergistic combination of surgery and immunotherapy in a mouse model of MPM. Two groups (n=5) of mice were vaccinated with CRS-207 or ANZ-100 concurrently with tumor cell inoculation. Booster vaccines were given after one week and at the time of cyto-reduction surgery to remove ~90% of tumor. Subsequent growth curves demonstrate the restoration of the antitumor activity of CRS-207 following surgical cyto-reduction. Arrow denotes vaccination with either CRS-207 or the empty vector control ANZ-100. * Signifies $p < 0.05$ and ** signifies $p < 0.01$.

Figure 5. Listeria vaccination increases CD8 T-cell lymphocyte infiltration and systemic populations more effectively than control vector

A. C57Bl/6 mice bearing flank tumors after surgery were randomized into four groups: (1) ANZ-100 treatment (control), (2) ANZ-100 with CD8 T-cell depletion, (3) CRS-207, or (4) CRS-207 with CD8 T-cell depletion. Effects of CRS-207 were eliminated in the absence of CD8 T cells. **B.** Winn assay results reveal that combination of surgery and Listeria function in a CD8 T-cell-dependent manner. CD8 T cells from mice receiving surgery and CSR-207 were superior in neutralizing fresh tumor cells in vivo when compared to those CD8 T cells

harvested from mice receiving ANZ-100 and surgery alone, and tumor cells alone (n=5 per group). * $P < 0.05$

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Figure 6. Surgical cytoreduction reduces tumor-associated immunosuppression

Flow cytometry of splenocyte populations obtained from tumor-naïve mice, mice bearing established tumors, or mice 3 days status post partial resection. Panel depicts myeloid derived suppressor cells (CD11b⁺ Gr1⁺). MDSCs increased with tumor growth, but were markedly reduced following cytoreduction surgery.