AD_____

Award Number: W81XWH-11-1-0384

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

PRINCIPAL INVESTIGATOR: David J. Chung, M.D., Ph.D.

CONTRACTING ORGANIZATION: Sloan-Kettering Institute for Cancer Research
New York, NY 10065-6007

REPORT DATE: July 2012

TYPE OF REPORT: Annual

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
July 2012	Annual	15 June 2011 – 14 June 2012

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0384

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
David J. Chung, M.D., Ph.D.

E-Mail: chungd1@mskcc.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Sloan-Kettering Institute for Cancer Research

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 on human dendritic cells (DCs) to optimize Listeria-based DC cancer vaccines. The project aims are: 1) Compare the activation and maturation of different human 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. 3) Augment the immunogenicity of Listeria-activated DCs by inhibiting the immunosuppressive enzyme, indoleamine 2,3-dioxygenase. During the initial period of funding, critical parameters and baseline readouts of Listeria infection of monocyte-derived DCs (moDCs) were identified and validated. Key findings include: 1) Listeria infection, including that mediated by attenuated strains, induces moDC maturation and activation. 2) Listeria-treated moDCs are functionally active as potent stimulators of T-cell proliferation. 3) Listeria treatment, as compared with standard inflammatory cytokine stimulation, does not promote the over-expression of inhibitory markers on moDCs. 4) Listeria treatment, as compared with standard inflammatory cytokine stimulation, does not potentiate the expansion of immune-dampening regulatory T-cells by moDCs. These findings confirm the immune-stimulatory properties of Listeria as a vaccine adjuvant. Studies of other DC subtypes are underway to identify the optimal DC for further study in Aims 2/3.

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	12	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

This project will provide key insights into the immunobiology of Listeria-induced activation of different human dendritic cell (DC) subsets and will impact military beneficiaries by addressing the FY10 PRCRP topic area of **Listeria vaccine for cancer**. This project will cover 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*. In addition, this project will assess immune responses to a melanoma-specific antigen and will evaluate indoleamine 2,3-dioxygenase (IDO)-mediated suppression of T cell and NK cell responses by Listeria-activated DCs. The project therefore has overlapping relevance to the topic area of **Melanoma and other skin cancers**, including the focus area of *evaluation of a key immunosuppressive mechanism*. The findings of this project will help identify an optimal Listeria-activated DC subset for clinical vaccine application and will provide important proof-of-principle for further enhancing antitumor immunogenicity by inhibiting IDO. The project will build on our laboratory and clinical experiences with DC-based immunotherapy, including vaccination strategies for melanoma.

BODY

As described in the approved Statement of Work (SOW), the objectives of this project are: (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).

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

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

1a) Local IRB approval granted. The use of human cells for research in this project is covered by an institutional biospecimen procurement protocol (MSKCC IRB #09-141 – "Collection of Human Biological Specimens from Patients for Research Studies").

Result/status: Completed.

1b) Approval from DoD ORP (months 2-6)

Result/status: Completed.

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).*

2a) Thawing, growing, and preparing Listeria strains for infecting DCs (months 2-12)

Result/status: Two attenuated Listeria stains, one deficient in listeriolysin (LLO), which is essential for vacuolar lysis and entry into the cytosol[1,2], and the other deficient in actin-assembly-inducing protein (ActA), which is required for bacterial spread to adjacent cells[1,2], were tested for infecting DCs. The two attenuated strains (LLO-deficient and ActA-deficient) were compared with wild-type Listeria for their ability to activate moDCs, as measured by the upregulation of maturation markers and stimulation of allogeneic T cells (see below).

>> Wild-type, LLO-deficient, and ActA-deficient Listeria were grown and prepared for use in the experiments outlined below.

2b) Generation of human DCs (months 2-12).

Result/status: Immature monocyte-derived DCs (moDCs) were successfully generated per standard methodology and validation using peripheral blood from healthy volunteers[3]. Initial attempts to generate CD34+ hematopoietic progenitor cell (HPC)-derived dermal-interstitial DCs (DDC-IDCs) and CD34+ HPC-derived Langerhans cells (LCs) were complicated by low cell yields, which was ultimately attributed to an issue with one of the cytokines required for expansion and is now resolved.

