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PRINCIPAL INVESTIGATOR: David J. Chung, MD, PhD

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

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> 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 treatment induces DC maturation and activation. 2) Listeria-treated DCs are functionally active, potent stimulators of T-cell proliferation. 3) Listeria treatment does not promote the over-expression of inhibitory markers on DCs. 4) Listeria treatment does not potentiate the expansion of immune-dampening regulatory T-cells by DCs. 5) Listeria-treated moDCs, without exogenous cytokine supplementation, are potent stimulators of antigen-specific CTLs. These findings confirm the immune-stimulatory properties of Listeria as a vaccine adjuvant. Studies of the mechanisms of Listeria-induced immunity and optimization of Listeria-based DC vaccines are ongoing.					
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## 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<sup>+</sup>/CD8<sup>+</sup> 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 and subsections of Tasks 2 and 3 were completed and/or addressed in the annual report covering the first year of the funding period from 06/15/2011 to 06/14/2012. By necessity of the experimental design and the timing of experiments, there are some areas of overlap between the different tasks. With this in mind, the research accomplishments during the second year of the funding period are as follows:**

**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 strains, one deficient in listeriolysin (LLO), which is essential for vacuolar lysis and entry into the cytosol<sup>1,2</sup>, and the other deficient in actin-assembly-inducing protein (ActA), which is required for bacterial spread to adjacent cells<sup>1,2</sup>, 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:** Experiments with monocyte-derived DCs (moDCs) were completed in year one. In year two, immature CD34<sup>+</sup> hematopoietic progenitor cell (HPC)-derived dermal-interstitial DCs (DDC-IDCs) and CD34<sup>+</sup> HPC-derived Langerhans cells (LCs) were successfully generated per standard methodology and validation using G-CSF-elicited CD34<sup>+</sup> HPCs obtained from healthy allogeneic donors already undergoing collection for transplantation<sup>3</sup>.

2c) T cell isolation for autologous mixed leukocyte reactions (months 12-24): pending

**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<sup>+</sup> 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:** Optimal dosing for infection of moDCs with wild-type, LLO-deficient, and ActA-deficient Listeria completed in year one. In year two, optimal dosing for infection of DDC-IDCs and LCs with the three Listeria strains was performed. Immature DDC-IDCs and LCs 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- $\alpha$ , IL-1 $\beta$ , IL-6, and PGE2<sup>3</sup>) to serve as uninfected controls.

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

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

Similar to our data for moDCs, infection of DDC-IDCs and LCs with wild-type, LLO-deficient, and ActA-deficient *Listeria* induces the activation and maturation of DDC-IDCs (**Figure 1**) and LCs (**Figure 2**). When compared with uninfected controls, *Listeria*-infected DDCs and LCs upregulate CD40, CD80, CD86, CD83, and CCR7. The degree of upregulation was equivalent between the three types of *Listeria* ( $p = \text{NS}$ ) and was comparable to that achieved with a standard combination of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and PGE2)<sup>3</sup> used to mature the DCs. Also, *Listeria* induces the expression of CD103. CD103 expression may represent functional specialization of DCs for gut-associated lymphoid tissue in response to *Listeria* infection<sup>8</sup>. Experiments to further delineate the functional specialization of CD103<sup>+</sup> DCs are ongoing.

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-1 $\beta$ , TNF- $\alpha$ , and/or IL-12p70) by intracellular cytokine secretion assay (Miltenyi).

Preliminary results show no significant difference in the levels of cytokine secretion between *Listeria*-treated DCs and untreated controls. Additional confirmatory experiments are ongoing.

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).

*Listeria*-infected DDC-IDCs and LCs are potent stimulators of *allogeneic* T cell proliferation (**Figure 3**). *Listeria*-infected DDC-IDCs and LCs without proinflammatory cytokine supplementation were equivalent to inflammatory cytokine-matured moDCs at inducing T cell proliferation, underscoring the immunologic potential of *Listeria* as vaccine adjuvants. Importantly, both attenuated *Listeria* strains used in this task retained their immune-stimulatory efficacy.

