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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⁺/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 and the majority of Tasks 2 and 3 were completed and/or addressed in the annual reports covering the first two years of the funding period, from 06/15/2011 to 06/14/2012 and from 06/15/2012 to 06/14/2013, respectively. 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 <u>third</u> 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).

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

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)[1, 2] by flow cytometry. The expression of CCR7, a chemokine receptor essential for trafficking of DCs to lymph nodes after vaccination[3], was also checked by flow cytometry. In addition, CD103 (integrin α E), which is expressed by a subset of DCs in response to Listeria infection[4], was assessed.

Similar to our data for moDCs, infection of DDC-IDCs and LCs with wild-type, LLO-deficient, and ActAdeficient Listeria induces the activation and maturation of DDC-IDCs (Figure 1 in annual report for year two) and LCs (Figure 2 in annual report for year two). 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 = NS) and was comparable to that achieved with a standard combination of inflammatory cytokines (TNF- α , IL-1 β , IL-6, and PGE2)[5] 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[6].

In mice, CD103⁺ DCs promote the conversion of naïve T cells into Tregs to dampen intestinal inflammation[7, 8]. We sorted DCs based on CD103 expression into CD103⁺ and CD103^{neg} fractions and compared the two populations for their ability to generate Tregs. Both populations induced Tregs above baseline, although we did not observe greater induction by CD103⁺ DCs (**Figure 1**).

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-1 β , TNF- α , and/or IL-12p70) by intracellular cytokine secretion assay (Miltenyi). Preliminary results show no significant differences in the levels of cytokine secretion

between Listeria-treated DCs and untreated controls. Additional confirmatory experiments are ongoing.

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[9, 10]. Listeria-mediated upregulation of IDO in moDCs was shown in one study[11]. It is unknown whether Listeria has a similar effect on IDO in other DC subtypes.

We previously reported that Listeria-infected moDCs demonstrated equivocal upregulation of IDO protein based on Western blot analysis. Repeat experiments demonstrate that WT and ActA-deficient Listeria induce IDO to a much greater extent than the LLO-deficient strain (**Figure 2**). 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 are planned to more fully evaluate this.

21) 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.

Listeria act by stimulating toll-like receptor 2 on DCs. This will be further evaluated after completion of other experiments (Task 4).

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.

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

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

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

Result/status: We have prioritized T cell studies, as their interactions with DCs are the more likely predominant response in the setting of active DC vaccination. In addition, NK cell yields and viability have been suboptimal to set-up experiments with reliable readouts. We have therefore postponed NK cell experiments but will revisit the feasibility of NK cell studies in the future if time/funds allow.

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

Result/status: T cells used in CTL assays harvested. NK cell experiments on hold (see Task 3d).

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, and the results were reported in the annual report for year two. Analysis of the other T cell subsets did not reveal any statistically significant differences between bulk T cells stimulated by Listeria-infected vs untreated DCs.

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

Result/status: See Task 3d.

- Task 4: Augment the immunogenicity of Listeria-activated DCs by inhibiting the immunosuppressive enzyme, indoleamine 2,3-dioxygenase (IDO). *Anticipated timeframe: months 16-36.*
 - 4a) Thawing, growing, and preparing Listeria strains for infecting DCs (months 16-36)

Result/status: Two attenuated Listeria stains, one deficient in listeriolysin (LLO), which is essential for vacuolar lysis and entry into the cytosol[12, 13], and the other deficient in actin-assembly-inducing protein (ActA), which is required for bacterial spread to adjacent cells[12, 13], 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.

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

Result/status: Experiments with monocyte-derived DCs (moDCs) were completed in year one. In year two, immature CD34⁺ hematopoietic progenitor cell (HPC)-derived dermal-interstitial DCs (DDC-IDCs) and CD34⁺ HPC-derived Langerhans cells (LCs) were successfully generated per standard methodology and validation using G-CSF-elicited CD34⁺ HPCs obtained from healthy allogeneic donors already undergoing collection for transplantation[5].

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

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.

NK cell experiments on hold (see Task 3d).

- 4d) NK cell isolation for autologous mixed leukocyte reactions (months 16-36): See Task 3d.
- 4e) Electroporation of tyrosinase-related protein-2 (TRP-2)-containing plasmid into Listeria (months 16-36):

Result/status: Electroporation of DCs with mRNA encoding TRP-2 was initially performed as previously described[14]. Due to suboptimal transfection efficiency, however, electroporation conditions were subsequently optimized, and results were published[15].

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

Result/status: A 40mM stock solution of 1-methyl-L-tryptophan (L-1MT; Sigma-Aldrich) was prepared for use in Task 4i.

