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14. ABSTRACT Our laboratory previously described the strategy of fusing chemokine receptor ligands to antigens in order to generate immunogenic DNA vaccines. In the present study, among a panel of chemokine receptor ligands tested, murine β -defensin2 (mBD2) was superior in inducing resistance to B16 melanoma when fused to gp100. We produced corresponding fusion proteins using both ovalbumin (OVA) and gp100 as model antigens. Superior cross-presentation by dendritic cells (DC) was observed for mBD2 fused antigens over unfused antigens <i>in vitro</i> . <i>In vivo</i> , we observed significant increases in the expansion of adoptively transferred antigen specific MHC class I, but not class II-restricted T cells after immunization with mBD2 fused OVA over OVA alone. This enhanced expansion of class I restricted T cells was Toll-like receptor 4 (TLR4) dependent, but CC chemokine receptor 6 (CCR6) independent. Superior tumor resistance was observed for mBD2-fusion protein vaccines, compared with unfused antigen, in both tumor models. These data suggest that production of mBD2 fusion protein vaccines are feasible and that they induce anti-tumor immunity, through a mechanism of TLR4-mediated uptake or uptake by an as yet unidentified receptor on DC.					
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Annual Report

1. Introduction

The ultimate goal of a cancer vaccine is to prevent or cure cancer effectively without causing collateral damage. Thus far, current cancer vaccines have not been successful therapeutic agents for cancer patients. Additionally, mechanisms of immunological tolerance to cancer make the generation of immune response to cancer difficult. Therefore, new and safe strategies which can break immune tolerance against tumor antigens are needed in order to improve the efficacy of current cancer vaccines.

Adjuvants, agents which help break immunological tolerance and enhance immune response, are a crucial component of cancer vaccines. In order to enhance the capability of the adjuvant to generate antigen specific immunity, many laboratories have described strategies whereby adjuvants or cytokines are fused to targeted antigen. We previously described the strategy of enhancing the immunogenicity of tumor antigens by fusion of antigens with a chemokine receptor ligand. Thus far, these vaccines have been shown to elicit antigen specific immunity in preclinical lymphoma models.

During this past year of funding, we sought to extend the work previously established by determining the feasibility of producing chemokine fusions as protein vaccines using two additional model tumor antigens. We focused on recombinant mBD2 fusion proteins and elucidated the role of mBD2 in cross-presentation, induction of antigen-specific T cell responses, and dependence on CCR6 versus TLR4.

2. Summary of Progress as related to Project Tasks (SOW)

TASK 1: DESIGN AND CREATE CANDIDATE DNA VACCINE CONSTRUCTS AND

TASK 2: SELECT THE OPTIMAL CHEMOKINE RECEPTOR LIGAND FOR FUSION WITH MODEL ANTIGENS

- **mBD2 and MIP-3 α are optimal chemokine receptor ligands for generating fusion vaccines.**

Our previous studies have shown that fusing tumor antigens with chemokines can target chemokine receptors found on immature DC and potentiated the immunogenicity of lymphoma and HIV antigens. To extend and further optimize this vaccine strategy towards clinical translation, we generated DNA vaccines that encode melanoma gp100 antigen fused with various chemokine receptor ligands including murine β -defensin2 (mBD2), MIP-3 α , MCP-3 and RANTES (Fig. 1A: DNA Vaccine Constructs) and compared them head-to-head for protective anti-tumor immunity against murine B16 melanoma to determine the optimal ligand for fusion vaccines. Consistent with our previous results, mBD2 and MIP-3 α fusion vaccines were the most potent in protecting mice from lethal dose tumor challenge (Table 1). No protection was observed in mice vaccinated with MCP-3 or RANTES fused gp100 DNA vaccine. These results were consistent with our earlier data using a lymphoma idiotypic as the model antigen. Given the additional advantage of mBD2 in activating TLR4, mBD2 was considered the optimal candidate for further development of corresponding fusion protein vaccines.