Having shown that *Listeria*-infected DCs retain their allo-stimulatory capacity, we next examined their ability to induce *autologous* T cell proliferation, as would be encountered in the setting of therapeutic cancer vaccination of patients. As shown in **Figure 4**, the auto-stimulatory capacity of moDCs is augmented by *Listeria* infection, a finding that has important implications for the optimization of DC-based immunotherapy.

Almost all previous DC vaccine trials have used monocyte-derived DCs (moDCs) in large part because monocyte precursors are easier to obtain and culture *in vitro* than CD34<sup>+</sup>-derived subsets, including LCs. LCs, however, are superior to moDCs and other conventional DC subsets at inducing Ag-specific CTLs against viral and tumor Ags *in vitro*<sup>3,9</sup>. When compared with moDCs, LCs secrete more IL15<sup>3,9,10</sup>, which in turn reduces IL2-induced T cell apoptosis and decreases regulatory T cell expansion during LC-mediated CTL generation<sup>11</sup>. LCs can break tolerance against WT1 by an IL15 $\alpha$ /IL15/pSTAT5-dependent mechanism<sup>11</sup>. Clinical trial data have shown greater efficacy of DC vaccines that contain LCs<sup>12</sup>, as well as greater tetramer reactivity stimulated by LCs when compared with moDCs<sup>13</sup>.

Our group has since discovered that LCs electroporated with Wilms' tumor 1 (WT1) mRNA, promote sustained presentation of antigenic peptides, which in conjunction with IL15R- $\alpha$ /IL15, induce robust autologous, WT1-specific CTLs<sup>11</sup>. The CTLs develop after just 7 days' stimulation without exogenous cytokine supplementation and lyse MHC-restricted targets, including primary WT1-expressing blasts from leukemia patients. MoDCs, in contrast, require exogenous IL15 to promote immune responses comparable to LCs. Interestingly, our data indicate that Listeria infection of moDC, similar to IL15, may confer augmented immune stimulatory capacity to moDCs, which if confirmed, would offer an alternative method for enhancing vaccine 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<sup>14,15</sup>) by DCs after Listeria-induced activation.

Similar to our results for moDCs, Listeria-infected DDC-IDCs and LCs do not upregulate PD-L1, PD-L2, B7-H3, or B7-H4 above levels induced by a standard combination of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , 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<sup>16,17</sup>. Listeria-mediated upregulation of IDO in moDCs was shown in one study<sup>18</sup>. 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.

We previously reported that Listeria-infected moDCs demonstrated equivocal upregulation of IDO protein based on Western blot analysis. Repeat experiments (Western blot for protein and PCR for mRNA) with moDCs, DDCs, and LCs 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 (Task 3).

**Task 3:** Define the induction of CD4<sup>+</sup>/CD8<sup>+</sup> T cell and NK cell responses to Listeria-activated DCs presenting a melanoma tumor-associated antigen (TAA).

*Anticipated timeframe: months 12-24.*

**For this task, we will focus on moDCs, which exhibited the most functional activation after Listeria infection in our allogeneic and autologous T cell proliferation experiments in Task 2.**

- 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)

**Result/status:** Completed (see Task 2b).

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

**Result/status:** Completed (see Task 2c).

- 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)

**Result/status:** Electroporation of DCs with mRNA encoding specific tumor-associated antigens is an effective non-viral method to stimulate T cell responses *in vitro* and *in vivo*<sup>19-26</sup>. This method of antigen loading, which facilitates processing and presentation of multiple class I and II MHC-restricted epitopes from the translated protein<sup>13</sup>, is more efficient than peptide pulsing and less problematic than retroviral transgenes, which carry the risk of genome integration<sup>27</sup>. mRNA electroporation also allows

individuals of any HLA type to process and present peptides tailored to their own MHC molecules. Electroporation of DCs with mRNA performed as previously described<sup>11,28</sup>.

