Award Number: W81XWH-11-1-0384

TITLE: Evaluation of Immune Responses Mediated by Listeria-Stimulated Human Dendritic Cells: Implications for Cancer Vaccine Therapy

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REPORT DATE: September 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2015	Final	15 June 2011 – 14 June 2015

4. TITLE AND SUBTITLE

Evaluation of Immune Responses Mediated by Listeria-Stimulated Human Dendritic Cells: Implications for Cancer Vaccine Therapy

5a. CONTRACT NUMBER
W81XWH-11-1-0384

5b. GRANT NUMBER
CA100463

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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New York, NY 10065-6007

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this project is to study the immunomodulatory effect of Listeria monocytogenes (Lm) on human dendritic cells (DCs) to optimize Lm-based DC cancer vaccines. The project aims are: 1) Compare the activation and maturation of different human DC subsets in response to Lm infection. 2) Define the induction of CD4+/CD8+ T-cell and NK cell responses to Lm-activated DCs presenting a melanoma tumor-associated antigen. 3) Augment the immunogenicity of Lm-activated DCs by inhibiting the immunosuppressive enzyme, indoleamine 2,3-dioxygenase. Key findings of the project include: 1) *Lm*, including attenuated strains, induces human DC maturation and activation. 2) *Lm* induces less inhibitory receptor expression on mature DCs than standard inflammatory cytokine stimulation. 3) *Lm*-activated DCs are potent stimulators of allogeneic and autologous T cell proliferation. 4) *Lm*-conditioned DCs induce robust T cell activation that is associated with inhibitory receptor upregulation, providing rationale for the inclusion of checkpoint inhibition to augment T cell responses. 5) *Lm* treatment, despite vigorous T cell activation, does not potentiate DC-mediated expansion of immune-dampening regulatory T cells. 6) LLO-deficient *Lm* induces less IDO in DCs than WT and ActA-deficient strains. 7) *Lm*-treated moDCs, without exogenous cytokine supplementation, induce melanoma antigen-specific CTLs. Collectively, these findings confirm the immune-stimulatory properties of *Lm*, lend further support for *Lm* as a DC vaccine adjuvant to optimize vaccines efficacy, and identify immune checkpoint blockade as a rational complement to *Lm*-mediated immune activation.

15. SUBJECT TERMS
Listeria, cancer vaccine, dendritic cells

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	14	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION
This project addressed the FY10 PRCRP topic area of **Listeria vaccine for cancer** by examining the immunobiology of Listeria-induced activation of different human dendritic cell (DC) subsets, and covered the following focus areas: *1) Induction and analysis of CD4+ and CD8+ T cell responses to tumor-restricted antigens; 2) Induction and maturation of DC responses to tumor antigens; and 3) Modulation of T cell and other effector cell trafficking*. This project also assessed immune responses to a melanoma-specific antigen and evaluated indoleamine 2,3-dioxygenase (IDO)-mediated suppression of T cell responses by Listeria-activated DCs. The project therefore had overlapping relevance to the topic area of **Melanoma and other skin cancers**, including the focus area of *evaluation of a key immunosuppressive mechanism*. The overall goal of this project was to identify an optimal Listeria-activated DC subset for clinical vaccine application, including vaccination strategies for melanoma, and to provide rationale for further enhancing antitumor immunogenicity by inhibiting IDO.

KEYWORDS
Listeria monocytogenes, cancer vaccine, dendritic cells

OVERALL PROJECT SUMMARY
As described in the approved Statement of Work (SOW), the objectives of this project were: (1) Compare the activation and maturation of different human dendritic cell (DC) subsets in response to Listeria infection; (2) Define the induction of $CD4^+/CD8^+$ T cell and NK cell responses to Listeria-activated DCs presenting a melanoma tumor-associated antigen (TAA); (3) Augment the immunogenicity of Listeria-activated DCs by inhibiting the immunosuppressive enzyme, indoleamine 2,3-dioxygenase (IDO).

Task 1: Seek regulatory approval on human anatomical substances from local IRB and DoD ORP.
Anticipated timeframe: months 2-6.

Result/status: Completed and addressed in the annual report covering the first year of the funding period, from 06/15/2011 to 06/14/2012.

Task 2: Compare the activation and maturation of different human DC subsets in response to Listeria infection.
Anticipated timeframe: months 2-12 (Note: exact start time will depend on completion of task 1b).

Result/status: Subsections 2a, 2b, 2c, 2d, 2e, 2i, and 2j completed and addressed in the annual report for year two, covering the funding period from 06/15/2012 to 06/14/2013.

Subsection 2f completed and addressed in the annual report for year three, covering the funding period from 06/15/2013 to 06/14/2014.

The research accomplishments during the fourth year of the funding period are as follows:

2g) Intracellular cytokine secretion assay to detect DC secretion of proinflammatory cytokines (months 3-12)

Result/status: Listeria-treated DCs were compared with untreated controls for secretion of proinflammatory cytokines (e.g., IL-1β, TNF-α, and/or IL-12p70) by intracellular cytokine secretion assay (Miltenyi). There were no significant differences in the levels of cytokine secretion between Listeria-treated DCs and untreated controls. Additional studies during year four of funding confirmed this finding.