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

Result/status: Optimal dosing for infection of DCs with wild-type, LLO-deficient, and ActA-deficient Listeria completed in years one and two. For Task 4, immature DCs 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-1 β , IL-6, and PGE2[5]) to serve as uninfected controls.

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

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

Result/status: T cells were successfully harvested for use in the experiments outlined below. NK cell experiments on hold (see Task 3d).

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

Result/status: DCs were electroporated with TRP-2 mRNA, or were mock electroporated with no mRNA and then matured with Listeria or inflammatory cytokines for 24 hours. The resulting mature DCs were added in serial 3-fold dilutions to a fixed number of purified autologous T cells and incubated for 7 days. At the end of culture, the cytolytic activity exerted by responder T lymphocytes was assessed in ⁵¹Cr release assays. ⁵¹Cr-labeled targets included TRP-2-expressing cell lines and a control TRP-2-negative cell line.

Initial results showed potent antigen-specific cytolytic activity of T cells stimulated by Listeria-treated moDCs that was comparable to that stimulated by LCs alone or by moDCs supplemented with IL15 (**Figure 3**). This finding, if confirmed, would have important implications for 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⁺-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*[5, 16]. When compared with moDCs, LCs secrete more IL15[5, 16, 17], which in turn reduces IL2-induced T cell apoptosis and decreases regulatory T cell expansion during LC-mediated CTL generation[14]. We previously showed that LCs can break tolerance against a self-differentiation antigen by an IL15R α /IL15/pSTAT5-dependent mechanism[14] and that this biological mechanism at least in part explains the potency of LCs vs moDCs.

Technical issues significantly delayed repeat experiments to confirm of our initial findings of Listeria infection augmenting the potency of moDCs to the level of LCs without exogenous IL15. We recently resolved the technical issues, but a repeat experiment did not replicate our initial findings. Moreover, treatment with the IDO inhibitor, 1-MT, did not enhance CTL activity in our assay, which may not be sufficiently sensitive to detect finer changes in CTL activity. In addition, the benefits of IDO blockade with 1-MT may involve other mechanisms of immune-modulation, like Treg induction. Repeat experiments 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. Repeat experiments are ongoing.

- 4k) NK cell analyses for phenotypic activation, proliferation, and cytotoxicty (months 16-36): See Task 3d.
- <u>Task 5</u>: 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: Ongoing.
 - 5b) Analyze data for each set of experiments outlined in Tasks 1-4 (months 1-36)Result/status: Ongoing.
 - 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 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 overexpression 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.
- WT and ActA-deficient Listeria induce IDO to much greater extent than LLO-deficient Listeria. 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.
- Listeria-treated moDCs, without exogenous cytokine supplementation, may be potent stimulators of antigenspecific CTLs (repeat experiments pending).

REPORTABLE OUTCOMES

One peer-reviewed publication has resulted during the funding period: 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**: p. 166.

It is anticipated that additional data generated from current ongoing experiments will result in another peer-reviewed manuscript.

CONCLUSION

Key findings from years 1-3 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 stimulatory of the expansion of immune-dampening regulatory T cells by moDCs. 6) WT and ActA-deficient Listeria induce IDO to much greater extent than LLO-deficient Listeria. 7) Listeria-treated moDCs, without exogenous cytokine supplementation, may be 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. Final confirmatory experiments 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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Figure 1: Regulatory T cell (Treg) induction by CD103⁺ and CD103^{neg} DCs. CD103 (integrin α E) expression by moDCs is induced by Listeria. **(A)** DCs were cytofluorographically sorted into CD103⁺ and CD103^{neg} fractions. **(B)** Treg induction by CD103⁺ and CD103^{neg} DCs was compared between the two sorted populations, using Treg induction by unsorted DCs as baseline reference. Representative dot plots are shown.



Figure 2: Indoleamine 2,3-dioxygenase (IDO) protein expression in moDCs after Listeria treatment. Immature moDCs treated with wild-type, ActA-deficient, or LLO-deficient Listeria were assessed for induction of IDO by Western blot. Untreated HeLa cells 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 \pm SD; **P* <0.05; ***P* <0.01; ****P* <0.001) showing relative IDO expression between groups vs cytokine-matured moDCs. Densitometry values for each group were normalized to α -tubulin (internal control).



Figure 3: Listeria-infected moDCs stimulate TRP-2-specific CTLs in vitro that kill melanoma cells. Immature moDCs were electroporated with TRP-2 mRNA, terminally matured with ActA-deficient Listeria 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 ⁵¹Cr release 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 \pm SEM of triplicate means from one experiment.