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

**Result/status:** Completed (see Task 2d).

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

**Result/status:** T cells used in CTL assays harvested. NK cell experiments are pending.

- 3h) Antigen-specific CTL response assessments, including intracellular cytokine secretion assay to detect IFN- $\gamma$  secretion and standard <sup>51</sup>Cr release assay (months 12-24).

**Result/status:** LCs are the most potent conventional DC subtype for stimulating CD8<sup>+</sup> CTLs *in vitro*. The potency of LCs is based on an IL15R- $\alpha$ /IL15/pSTAT5-dependent mechanism<sup>11</sup>. MoDCs, in contrast, require exogenous IL15 to promote immune responses comparable to LCs<sup>11</sup>. Based on our observation that Listeria-infected moDCs induce augmented autologous T cell proliferation vs cytokine-matured moDCs, we assessed whether Listeria infection would promote stimulation of CTLs to the same degree as supplemental IL15.

After primary stimulation *in vitro* for only 7 days without exogenous IL15, Listeria-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. The mechanism for enhanced CTL generation mediated by Listeria is under evaluation.

- 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<sup>16</sup>.

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<sup>+</sup>CD25<sup>bright</sup>Foxp3<sup>+</sup>CD127<sup>neg</sup> 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 cytotoxicity (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- $\gamma$  secretion and standard <sup>51</sup>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 cytotoxicity (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, DDC, and LC maturation and activation.
- Listeria-treated DCs are functionally active, potent stimulators of allogeneic T cell proliferation.
- Listeria-treated moDCs are potent stimulators of autologous T cell proliferation.
- Listeria treatment, as compared with standard inflammatory cytokine stimulation, does **not** promote the over-expression of inhibitory markers on DCs.
- Listeria treatment, as compared with standard inflammatory cytokine stimulation, does **not** potentiate the expansion of immune-dampening regulatory T cells by DCs.
- Listeria-treated moDCs, without exogenous cytokine supplementation, are potent stimulators of antigen-specific CTLs.