2k) Analysis for indoleamine 2,3-dioxygenase expression and activity (months 3-12)

Result/status: The immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) can impede immunity mediated by activated DCs[1, 2]. Listeria-mediated upregulation of IDO in moDCs was shown in one study[3]. It is unknown whether Listeria has a similar effect on IDO in other DC subtypes. In the annual report for year three, we reported that WT and ActA-deficient Listeria induce IDO to a greater extent than the LLO-deficient strain. This has potential bearing on the choice of attenuated Listeria for vaccine production, as a minimally IDO-inducing strain would mediate less Treg generation, which in turn might be advantageous in the setting of active DC-based vaccination. Additional studies during year four of funding confirmed this finding.

2l) Phos-Flow analysis of DCs for phosphorylated STAT3 & other signaling pathways (months 3-12)

Result/status: These experiments proved more challenging than expected, with initial studies showing variable results, requiring methods optimization. We hope to complete these studies in the future if time/other funds allow.

Task 3: Define the induction of $CD4^+$/$CD8^+$ T cell and NK cell responses to Listeria-activated DCs presenting a melanoma tumor-associated antigen (TAA).
Anticipated timeframe: months 12-24.

Result/status: Subsections 3a, 3b, 3c, 3e, 3f, and 3h completed and addressed in the annual report for year two, covering the funding period from 06/15/2012 to 06/14/2013.

Subsections 3i and 3g completed and addressed in the annual report for year three, covering the funding period from 06/15/2013 to 06/14/2014.

Subsections 3d and 3j were not performed, as NK cell yields and viability were suboptimal to set-up experiments with reliable readouts. We therefore prioritized T cell studies, as their interactions with DCs are the more likely predominant response in the setting of active DC vaccination. We hope to revisit the feasibility of NK cell studies in the future if time/other funds allow.

Task 4: Augment the immunogenicity of Listeria-activated DCs by inhibiting the immunosuppressive enzyme, indoleamine 2,3-dioxygenase (IDO).
Anticipated timeframe: months 16-36.

Result/status: Subsections 4a, 4b, 4c, 4e, 4f, 4g, and 4h completed and addressed in the annual report for year three, covering the funding period from 06/15/2013 to 06/14/2014.

Similar to Task 3, subsections 4d and 4k were not performed, as NK cell yields and viability were suboptimal to set-up experiments with reliable readouts.

The research accomplishments during the fourth year of the funding period are as follows:

4i) Antigen-specific CTL response assessments, including intracellular cytokine secretion assay to detect IFN-γ secretion and standard ^{51}Cr release assay (months 16-36)

Result/status: As described in the annual report for year three, initial experiments indicated that Listeria-treated moDCs were comparable to LCs alone and moDCs supplemented with IL15 at inducing antigen-specific CTLs. Repeat studies, however, produced equivocal results. Subsequently, studies performed during year four of funding show that Listeria-treated moDCs induce antigen-specific CTL activity, but not to the same degree as LCs alone and moDCs supplemented with IL15. Experiments to gain mechanistic insight(s) for this difference, including the effects of concurrent inhibition of IDO with 1MT (or siRNA), are ongoing.

4j) Flow cytometry analysis of T cells subsets (e.g., naïve, central memory, effector memory, Th1, Th2, Th17, and Treg) (months 16-36)

Result/status: Analysis of T cell subsets did not reveal any statistically significant differences between bulk T cells stimulated by Listeria-infected vs untreated DCs. Additional studies during year four of funding confirmed this finding.

Task 5: Conduct data analysis and prepare report to CDMRP at the end of the performance period.
Anticipated timeframe: months 1-36.

5a) Collect data for each set of experiments outlined in Tasks 1-4 (months 1-36)

Result/status: Completed, with exceptions noted above.

5b) Analyze data for each set of experiments outlined in Tasks 1-4 (months 1-36)

Result/status: Completed, with exceptions noted above.

5c) Organize and prepare report to CDMRP at the end of the performance period (months 30-36)

Result/status: Requisite annual reports filed.

KEY RESEARCH ACCOMPLISHMENTS

- *Listeria monocytogenes (Lm)*, including attenuated strains, induces human DC maturation and activation.
- *Lm* induces less inhibitory receptor expression on mature DCs than standard inflammatory cytokine stimulation.
- *Lm*-activated DCs are potent stimulators of allogeneic and autologous T cell proliferation.
- *Lm*-conditioned DCs induce robust T cell activation that is associated with inhibitory receptor upregulation, providing rationale for the inclusion of checkpoint inhibition to augment T cell responses.
- *Lm* treatment, despite vigorous T cell activation, does not potentiate DC-mediated expansion of immune-dampening regulatory T cells.
- LLO-deficient *Lm* induces less IDO in DCs than WT and ActA-deficient strains.
- *Lm*-treated moDCs, without exogenous cytokine supplementation, induce melanoma antigen-specific CTLs.

CONCLUSION

This study confirms the immune-stimulatory properties of *Lm*, lends further support for *Lm* as a DC vaccine adjuvant to optimize vaccine efficacy, and identifies immune checkpoint blockade as a rational complement to *Lm*-mediated immune activation.