2c) T cell isolation for allogeneic mixed leukocyte reactions (months 2-12)

Result/status: T cells used in this project are tissue culture plastic non-adherent lymphocytes, further purified by elution over nylon wool columns. This method avoids T cell activation and typically achieves >95% CD3+ T cell purity.

>> T cells were successfully isolated for use in the experiments outlined below.

2d) Listeria infection of DCs (months 2-12)

Result/status: Immature moDCs were incubated with Listeria in 24-well plates for 1 hour at 37°C. DCs were infected at several multiplicities of infection (MOI) to determine optimal dosing. Extracellular bacteria were removed by washing, and DCs were cultured for 36 hours. A separate cohort of DCs was matured with a standard combination of inflammatory cytokines (TNF-α, IL-1β, IL-6, and PGE2[3]) to serve as uninfected controls.

>> Optimal dosing for infection of moDCs with wild-type, LLO-deficient, and ActA-deficient Listeria completed. Infection of other DC subtypes is ongoing.

2e) Harvesting DCs after Listeria infection for analysis (months 3-12)

Result/status: Listeria-treated DCs were harvested for further analysis and/or use in other assays as described below.

2f) Flow cytometry analysis of DCs for co-stimulatory and maturation markers (months 3-12)

Result/status: Listeria-treated moDCs were compared with untreated controls for the upregulation of co-stimulatory and maturation markers (e.g., CD40, CD80, CD86, CD83)[4,5] by flow cytometry. The expression of CCR7, a chemokine receptor essential for trafficking of DCs to lymph nodes after vaccination[6], was also checked by flow cytometry. In addition, CD103 (integrin αE), which is expressed by a subset of DCs in response to Listeria infection[7], was assessed.

>> As shown in Figure 1 (Supporting data), infection of moDCs with wild-type, LLO-deficient, and ActA-deficient Listeria induces the activation and maturation of moDCs. When compared with uninfected controls, Listeria-infected moDCs upregulate CD40, CD80, CD86, CD83, and CCR7. The degree of upregulation was equivalent between the three types of Listeria (p = NS) and was comparable to that achieved with a standard combination of inflammatory cytokines (TNF-α, IL-1β, IL-6, and PGE2)[3] used to mature moDCs.

Of note, treatment of moDCs with Listeria induces the expression of CD103, which is not detected on moDCs in response to inflammatory cytokine stimulation. CD103 expression may represent functional specialization of moDCs for gut-associated lymphoid tissue in response to Listeria infection[8]. Experiments to further delineate the functional specialization of CD103+ DCs are in progress.

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

Result/status: Listeria-treated DCs will be 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).

>> Experiments are ongoing.

2h) Analysis of cyclic diadenosine monophosphate (c-di-AMP) levels by DCs (months 3-12)

Result/status: Cyclic diadenosine monophosphate (c-di-AMP)-mediated signaling is one of the mechanisms by which Listeria induces host immunity[9]. It was therefore proposed in the original SOW that differences in activation between Listeria-treated and untreated control DCs would be correlated with changes in c-di-AMP levels by high-resolution mass spectrometry.

>> These experiments have not been done, as no significant difference in activation was observed between treated and untreated cells.

2i) T cell proliferation assays/allogeneic mixed leukocyte reactions (months 3-12)

Result/status: DC function was assessed by comparing the ability of Listeria-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 2 (Supporting data), Listeria-infected moDCs are potent stimulators of T cell proliferation. Listeria-infected moDCs were consistently superior to inflammatory cytokine-matured moDCs at inducing T cell proliferation, thus underscoring the immunologic potential of Listeria as vaccine adjuvants. Importantly, both attenuated Listeria strains used in this task retained their immune-stimulatory efficacy.

2j) Flow cytometry analysis of DCs for inhibitory co-stimulatory markers (months 3-12)

Result/status: Activated DCs also co-express inhibitory factors that can blunt their immunostimulatory properties. Using flow cytometry, we assessed the expression of *inhibitory* molecules (e.g., PD-L1, PD-L2, B7-H3, B7-H4[10,11]) by moDCs after Listeria-induced activation.