## 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.

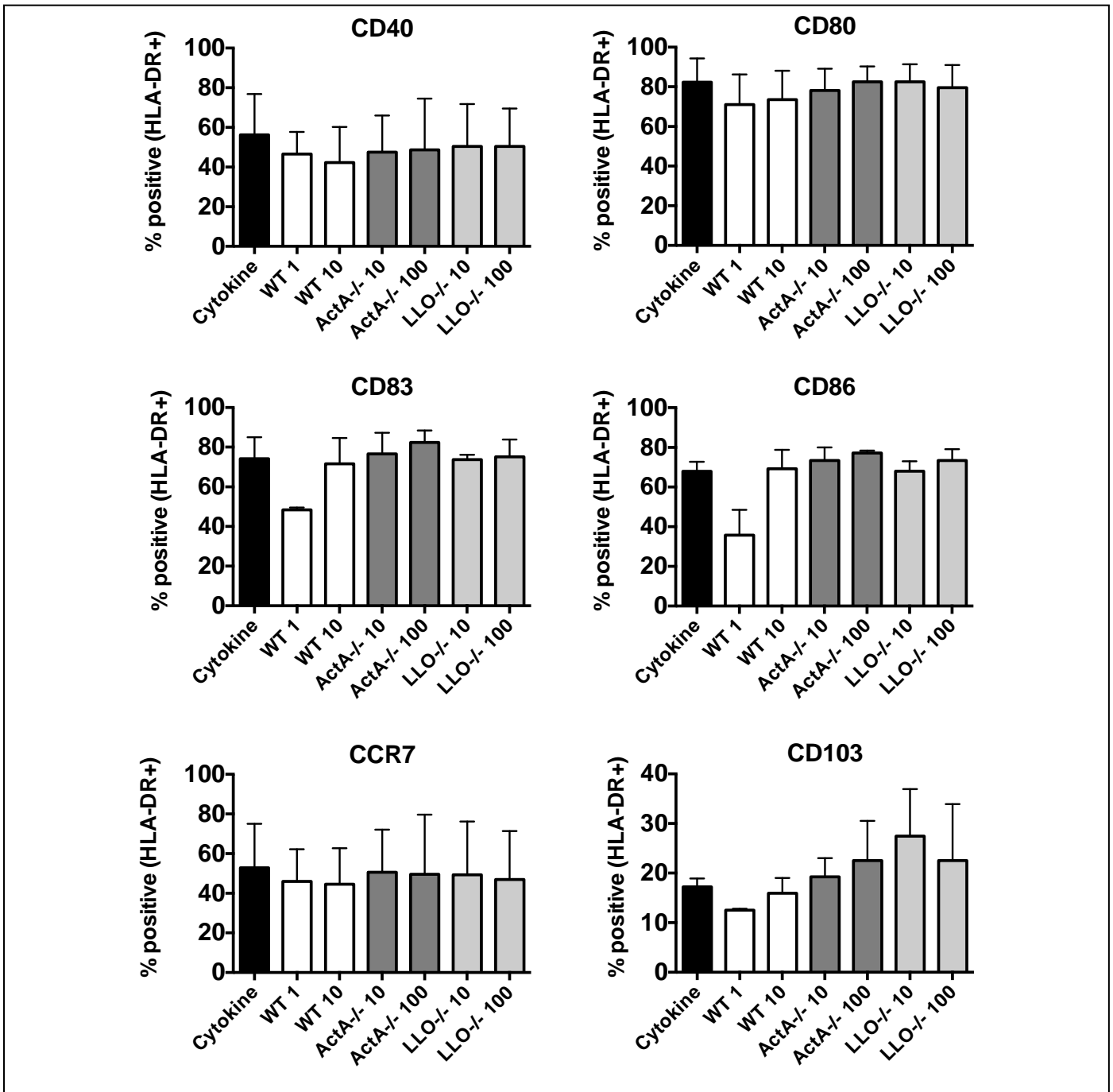
## CONCLUSION

Since the previous annual report, critical parameters and baseline readouts of Listeria infection of DDCs and LCs were identified and validated. Key findings from years 1-2 include: 1) Listeria infection, including that mediated by attenuated strains, induces moDC, DDC, and LC maturation and activation. 2) Listeria-treated DCs are functionally active, potent stimulators of T cell proliferation. 3) Listeria-treated moDCs are potent stimulators of autologous T cell proliferation. 4) Listeria treatment, as compared with standard inflammatory cytokine stimulation, does **not** promote the over-expression of inhibitory markers on DCs. 5) Listeria treatment, as compared with standard inflammatory cytokine stimulation, does **not** potentiate the expansion of immune-dampening regulatory T cells by moDCs. 6) Listeria-treated moDCs, without exogenous cytokine supplementation, are potent stimulators of antigen-specific CTLs. These findings confirm the immune-stimulatory properties of Listeria and lend further support for Listeria as a DC vaccine adjuvant. Experiments for the upcoming funding period will further explore the role of Listeria in augmenting the immunity of moDCs, which are the most commonly used DC in clinical trials, as a means to optimize DC-based cancer vaccines.