PUBLICATIONS, ABSTRACTS, and PRESENTATIONS

Peer-reviewed publication: Chung, D.J., et al., *Langerhans-type and monocyte-derived human dendritic cells have different susceptibilities to mRNA electroporation with distinct effects on maturation and activation: implications for immunogenicity in dendritic cell-based immunotherapy.* J Transl Med, 2013. **11**: 166-175. PMID: 23837662.

Additional manuscript in preparation for submission to peer-reviewed journal, pending completion of Task 4i.

INVENTIONS, PATENTS, and LICENSES

None

REPORTABLE OUTCOMES

- Listeria-activated human dendritic cells for cancer vaccine therapy

OTHER ACHIEVEMENTS

None

REFERENCES

1. Chung, D.J., et al., *Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells.* Blood, 2009. **114**(3): p. 555-63.
2. Munn, D.H. and A.L. Mellor, *Indoleamine 2,3-dioxygenase and tumor-induced tolerance.* J Clin Invest, 2007. **117**(5): p. 1147-54.
3. Popov, A., et al., *Indoleamine 2,3-dioxygenase-expressing dendritic cells form suppurative granulomas following Listeria monocytogenes infection.* J Clin Invest, 2006. **116**(12): p. 3160-70.

APPENDICES

Final report project summary (see page 7)

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Project PI. No other personnel received salary support.

ADDENDIX - FINAL REPORT PROJECT SUMMARY (CA100463)

The goal of this project was to study the immunomodulatory effect of *Listeria monocytogenes (Lm)* on human dendritic cells (DCs) to identify the optimal *Lm*-activated DC subset for clinical vaccine development and to enhance antitumor immunogenicity by identifying/inhibiting mechanisms of immune suppression. Specific aims included: 1) Compare the activation and maturation of different human DC subsets in response to *Lm* infection. 2) Define the induction of $CD4^+$/$CD8^+$ T cell and NK cell responses to *Lm*-activated DCs presenting a melanoma tumor-associated antigen. 3) Augment the immunogenicity of *Lm*-activated DCs by inhibiting the immunosuppressive enzyme, indoleamine 2,3-dioxygenase.

Background

Cancer vaccines induce measurable tumor-specific immune responses in many patients, but meaningful clinical responses are infrequent. DCs are the most potent antigen-presenting cells of the immune system, are critical to the onset of immunity[1-4], and have demonstrated feasibility as vaccines in numerous studies[5,6]. The intracellular bacterium *Lm* is a potent stimulator of innate and adaptive immunity[7], in large part through DC activation[8-10]. Vaccination with DCs activated by *Lm* therefore offers a promising strategy to improve vaccine efficacy. The rational design of therapeutic cancer vaccines using this approach mandates a more comprehensive understanding of the immunobiology of *Lm*-DC interactions.

Materials and Methods

Human blood samples

Collection and use of biospecimens followed protocols approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center (MSKCC). Leukocyte concentrates (buffy coats) from healthy donors were purchased from the New York Blood Center (NYBC) and centrifuged over Ficoll-Paque PLUS (GE Healthcare) to isolate peripheral blood mononuclear cells (PBMCs). G-CSF-elicited peripheral blood leukapheresis products were obtained from healthy donors undergoing allogeneic transplantation and were similarly centrifuged over Ficoll-Paque PLUS to isolate mononuclear cells (MNCs). $CD34^+$ hematopoietic progenitor cells (HPCs) were positively selected from MNCs using immunomagnetic beads and separation columns according to the manufacturer's protocol (Miltenyi Biotec).

Media, serum, noncytokine reagents

Complete RPMI 1640 (Media Laboratory, MSKCC) was supplemented with 10 mM HEPES, 100 U/mL penicillin/streptomycin (Gibco), 50 μM β-mercaptoethanol (Gibco), 2mM L-glutamine (Corning Cellgro), and heat-inactivated pooled human serum (1 or 10% v/v) (Atlanta Biologicals). X-VIVO 15 (Lonza) was supplemented only with 100 U/mL penicillin/streptomycin. All media and reagents were sterile and endotoxin-free.

Cytokines

All cytokines used were sterile, recombinant, endotoxin-, pyrogen-, mycoplasma-, and carrier-free, as previously published[2].

Isolation of T lymphocytes

T cells were purified from tissue culture plastic-nonadherent PBMCs by nonadherence and elution from nylon wool columns (Polysciences), as published[2].

Dendritic cell generation and maturation

Monocyte-derived dendritic cells (moDCs) were generated from tissue culture plate-adherent PBMCs and Langerhans cells (LCs) were generated from $CD34^+$ HPCs exactly as previously published[2]. Cells were then incubated with inflammatory cytokines for 2 days to induce maturation.

Infection of dendritic cells with *Listeria monocytogenes*

Wild-type *Listeria monocytogenes (Lm)* and attenuated strains deficient in actin-assembly inducing protein (ActA) or listeriolysin O (LLO) were cultured in Brain Heart Infusion Media (BD Bacto) to log phase growth. Immature moDCs and LCs were infected with *Lm* at an MOI between 1 to 100 in RPMI 1640 with 1% heat-inactivated pooled human serum and serum-free X-VIVO 15, respectively, without penicillin/streptomycin. After 1 hour, cells were washed twice with PBS supplemented with 200 U/mL penicillin/streptomycin to remove free-floating bacteria. Cells were subsequently incubated with IL4 and GM-CSF for two additional days to allow for maturation.