Table 1

DNA Vaccine	Median Survival Time (Days*)	Log-rank p-value vs PBS
mBD2-gp100F	32.000	.017
MIP-3 α -gp100F	32.000	.011
MCP-3-gp100F	28.000	.651
RANTES-gp100F	30.000	.862
PBS	25.000	-

*Days after lethal dose tumor challenge

Table 1. Testing various chemokine candidates for induction of anti-tumor immunity when fused to gp100 as DNA vaccines. Fourteen C57BL/6 mice per group received three 50 μ g of individual plasmid chemokine vaccine (Fig 1A: DNA Vaccine Constructs) or PBS mock vaccine intramuscularly at two week intervals. Two weeks after final vaccination, all mice were challenged with a lethal dose of 1x10⁵ B16 melanoma cells by intraperitoneal injection and were followed for survival. Survival differences between groups were analyzed by log-rank test. Median survival times along with log-rank test p-value for respective chemokine vaccine versus PBS have been charted on table.

• Production of recombinant fusion proteins

One of the limitations of naked DNA vaccines is weak potency. Protein vaccines usually induce a stronger immune response over DNA vaccines; therefore, we focused our development efforts on the corresponding fusion protein vaccines. However, while protein vaccines effectively induce humoral responses, induction of antigen-specific cytotoxic T cells is generally suboptimal. We reasoned that chemokine receptor-mediated antigen capture may provide an alternative to overcome this disadvantage. To test this hypothesis, we first generated recombinant proteins fusing mBD2 with chicken ovalbumin (OVA) (Fig.1A: Protein Vaccine Constructs).

Desired recombinant proteins were expressed in a SF9 insect cell/baculovirus expression system whereby insect cells were infected with target specific viruses at an M.O.I. of 2 and harvested 48 hours post infection. Via an optimized multi-step protein purification process including buffer exchange, recombinant proteins were extracted from harvested cells, were

further purified for target protein using a His tag protein binding column, and removed of endotoxin using an endotoxin removing column. We obtained higher yields (~1500 μ g of purified protein per 10⁸ virus infected SF9 cells) of mBD2-OVA protein (Fig. 1B) versus poor yielding (~250 μ g of purified protein per 10⁸ virus infected SF9

Figure 1

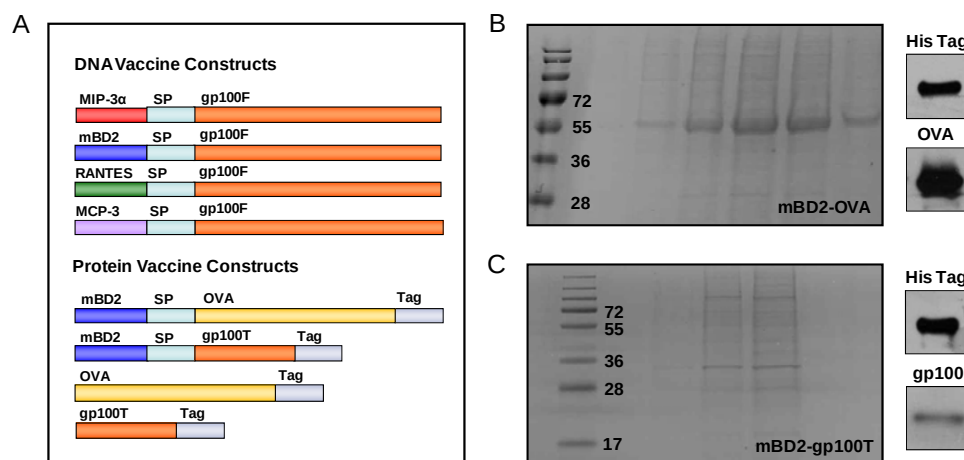


Figure 1. Schematics for chemokine fusion DNA and protein vaccine constructs along with tumor challenge survival times for DNA chemokine fusion vaccine candidates. (A) For DNA vaccines, the cDNA encoding melanoma associated antigen, full length human gp100 (gp100F) was genetically fused with chemokines including murine MIP-3 α , mBD2, murine RANTES, and human MCP-3. A spacer (SP) was added for proper separation and folding of chemokine and antigen protein. For protein vaccines, insect cell derived recombinant protein vaccines were expressed from SF9 cells infected with Gateway adapted BaculoDirect baculovirus. Proteins were tagged with C-terminal V5-6xHis tag (Tag) and Ni-NTA columns were used to purify protein. Model antigen, ovalbumin (OVA) and melanoma associated antigen truncated human gp100 (gp100T) were chosen for recombinant protein production. Proteins for antigens fused to murine β -defensin2 (mBD2) as well as unfused antigens were produced. (B) mBD2-OVA (48.5 kDa) protein in SDS-PAGE was stained with Coomassie dye to verify protein purity. Also, protein identity was confirmed via western blot for proteins with 6xHis tag (His Tag) antibody and also corresponding OVA antibody. (C) mBD2-gp100T (29.8 kDa) protein in SDS-PAGE was stained with Coomassie dye (Coomassie) to verify protein purity. Also, protein identity was confirmed via western blot for proteins with 6xHis tag (His Tag) antibody and also corresponding gp100 antibody.