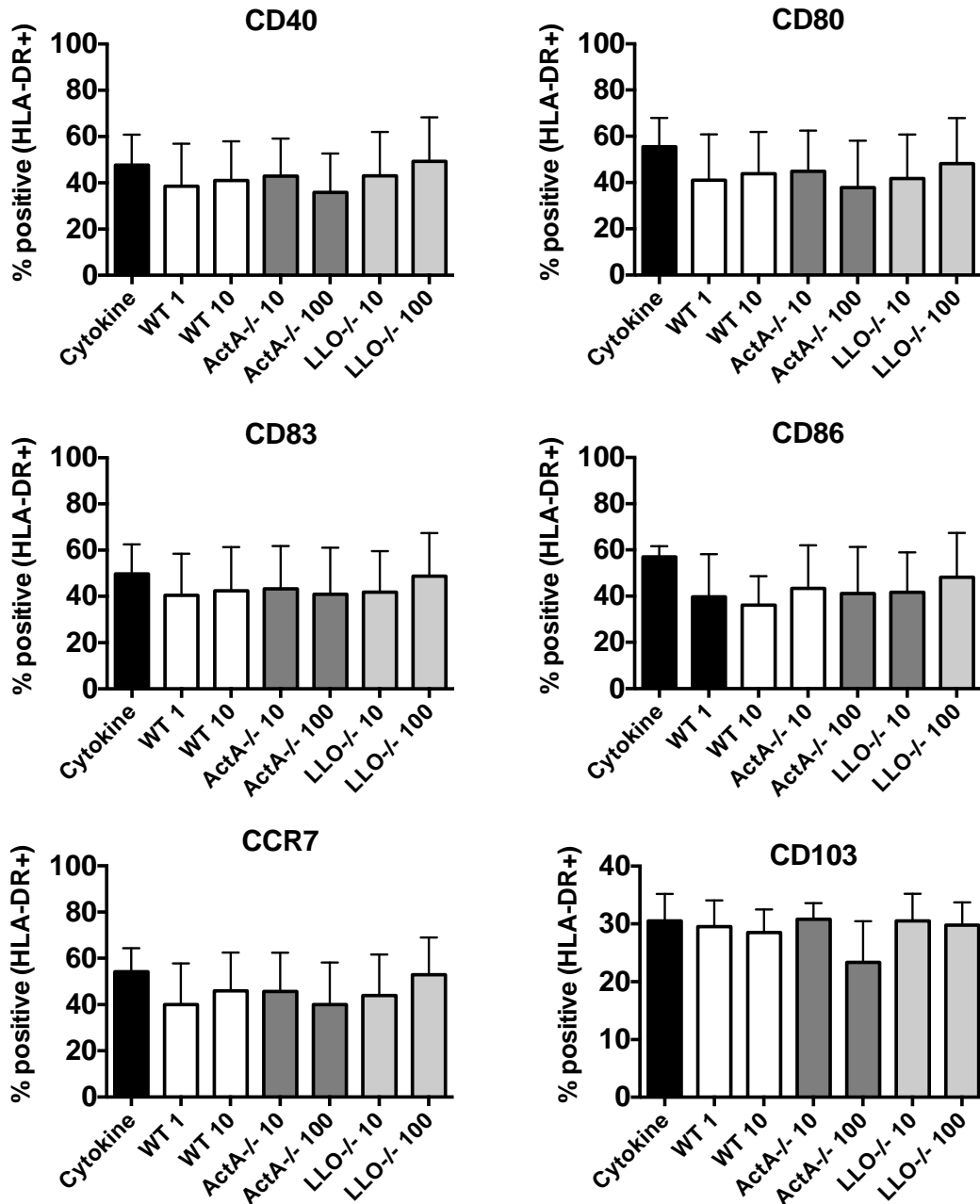
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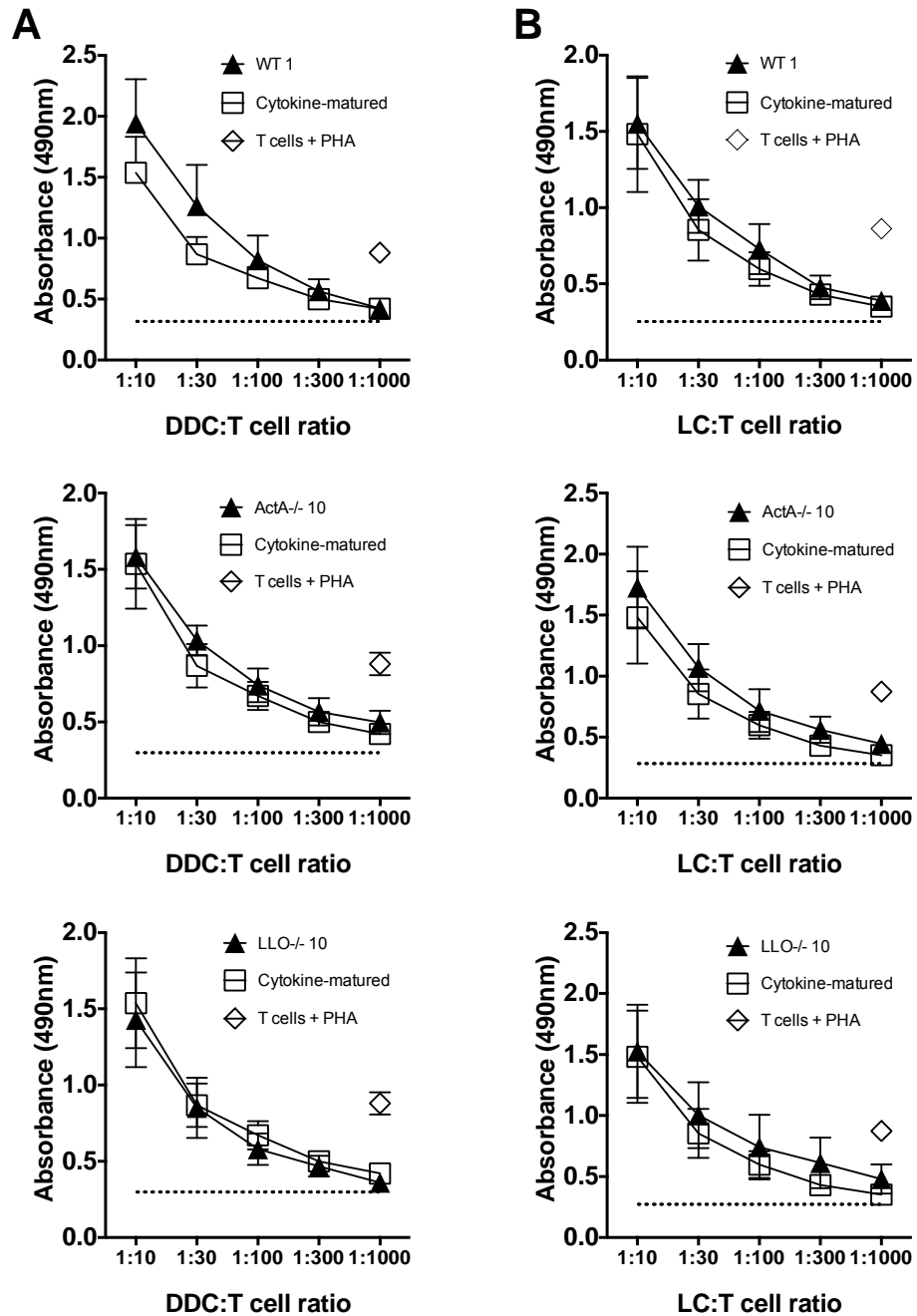
Supporting Data