Phenotypic analysis using flow cytometry
FITC-, PE-, PE-Texas Red–, ECD-, APC-, PE-Cy7–, PerCP-eFluor 710, AF700, BV650, and BV785-conjugated mouse and rat anti-human mAbs incubated with cells included anti-CD80, anti-CD86, anti–Ki-67, anti-PD-L1/CD274, anti-PD-L2/CD273 (BD Pharmigen), anti-CD83, anti-HLA-DR (Beckman Coulter), anti-CD127, anti-EOMES, anti-FoxP3, anti-ICOS/CD278, anti–LAG-3, anti–PD-1, (eBioscience), anti-CCR7, anti–TIM-3 (R&D Systems), anti-CD4 (Invitrogen) and anti-CD3, anti-CD8, anti-T-bet (BioLegend). Nonreactive isotype-matched antibodies (BD Pharmigen; eBioscience; R&D Systems) were used as controls. Intracellular staining of FoxP3, EOMES, and T-bet were performed using a kit (eBiosciences) according to the manufacturers' instructions. Dead cells were excluded from analysis using the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies). An FC 500 (Beckman Coulter) or an LSRFortessa (Becton Dickinson) flow cytometer was used to collect at least 20,000 live events for each sample. FlowJo software (TreeStar) was used to analyze flow cytometric data.

Mixed leukocyte reactions
Mature moDCs or LCs were separately added in serial doses (1:10 to 1:1000, moDC:T) to triplicate wells of 1×10^5 allogeneic or autologous T cells in 96 round-bottomed well plates (Corning Life Sciences), as previously described[11]. Final volume was 100 µL/well of complete RPMI 1640 with 10% heat-inactivated pooled human serum. After 5 days, responder T cell proliferation was measured by colorimetric assay according to manufacturer's instructions (CellTiter96 Aqueous One Solution Cell Proliferation Assay MTS; Promega). Additional T cells were phenotyped after 7 days in culture, or harvested and restimulated for 7 more days with autologous moDCs at a 1:10 ratio prior to measurement of cytolytic activity.

Western blot for IDO protein expression
IDO expression in moDCs was determined by Western blotting, as previously described[11]. Mouse anti-human GAPDH (Ambion) was used as a loading control.

mRNA electroporation of moDCs
For TRP2 mRNA transcription, the pGEM-4Z/WT1 plasmid was linearized with HindIII (New England Biolabs) before mRNA transcription in vitro, which was performed with SP6 RNA polymerase (mMessage mMachine SP6 kit; Ambion). Agarose gel electrophoresis confirmed production of full-length capped mRNA, and spectrophotometry measured mRNA concentration. Immature moDCs were electroporated with TRP2 mRNA on day 5-6. After electroporation, cells were immediately transferred to culture and terminally matured by exposure to inflammatory cytokines for 48 hours[2].

Cytolytic T lymphocyte (CTL) assays
Mature TRP2 mRNA-electroporated autologous moDCs were added in serial doses to triplicate wells containing 1×10^5 T cells in a 96 round-bottomed well plate (Corning Life Sciences). Final volume was 100 µL/well of RPMI-10% heat-inactivated, autologous serum, supplemented with recombinant human IL15 (10 ng/mL; R&D Systems). After 7 days of moDC-T cell culture, $5x10^3$ target cells were added directly to each well, and cytolytic activity exerted by responder T lymphocytes was assessed after 4 to 6 hours with a colorimetric CTL assay [12]. These data represented the total cytolytic activity generated in each culture according to the primary stimulation conditions, rather than per number of effector T cells irrespective of their frequency in the primary cultures. Target cells were SK-MEL-37 cells. HELA cells served as a negative control.

Statistics
Unpaired t-tests were used to explore mean differences in expression between categories. *P* values less than 0.05 were considered statistically significant. Prism 6 (GraphPad) was used to calculate statistics.

Results

***L. monocytogenes* (*Lm*) induces DC maturation to levels comparable to inflammatory cytokines.**
Immature moDCs and LCs were treated with wild-type (WT), ActA-deficient, or LLO-deficient *Lm* for one hour and then returned to culture for an additional two days. DCs were then harvested and assessed by flow cytometry for the upregulation of the maturation/activation markers, CD83, CD80, CD86, and CCR7 [2,13], and the immune inhibitory receptors, programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2). As shown in **Figure 1A-B**, *Lm* treatment efficiently activates and matures DCs. The degree of upregulation was equivalent between the three types of *Lm* (p = NS) and was comparable to that achieved with a standard combination of inflammatory cytokines (TNF-α, IL-1β, IL-6, and PGE2)[2] used to mature DCs. *Lm*-infected moDCs and LCs do not upregulate

PD-L1 and PD-L2 above levels induced by standard inflammatory cytokines. These findings indicate that Listeria-mediated activation of DCs (vs standard cytokine-mediated activation) does not stimulate overly robust inhibitory receptor expression that could potentially blunt a DC vaccine-based immune response.

Of note, treatment of moDCs with *Lm* induces the expression of CD103 (**Figure 1C**), which is not detected on moDCs in response to inflammatory cytokine stimulation. CD103 expression may reflect functional specialization of moDCs for gut-associated lymphoid tissue in response to *Lm* infection[14]. CD103 upregulation was not seen for LCs after *Lm* treatment (data not shown). Experiments to further delineate the functional specialization of $CD103^+$ DCs are warranted.