>> As shown in Figure 3 (Supporting data), Listeria-infected moDCs do not upregulate PD-L1, PD-L2, B7-H3, or B7-H4 above levels induced by a standard combination of inflammatory cytokines (TNF-α, IL-1β, IL-6, and PGE2). These findings indicate that Listeria-mediated activation of DCs (vs standard cytokine-mediated activation) does not stimulate overly robust inhibitory molecule expression that could potentially blunt a vaccine-based immune response.

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[12,13]. Listeria-mediated upregulation of IDO in moDCs was shown in one study[14]. It is unknown whether Listeria has a similar effect on IDO in other DC subtypes. We will therefore compare the different DCs after Listeria infection for IDO expression.

>> A preliminary assessment of Listeria-infected moDCs (n=1) demonstrated equivocal upregulation of IDO protein based on Western blot analysis. Repeat experiments (Western blot for protein and PCR for mRNA) with moDCs and other DC subtypes are ongoing.

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

Result/status: Standard methods now exist to detect phosphorylated proteins by flow cytometry (Phos-Flow, BD Biosciences). This methodology can be used to detect differences in signaling pathways (ex: IRF3 vs MyD88/TRIF vs MAVS49) after Listeria infection that confer immunogenic vs tolerogenic properties on DCs.

>> Not yet performed. Awaiting completion of other experiments (see above).

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.

>> Note: Majority of this task is scheduled for completion during year two of funding.

3a) Thawing, growing, and preparing Listeria strains for infecting DCs (months 12-24)

Result/status: Completed (see Task 2a).

3b) Generation of human DCs (months 12-24): pending

3c) T cell isolation for autologous mixed leukocyte reactions (months 12-24): pending

3d) NK cell isolation for autologous mixed leukocyte reactions (months 12-24): pending

3e) Electroporation of tyrosinase-related protein-2 (TRP-2)-containing plasmid into Listeria (months 12-24): pending

3f) Listeria infection of DCs (months 12-24): pending

3g) Harvesting T cells and NK cells for analysis (months 12-24): pending

3h) Antigen-specific CTL response assessments, including intracellular cytokine secretion assay to detect IFN-γ secretion and standard ^{51}Cr release assay (months 12-24): pending

3i) Flow cytometry analysis of T cells subsets (e.g., naïve, central memory, effector memory, Th1, Th2, Th17, and Treg) (months 12-24)

Result/status: Treg analysis has been performed. We previously showed that human moDCs upregulate the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) with maturation and expand potent regulatory T cells (Tregs) in an IDO-dependent manner[12].

>> As shown in Figure 4 (Supporting data), Listeria-treated moDCs induce regulatory T cell (Treg) expansion to levels comparable to cytokine-matured moDCs. Priming of resting bulk T cells with autologous, Listeria-treated moDCs in the absence of exogenous cytokines results in the expansion of $CD4^{+}CD25^{bright}Foxp3^{+}CD127^{neg}$ Tregs. Thus, Listeria-infected moDCs support, but do not potentiate, the generation of Tregs by moDCs. The mechanism of Treg induction, including the potential role of IDO, will be further explored in Task 4.

3j) NK cell analyses for phenotypic activation, proliferation, and cytotoxicty (months 12-24): pending

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

>> Note: Majority of this task is scheduled for completion during year three of funding.

4a) Thawing, growing, and preparing Listeria strains for infecting DCs (months 16-36)

Result/status: Completed (see Task 2a).

4b) Generation of human DCs (months 16-36): pending

4c) T cell isolation for autologous mixed leukocyte reactions (months 16-36): pending

4d) NK cell isolation for autologous mixed leukocyte reactions (months 16-36): pending

4e) Electroporation of tyrosinase-related protein-2 (TRP-2)-containing plasmid into Listeria (months 16-36): pending

4f) Preparation of IDO inhibitor, 1-methyl-L-tryptophan (1-MT) (months 16-36): pending

4g) Listeria infection of DCs (months 16-36): pending

4h) Harvesting T cells and NK cells for analysis (months 16-36): pending

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): pending

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

4k) NK cell analyses for phenotypic activation, proliferation, and cytotoxicty (months 16-36): pending

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 1-3 (months 1-36)

Result/status: Ongoing.