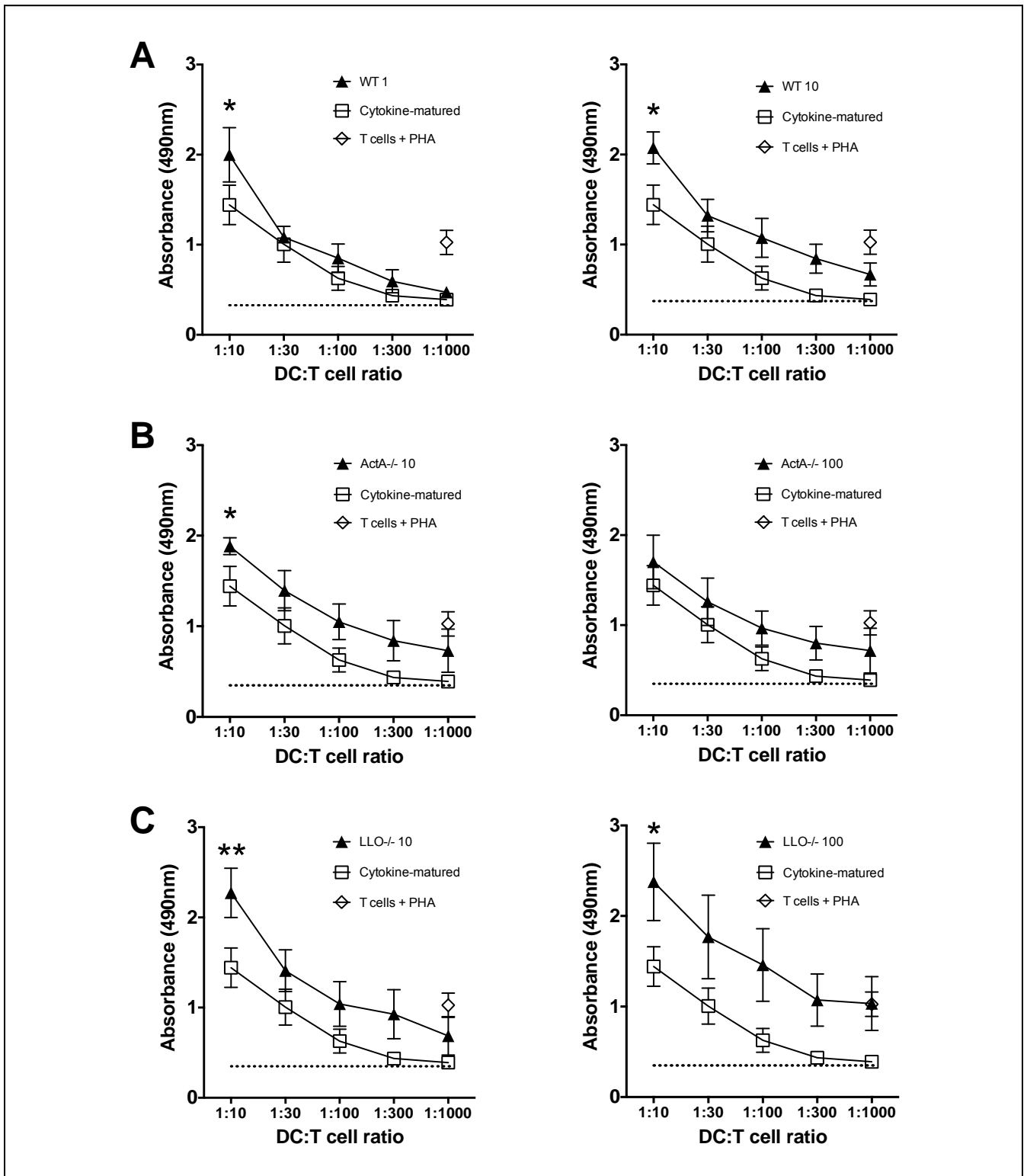
**Figure 1: Listeria treatment induces DDC maturation and activation.** Immature DDCs treated with wild-type, ActA-deficient, or LLO-deficient Listeria were assessed by flow cytometry for expression of CD40, CD80, CD83, CD86, and CCR7. Controls were untreated cytokine-matured DDCs. There was no significant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