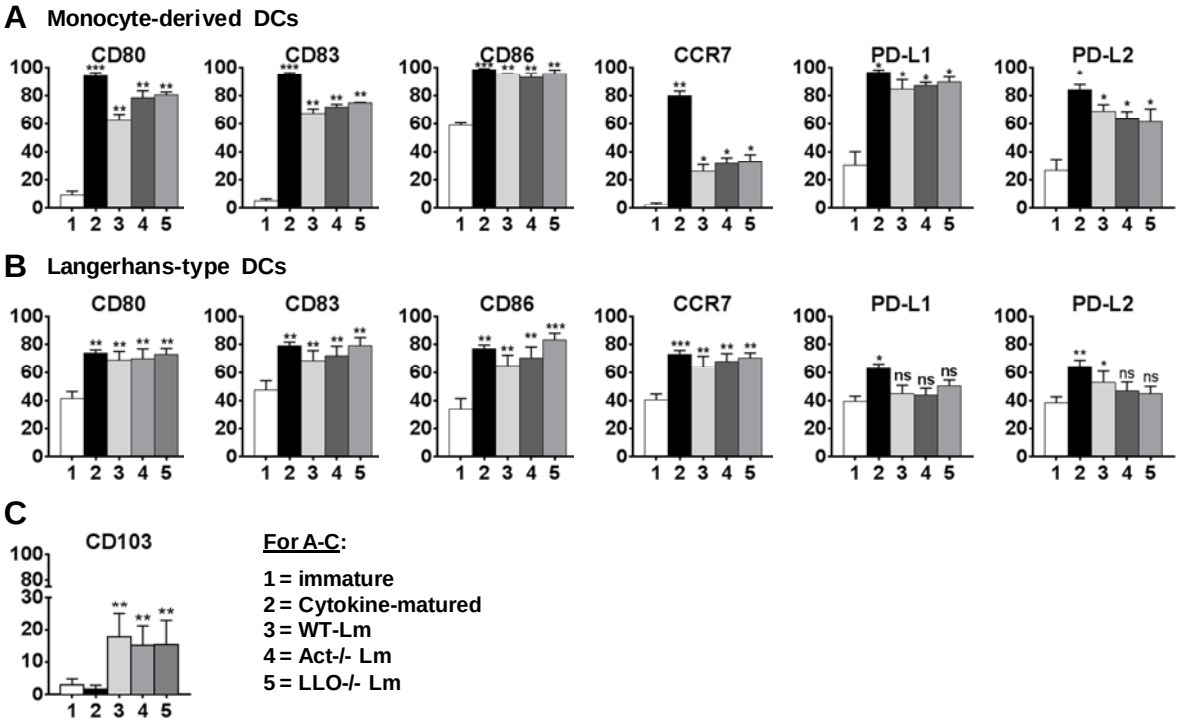

Figure 1: *L. monocytogenes* (*Lm*) conditioning efficiently induces DC maturation. Immature moDCs **(A)** and LCs **(B)** treated with wild-type (WT), ActA-deficient, or LLO-deficient *Lm* were assessed by flow cytometry for expression of CD80, CD83, CD86, CCR7, PD-L1, and PD-L2. Y-axis indicates percentage of positive cells for each marker. Positive controls were untreated cytokine-matured DCs. Pooled data (mean ± SD, n = 3 independent experiments) are shown. **P* < .05, ***P* < .01, and ****P* < .001.

***Lm*-treated DCs are potent stimulators of allogeneic and autologous T cell proliferation *in vitro*.**
DC function was assessed by comparing the ability of *Lm*-treated DCs (vs uninfected controls) to stimulate the proliferation of allogeneic T cells in a mixed leukocyte reaction (MLR), which is a standard assay for DC function. After 4-5 days, proliferation was measured by a colorimetric proliferation assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega).

As shown in **Figure 2A-B**, *Lm*-infected moDCs and LCs without proinflammatory cytokine supplementation were equivalent to inflammatory cytokine-matured DCs and LCs at inducing <u>*allogeneic*</u> T cell proliferation, underscoring the immunologic potential of *Lm* as a vaccine adjuvant. Importantly, both attenuated strains of *Lm* retained their immune-stimulatory efficacy.

Having demonstrated that *Lm*-infected DCs retain their allo-stimulatory capacity, we next examined their ability to induce <u>*autologous*</u> T cell proliferation, which would be more physiologically relevant in the setting of therapeutic cancer vaccination of patients. As shown in **Figure 2C-D**, the auto-stimulatory capacity of *Lm*-activated LCs is comparable to cytokine-matured cells and for moDCs is augmented by Lm infection, a finding that has important implications for optimizing DC-based immunotherapy.