cells) mBD2-gp100T protein (Fig. 1C). Identity of the purified proteins were verified by Western blot (Fig. 1B and 1C), MALDI-TOF (Data not shown), and protein predicted size check by Coomassie stain on SDS-PAGE (Fig. 1B and 1C).

TASK 3: ESTABLISH AND CHARACTERIZE AN A20 LYMPHOMA IDIOTYPE-SPECIFIC CD8+ T-CELL LINE.

These studies in the lymphoma model are currently being postponed, as we prioritized the studies below using model systems for which TCR transgenic mice already are available as a source of clonal CD8+ antigen-specific T cells.

TASK 4: INVESTIGATE MECHANISMS BY WHICH ANTIGEN IS CROSS-PRESENTED TO CD8+ T CELLS

- **mBD2-OVA fusions facilitated antigen cross-presentation in a CCR6 independent manner.**

Bone marrow derived DC (BM-DC) pulsed with mBD2-OVA fusion proteins were superior in the stimulation of OVA-specific CD8+ T cells (OT-I) as assessed by IFN- γ production compared to OT-I cells stimulated by DC pulsed with PBS or mBD2 fused with an irrelevant antigen (mBD2-gp100T) for 5.5 hours (Fig. 2). This immunological effect on T cell activation was clearly dependent on fusing the antigen with mBD2, as unfused OVA proteins generated by the same approach only resulted in a marginal level of IFN- γ secretion by OT-I cells (Fig. 2). With prolonged stimulation (8 hours), elevated levels of IFN- γ release were observed by both OVA and mBD2-OVA stimulated T cells, those stimulated by mBD2-OVA were still significantly higher (Data not shown).

Given that mBD2 is a ligand for CCR6, we hypothesized that the fusion protein-induced antigen cross-presentation would be CCR6 dependent. Thus, we performed the same experiment using BM-DC generated from CCR6 deficient mice. To our surprise, when we compared the levels of IFN- γ released by effector T cells stimulated with mBD2-OVA-pulsed CCR6 deficient or wild type BM-DC, there was no difference, suggesting that enhanced IFN- γ secretion induced by mBD2 is not dependent on CCR6 (Fig. 2).