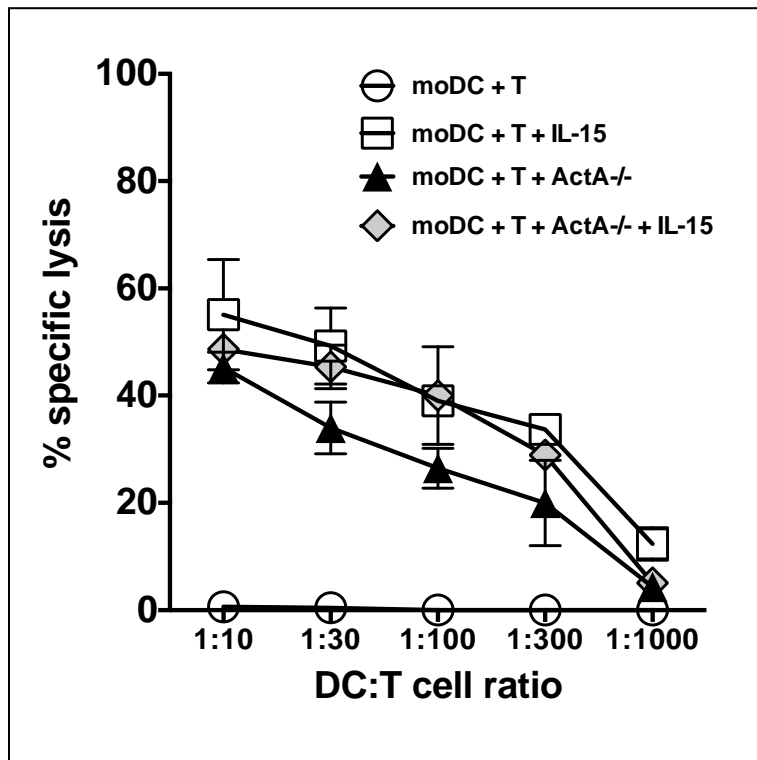
**Figure 2: Listeria treatment induces LC maturation and activation.** Immature LCs treated with wild-type, ActA-deficient, or LLO-deficient Listeria were assessed by flow cytometry for expression of CD40, CD80, CD83, CD86, and CCR7. Controls were untreated cytokine-matured LCs. There was no significant difference between treated and untreated groups, as shown by fold change compared with immature LCs. In contrast, CD103 (integrin  $\alpha$ E) expression, which can be induced by Listeria, was increased after infection. Data are representative of 3 experiments.