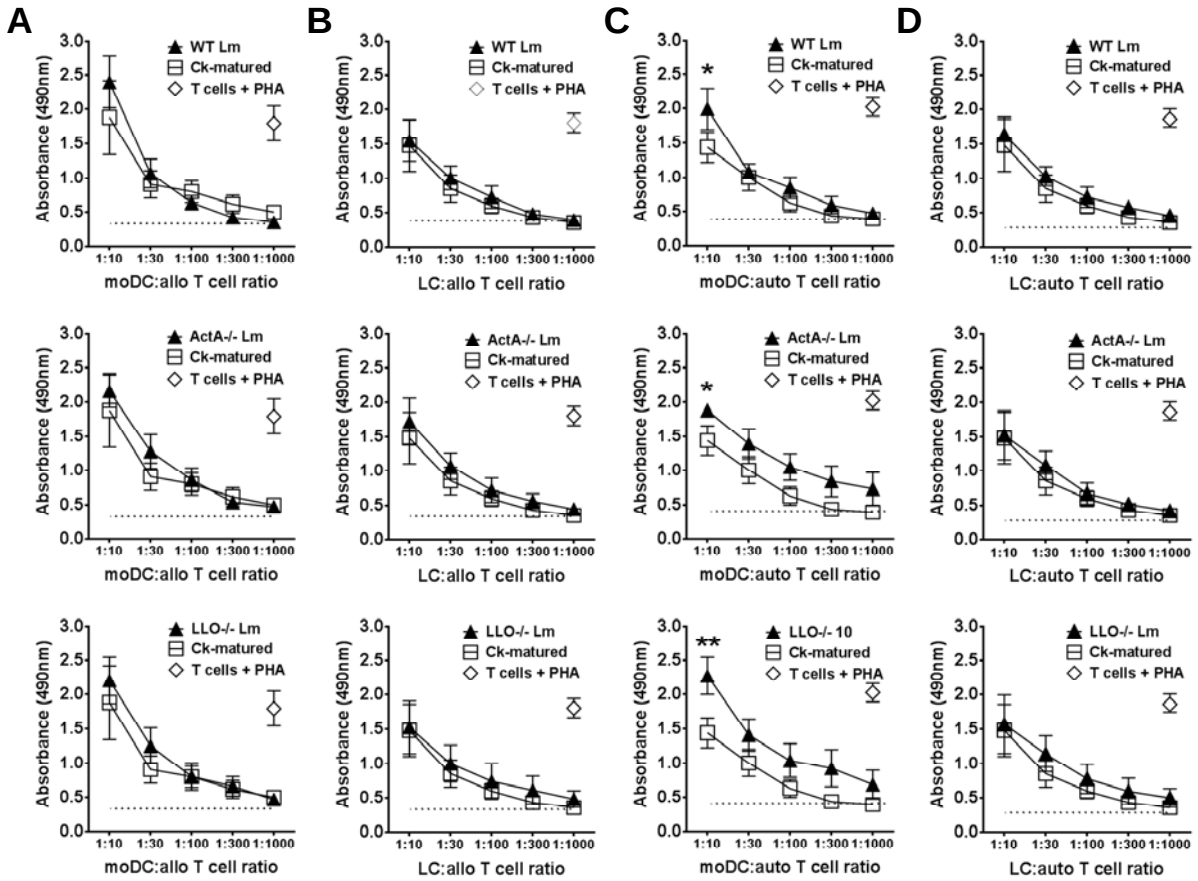

Figure 2: *Lm*-treated DCs are potent stimulators of allogeneic and autologous T cell proliferation *in vitro*. Immature moDCs (A and C) and LCs (B and D) were treated with wild-type (WT), ActA-deficient, or LLO-deficient *Lm* and then cultured with allogeneic (A and B) or autologous (C and D) T cells for five days in MLRs. DC:T ratios ranged from 1:10 to 1:1000. T cell proliferation was measured by a colorimetric assay (triplicate means ± SEM, n = 3 independent experiments). Dotted line represents unstimulated T cells. **P* < .05 and ***P* < .01, relative to cytokine-matured DC control.

T cell activation mediated *Lm*-treated DCs is accompanied by the upregulation of inhibitory receptors. ICOS is a T cell specific molecule that is expressed only after cellular activation and is required for optimal antitumor immune responses[15]. We assessed ICOS expression by T cells after autologous culture in mixed leukocyte reactions with DCs treated with LLO-deficient *Lm*. Both $CD4^+ICOS^+$ and $CD8^+ICOS^+$ T cells were increased to a greater extent after exposure to DCs treated with *Lm*, compared with cytokine-matured DCs, indicating more efficient T cell activation with *Lm* conditioning of DCs (**Figure 3A**).

Suppressor mechanisms operating under normal physiologic conditions to modulate immunity can also contribute to immune evasion by cancer cells. Immune checkpoint pathways dampen immune responses and can contribute to persistent and/or relapsed malignancy[16]. To assess this key regulatory axis in our system, we compared T cells after culture with *Lm*-treated DCs for expression of the inhibitory receptors LAG-3, PD-1, and TIM-3. $CD4^+$ and $CD8^+$ T cells had greater expression of each inhibitory receptor after stimulation by *Lm*-treated DCs (**Figure 3B**). T-bet and EOMES are two key transcription factors for $CD8^+$ T cell differentiation and function, and the co-expression of each with PD-1 is differentially linked to potential for revival, with $T\text{-bet}^+PD\text{-}1^+$ cells more responsive than $EOMES^+PD\text{-}1^+$ cells to checkpoint blockade[17]. As shown in **Figure 3C**, $T\text{-bet}^+PD\text{-}1^+$ cells represent the majority of *Lm* pathway activated $CD8^+$ T cells. Collectively, these findings provide rationale for the inclusion of checkpoint inhibition to revive the reservoir of $T\text{-bet}^+PD\text{-}1^+$ cells and further augment T cell responses in the setting of *Lm*-mediated immune stimulation.