Figure 2

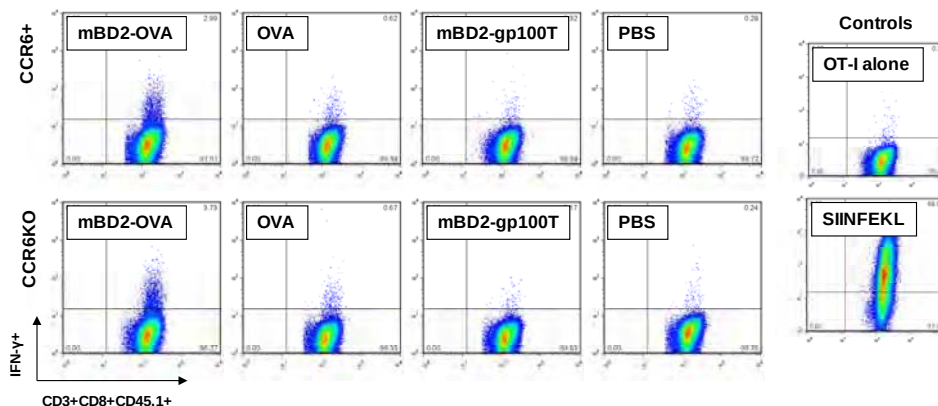


Figure 2. Cross-presentation is enhanced by chemokine-antigen fusion and is independent of CCR6. RBC lysed splenocytes from OT-I transgenic mice were cultured for 8 days in the presence of OVA₂₅₇₋₂₆₄ peptide and IL-2 to enrich for OVA specific T cells. The OT-I T cells were then co-cultured with bone marrow derived DC (BM-DC) from C57BL/6 or CCR6 deficient mice loaded with mBD2-OVA, OVA, or mBD2-gp100T (mBD2 fused with irrelevant antigen) protein for 5.5 hours at a ratio of 5:1 respectively. OT-I cells stimulated by the antigenic peptide OVA₂₅₇₋₂₆₄ (SIINFEKL) served as positive control. Negative controls included untreated OT-I cells or OT-I cells stimulated with DC with no protein loading (PBS). IFN- γ production by OT-I cells was assessed by intracellular staining of IFN- γ measured by flow cytometry. OT-I cells were gated for live lymphocytes and on the CD3+CD8+CD45.1+ population. Data represent 3 independent experiments with 3 replicates per group.

- **Vaccination with mBD2-OVA fusion proteins stimulated the expansion of adoptively transferred antigen specific CD8+, but not CD4+ T cells.**

We next investigated the potential of mBD2-OVA vaccination to boost expansion of transferred OVA-specific CD8+ T cells *in vivo*. T cells derived from OT1+CD45.1+ transgenic mice were adoptively transferred into wild type C57BL/6 mice (CD45.2+ background), followed by vaccination the same day. The results revealed that single subcutaneous administration of 150 µg recombinant mBD2-OVA protein significantly stimulated the proliferation of adoptively transferred OT-I cells, determined by flow cytometry. The peak T-cell expansion appeared at about 5 days after vaccination (Fig. 3A). Although, vaccination with unfused OVA protein also was associated with OT-I cell expansion when compared to PBS controls; the level of expansion was significantly lower than that of mBD2-OVA (Fig. 3A). Unexpectedly, mBD2-OVA vaccination failed to enhance the proliferation of transferred OVA-specific CD4+ T cells derived from OT-II+CD45.1+ transgenic mice (Fig. 3B). Together, these data suggest that mBD2-OVA fusion proteins are also cross-presented *in vivo*.

Figure 3

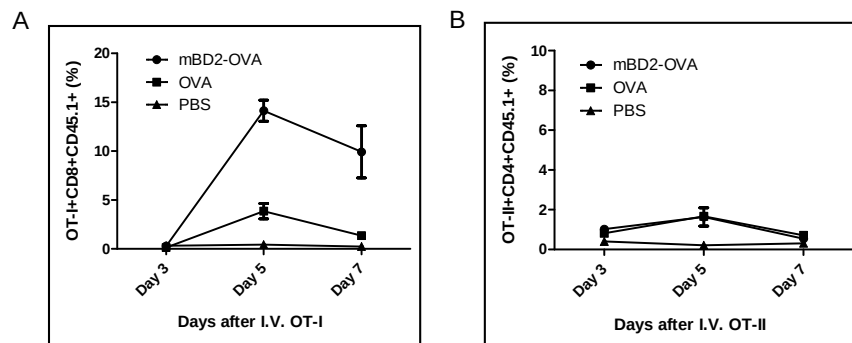


Figure 3. Superior expansion of transferred OT-I, but not OT-II, cells *in vivo* following vaccination with mBD2-OVA versus OVA protein. Five C57BL/6 mice per group received freshly isolated splenocytes (~1-2x10⁶ cells) intravenously from OT-I+CD45.1+ (A) or OT-II+CD45.1+ (B) transgenic mice followed by subcutaneous immunization with 150µg mBD2-OVA or OVA recombinant protein, or PBS. Mice were tail bled 3, 5, and 7 days post immunization. Blood was stained with appropriate fluoro-chrome labeled CD8 or CD4 and CD45.1 antibodies and analyzed by flow cytometry. Differences between groups were statistically analyzed by 2 tailed student t-test. The data is shown as percentage CD45.1+ cells in the CD8+ or CD4+ population ± standard error of the mean. (A) Data represent 8 independent experiments consisting of five mice per group. (B) Data represent 2 independent experiments consisting of five mice per group.