5b) Analyze data for each set of experiments outlined in Tasks 1-3 (months 1-36)

Result/status: Ongoing.

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

KEY RESEARCH ACCOMPLISHMENTS

- Listeria infection, including that mediated by attenuated strains, induces moDC maturation and activation.
- Listeria-treated moDCs are functionally active as potent stimulators of T cell proliferation.
- Listeria treatment, as compared with standard inflammatory cytokine stimulation, does ***not*** promote the over-expression of inhibitory markers on moDCs.
- Listeria treatment, as compared with standard inflammatory cytokine stimulation, does ***not*** potentiate the expansion of immune-dampening regulatory T cells by moDCs.

REPORTABLE OUTCOMES

None to date. It is anticipated that additional data generated from current ongoing experiments will result in manuscripts, abstracts, and presentations in the near future.

CONCLUSION

During the initial period of funding, critical parameters and baseline readouts of Listeria infection of moDCs were identified and validated. Key findings include: 1) Listeria infection, including that mediated by attenuated strains, induces moDC maturation and activation. 2) Listeria-treated moDCs are functionally active as potent stimulators of T cell proliferation. 3) Listeria treatment, as compared with standard inflammatory cytokine stimulation, does ***not*** promote the over-expression of inhibitory markers on moDCs. 4) Listeria treatment, as compared with standard inflammatory cytokine stimulation, does ***not*** potentiate the expansion of immune-dampening regulatory T cells by moDCs. These findings confirm the immune-stimulatory properties of Listeria and lend further support for Listeria as a DC vaccine adjuvant. Additional studies of proinflammatory cytokine secretion, indoleamine 2,3-dioxygenase expression/activity, and signaling pathways are ongoing. Examination of the other DC subtypes (e.g., DDC-IDCs, LCs, pDCs) is ongoing and is anticipated to proceed quickly to complete Task 2. These experiments will define the degree of activation and maturation of the different DC subtypes after Listeria infection and will assist in the selection of the optimal DC (i.e., fully activated, mature, and functionally potent) for further study in Tasks 3 and 4. If significant differences in Listeria-induced activation are not observed between the DC subsets, we will focus our subsequent studies on LCs, which have demonstrated superiority over other known conventional or myeloid human DC subtypes for inducing antigen-specific cytotoxic T lymphocytes (CTLs)[3]. Collectively, the studies in this project will provide important insights into the development and optimization of human DC-based cancer vaccines.

REFERENCES

1. Pamer EG. Immune responses to Listeria monocytogenes. Nat Rev Immunol. 2004;4:812-823.
2. Portnoy DA, Chakraborty T, Goebel W, Cossart P. Molecular determinants of Listeria monocytogenes pathogenesis. Infect Immun. 1992;60:1263-1267.
3. Ratzinger G, Baggers J, de Cos MA, et al. Mature human Langerhans cells derived from CD34+ hematopoietic progenitors stimulate greater cytolytic T lymphocyte activity in the absence of bioactive IL-12p70, by either single peptide presentation or cross-priming, than do dermal-interstitial or monocyte-derived dendritic cells J Immunol. 2004;173:2780-2791(Erratum in J Immunol. 2005; 2174:3818).
4. Rossi M, Young JW. Human dendritic cells: potent antigen-presenting cells at the crossroads of innate and adaptive immunity. J Immunol. 2005;175:1373-1381.
5. Reis e Sousa C. Dendritic cells in a mature age. Nat Rev Immunol. 2006;6:476-483.
6. Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of immunity. Immunol Rev. 2003;195:58-71.
7. Edelson BT, Bradstreet TR, Hildner K, et al. CD8alpha(+) dendritic cells are an obligate cellular entry point for productive infection by Listeria monocytogenes. Immunity. 2011;35:236-248.
8. del Rio ML, Bernhardt G, Rodriguez-Barbosa JI, Forster R. Development and functional specialization of CD103+ dendritic cells. Immunol Rev. 2010;234:268-281.
9. Woodward JJ, Iavarone AT, Portnoy DA. c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science. 2010;328:1703-1705.
10. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. Nat Rev Immunol. 2002;2:116-126.
11. Yi KH, Chen L. Fine tuning the immune response through B7-H3 and B7-H4. Immunol Rev. 2009;229:145-151.
12. Chung DJ, Rossi M, Romano E, et al. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. Blood. 2009;114:555-563.
13. Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest. 2007;117:1147-1154.
14. Popov A, Abdullah Z, Wickenhauser C, et al. Indoleamine 2,3-dioxygenase-expressing dendritic cells form suppurative granulomas following Listeria monocytogenes infection. J Clin Invest. 2006;116:3160-3170.