**Figure 3: Listeria-treated DDCs and LCs are potent inducers of allogeneic T cell proliferation.** Immature DDCs (A, left panels) and LCs (B, right panels) were treated with wild-type (WT), ActA-deficient, or LLO-deficient Listeria and then cultured with allogeneic T cells for five days in allogeneic MLRs. DC:T ratios ranged from 1:10 to 1:1000. T cell proliferation was measured by a colorimetric assay (triplicate means  $\pm$  SEM, n = 3 independent experiments). Dotted line represents T cells alone.



**Figure 4: Listeria-treated moDCs are potent stimulators of autologous T cell proliferation.** Immature moDCs were treated with two concentrations of wild-type (A), ActA-deficient (B), or LLO-deficient (C) Listeria and then cultured with autologous T cells for seven days in autologous MLRs. DC:T ratios ranged from 1:10 to 1:1000. T cell proliferation was measured by a colorimetric assay (triplicate means  $\pm$  SEM,  $n = 3$  independent experiments). Dotted line represents T cells alone. \* $P < .05$  and \*\* $P < .01$ , relative to cytokine-matured moDC control.



**Figure 5: Listeria-infected moDCs stimulate TRP2-specific CTLs *in vitro* that kill melanoma cells.**

Immature moDCs were electroporated with TRP2 mRNA and then infected with attenuated ActA Listeria (▲ and ◇) or terminally matured by a combination of inflammatory cytokines (O and □). Cells were then added in serial doses to triplicate microwells containing  $1 \times 10^5$  autologous T cells and cultured with (□ and ◇) or without (O and ▲) exogenous IL15 (10ng/ml) for 7 days. Antigen-specific target cell lysis by CTLs stimulated by the moDCs under the different conditions was evaluated using a flow cytometry-based assay. Target cells were SK-MEL-37 cells (TRP2<sup>+</sup> melanoma cell line). 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  $\pm$  SEM of triplicate means from one experiment.