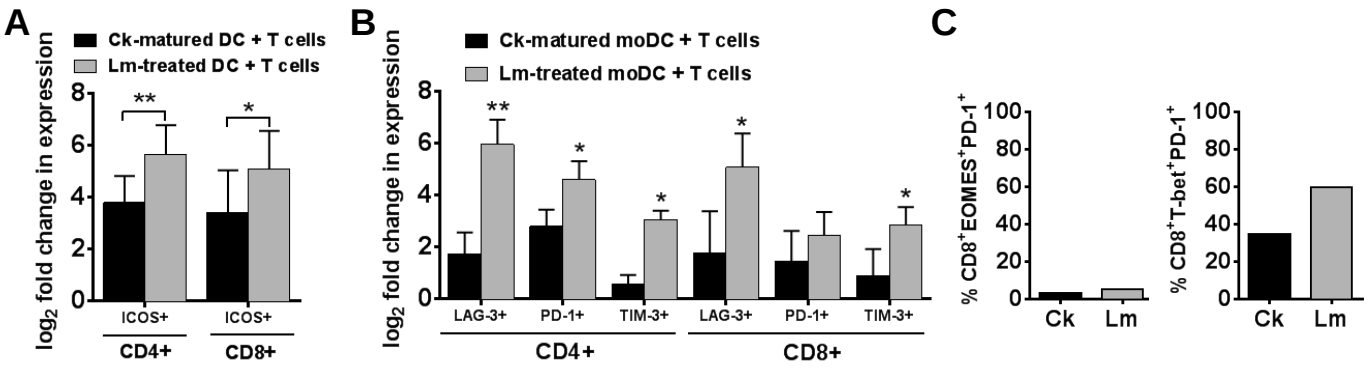

Figure 3: T cell activation by *Lm*-treated DCs is accompanied by the upregulation of inhibitory receptors. Immature moDCs treated with LLO-deficient *Lm* were co-cultured with autologous T cells in mixed leukocyte reactions. DC to T cell ratio was 1:10. After 6 days, T cells were harvested and analyzed by flow cytometry for expression of **(A)** ICOS, **(B)** inhibitory receptors (CTLA-4, PD-1, LAG-3, and TIM-3), and **(C)** EOMES and T-bet. Pooled data (mean ± SD) from at least 3 patients are shown. **P* < .05 and ***P* < .01, relative to T cells cultured with cytokine-matured DCs.

Lm-treatment of DCs does not potentiate regulatory T cell expansion or IDO expression.

The balance between regulatory T cells (Tregs) and activated effector $CD8^+$ T cells shapes antitumor immune responses and the efficacy of immune-based interventions[18]. As shown in **Figure 4A**, priming of resting bulk T cells with autologous, *Lm*-treated moDCs in the absence of exogenous cytokines results in the expansion of $CD4^+CD25^{bright}CD127^{neg}$ Tregs to levels comparable to cytokine-matured moDCs. Thus, *Lm*-infected moDCs support, but do not potentiate, the generation of Tregs by moDCs.

The immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) can impede immunity mediated by activated DCs[11,19]. We previously showed that human moDCs upregulate the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) with maturation and expand potent Tregs in an IDO-dependent manner[11]. As shown in **Figure 4B**, WT and ActA-deficient *Lm* induce IDO to a much greater extent than the LLO-deficient strain. This has potential bearing on the choice of attenuated *Lm* for vaccine production, as a minimally IDO-inducing strain should promote less inhibition of T cell proliferation by direct tryptophan depletion and by indirect mechanisms, like Treg generation and other related factors, which would be advantageous in the setting of active DC-based vaccination.