Supporting Data

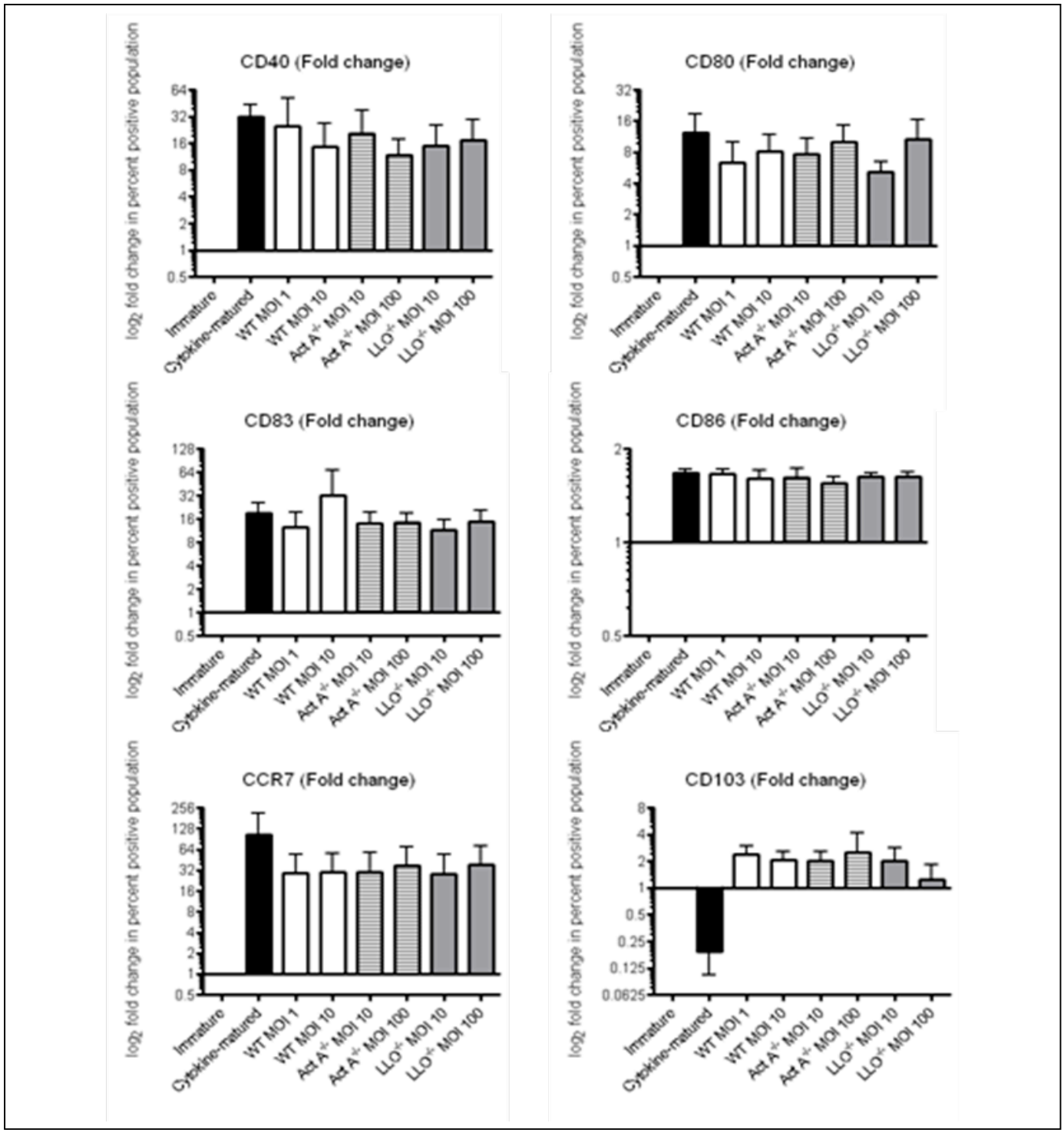

Figure 1: Listeria treatment induces moDC maturation and activation. Immature moDCs treated with wild-type, ActA-deficient, or LLO-deficient Listeria were assessed by flow cytometry for expression of CD40, CD80, CD83, CD86, and CCR7. Controls were untreated cytokine-matured moDCs. There was no significant difference between treated and untreated groups, as shown by fold change compared with immature moDCs. In contrast, CD103 (integrin αE) expression, which can be induced by Listeria, was increased after infection. Data are representative of 3 experiments.

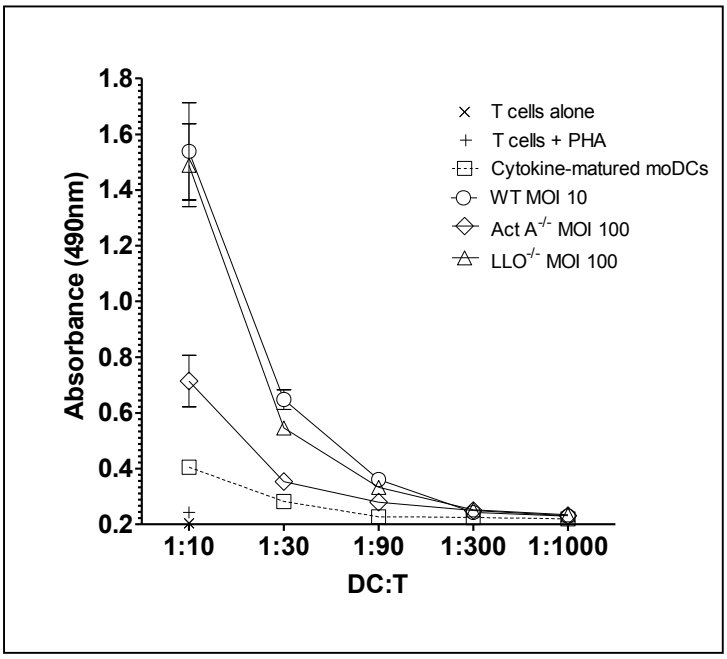

Figure 2: Listeria-treated moDCs are potent stimulators of T cell proliferation. Immature moDCs treated with wild-type, ActA-deficient, or LLO-deficient Listeria were co-cultured with allogeneic T cells in mixed leukocyte reactions. DC to T cell ratio ranged from 1:10 to 1:1000. After 4-5 days in culture, responder T cell proliferation was measured by a colorimetric proliferation assay and compared with controls containing cytokine-matured moDCs. Additional controls were T cells alone and T cells treated with PHA. Data are representative of 3 experiments.