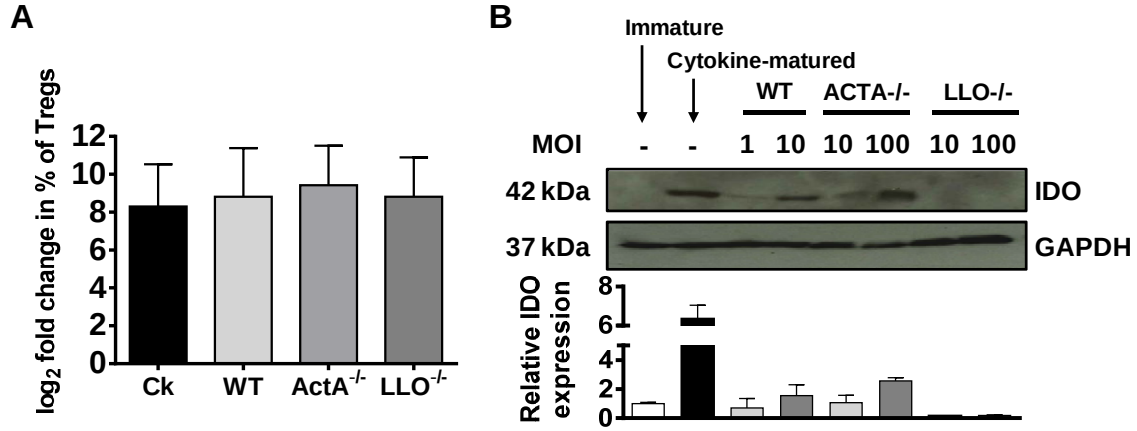

Figure 4: Regulatory T cell induction and indoleamine 2,3-dioxygenase (IDO) protein expression in moDCs after *Lm* treatment. **(A)** Immature moDCs treated with wild-type, ActA-deficient, or LLO-deficient *Lm* were co-cultured with autologous T cells in mixed leukocyte reactions. DC to T cell ratio was 1:10. After 6 days, T cells were harvested and the percentage of Tregs ($CD4^+CD25^{bright}CD127^{neg}$) was determined. Treg expansion is shown as fold change compared with Treg induction by immature moDCs. Data are representative of 5 experiments. **(B)** Immature moDCs treated with wild-type, ActA-deficient, or LLO-deficient *Lm* were assessed for induction of IDO by Western blot. Immature moDCs and cytokine-matured moDCs served as negative and positive controls, respectively. A representative blot from 1 experiment is shown along with pooled densitometry data from 3 separate experiments (mean ± SD) showing relative IDO expression between groups. Densitometry values for each group were normalized to GAPDH (internal control).

Lm-treatment of moDCs to induce cytotoxic T lymphocytes

LCs, by an IL15-dependent mechanism[20], are superior to moDCs and other conventional DC subsets at inducing antigen-specific cytotoxic T lymphocytes (CTLs) against viral and tumor Ags *in vitro*[2,13]. Almost all DC vaccine trials, however, use moDCs in large part because monocyte precursors are easier to obtain and culture *in vitro* than $CD34^+$-derived subsets, including LCs. The addition of exogenous IL15 to moDCs promotes immune responses comparable to LCs[20]. Based on our observation that *Lm*-infected moDCs induce augmented autologous T cell proliferation vs cytokine-matured moDCs (**Figure 2C**), we assessed whether *Lm* infection would promote stimulation of cytotoxic T lymphocytes (CTLs) to the same degree as supplemental IL15.

In a pilot experiment, after primary stimulation *in vitro* for only 7 days without exogenous IL15, Lm-infected moDCs demonstrated potent stimulation of CTLs against TRP2-expressing tumor cell lines (**Figure 5**), which was comparable to that achieved with the addition of IL15. Repeat studies, however, produced equivocal results, possibly due to donor variability and/or as yet unresolved technical issues of the assay. Currently, it appears that *Lm*-treated moDCs induce antigen-specific CTL activity, but not to the same degree as LCs alone or moDCs supplemented with IL15. Experiments to gain mechanistic insight(s) for this difference, including the effects of concurrent inhibition of IDO with 1MT (or siRNA), are ongoing.

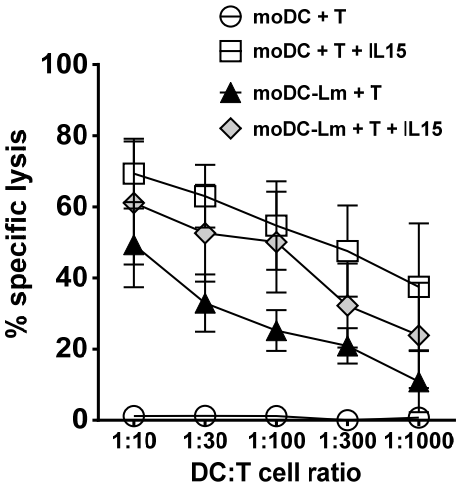

Figure 5: *Lm*-infected moDCs stimulate TRP-2-specific CTLs *in vitro* that kill melanoma cells. Immature moDCs were electroporated with TRP-2 mRNA, terminally matured with *Lm* or with a combination of inflammatory cytokines, and then added in serial doses to triplicate microwells containing 1×10^5 T cells and cultured without exogenous IL15 for 7 days. Antigen-specific target cell lysis by CTLs was evaluated using a colorimetric CTL assay. Target cells for were SK-MEL-37 cells. Specific lysis is plotted against the y-axis with respect to the conditions of primary stimulation shown along the x-axis. Data points are the averages ± SEM of triplicate means from one experiment.

Conclusion

Key findings of the project include: 1) *Lm*, including attenuated strains, induces human DC maturation and activation. 2) *Lm* induces less inhibitory receptor expression on mature DCs than standard inflammatory cytokine stimulation. 3) *Lm*-activated DCs are potent stimulators of allogeneic and autologous T cell proliferation. 4) *Lm*-conditioned DCs induce robust T cell activation that is associated with inhibitory receptor upregulation, providing rationale for the inclusion of checkpoint inhibition to augment T cell responses. 5) *Lm* treatment, despite vigorous T cell activation, does not potentiate DC-mediated expansion of immune-dampening regulatory T cells. 6) LLO-deficient *Lm* induces less IDO in DCs than WT and ActA-deficient strains. 7) *Lm*-treated moDCs, without exogenous cytokine supplementation, induce melanoma antigen-specific CTLs. Collectively, these findings confirm the immune-stimulatory properties of *Lm*, lend further support for *Lm* as a DC vaccine adjuvant to optimize vaccine efficacy, and identify immune checkpoint blockade as a rational complement to *Lm*-mediated immune activation.

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