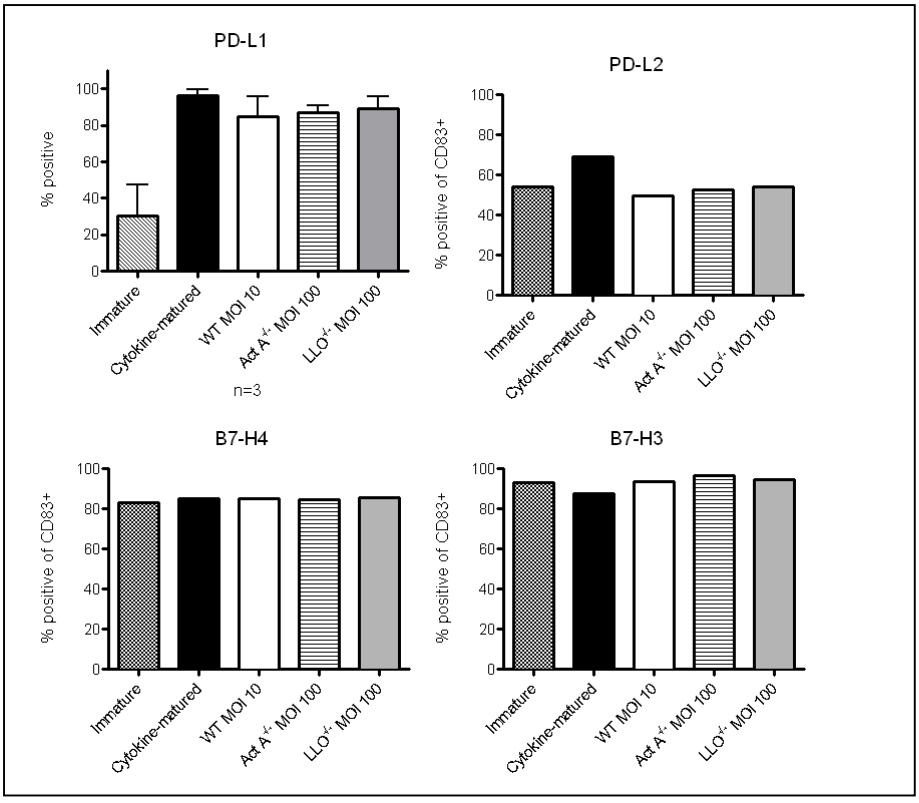

Figure 3: Listeria-treatment induces expression of inhibitory markers on moDCs to levels comparable to cytokine-matured moDCs. Immature moDCs treated with wild-type, ActA-deficient, or LLO-deficient Listeria were assessed by flow cytometry for expression of PD-L1, PD-L2, B7-H3, and B7-H4. Controls were untreated cytokine-matured moDCs and untreated immature moDCs. There was no significant difference in inhibitory marker upregulation between Listeria-treated and cytokine-matured groups.

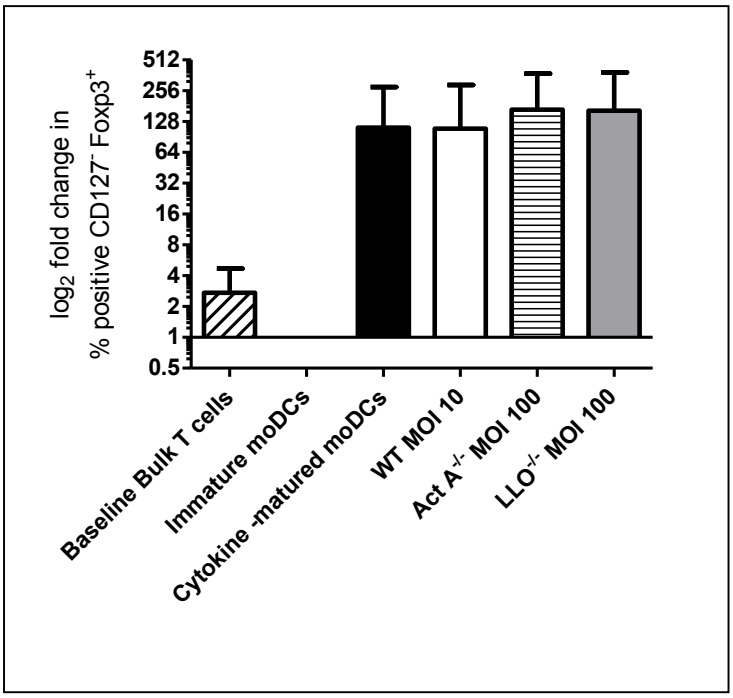

Figure 4: Listeria-treated moDCs induce regulatory T cell (Treg) expansion to levels comparable to cytokine-matured moDCs. Immature moDCs treated with wild-type, ActA-deficient, or LLO-deficient Listeria were co-cultured with autologous T cells in mixed leukocyte reactions. DC to T cell ratio was 1:10. After 6 days, T cells were harvested and the percentage of Tregs ($CD4^{+}CD25^{bright}Foxp3^{+}CD127^{neg}$) was determined. Extent of Treg expansion is shown as fold change compared with immature moDCs. Data are representative of 5 experiments.