TASK 5: DETERMINE WHETHER CO-ADMINISTRATION OF CHEMOKINE-ANTIGEN VACCINES WITH IMMUNE ADJUVANTS CAN IMPROVE VACCINE POTENCY

- **Toll-like receptor 4 is required for cross-priming T cells by vaccination with protein.**

Consistent with our *in vitro* data that suggested CCR6 independent cross-presentation of mBD2-OVA fusion protein (Fig. 2), expansion of OT-I T cells after adoptive transfer and vaccination was comparable in wildtype and CCR6 deficient hosts (Fig. 4A). However, T-cell expansion was dramatically reduced in mice deficient of TLR4 under the same experimental conditions (Fig. 4B). To further explore the involvement of TLR4, we vaccinated wildtype OT-I recipients with mBD2-OVA or OVA, either together with MPL, a TLR4 agonist (21) as a vaccine adjuvant, or alone. The addition of MPL boosted T-cell expansion in mice vaccinated with unfused OVA but not with mBD2-OVA fusion proteins (Fig. 4C), highlighting the role of TLR4 on cross-priming OT-I T cells.

Figure 4

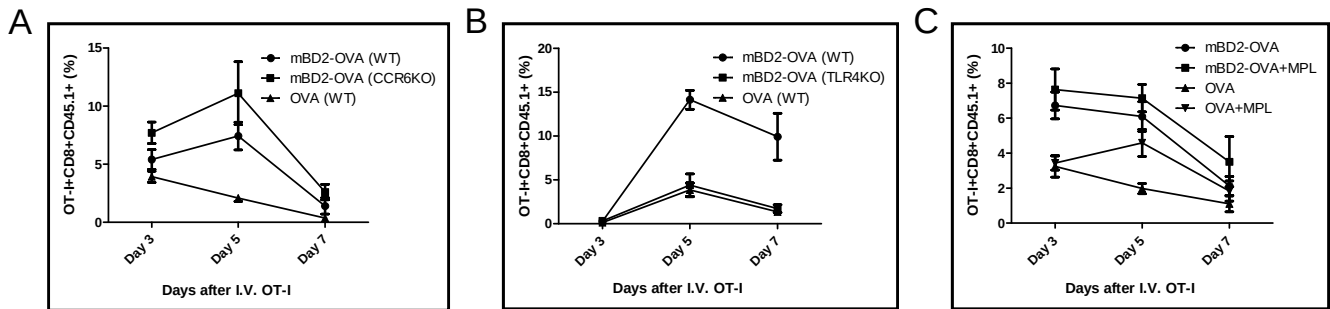


Figure 4. Expansion of antigen-specific T cells by vaccination with mBD2-OVA fusion protein is dependent on TLR4, but not CCR6. Five C57BL/6 wildtype (WT), CCR6 deficient (CCR6KO) (A), or TLR4 deficient (TLR4KO) (B) mice per group received freshly isolated splenocytes intravenously ($\sim 1-2 \times 10^6$ cells) from OT-I+CD45.1+ transgenic mice. The adoptive T cell transfer was combined with subcutaneous immunization with 150 μ g mBD2-OVA recombinant protein, and the percentages of CD8+CD45.1+ cells in the blood were determined on days 3, 5, and 7 as in Figure 3A. Data represent 2 independent experiments consisting of five mice per group. Five WT mice per group were vaccinated with mBD2-OVA or OVA after T cell transfer as above, either with or without 50 μ g MPL mixed with fusion protein (C).

- **Vaccination with mBD2-OVA fusion protein elicited protective anti-tumor immunity.**

Five mice per group first received adoptively transferred OT-I splenocytes, followed by vaccination with mBD2-OVA or OVA protein, and then challenge with a lethal dose of OVA-expressing B16 melanoma cells (B16-OVA) one week later. The OT-I and mBD2-OVA combination showed a potent effect on protecting mice from tumor challenge, achieving a long-term survival in all mice (Fig 5.) This anti-tumor protection was significantly greater than that achieved by OT-I transfer and vaccination with unfused OVA protein, which showed only 50% protection ($P < 0.0003$) or no vaccination ($P < 10^{-9}$) (Fig. 5). However, without adoptive OT-I cell transfer, differences in survival could be seen between mBD2-OVA or OVA protein immunized mice versus PBS immunized mice but not between mBD2-OVA versus OVA (Data not shown).

Figure 5

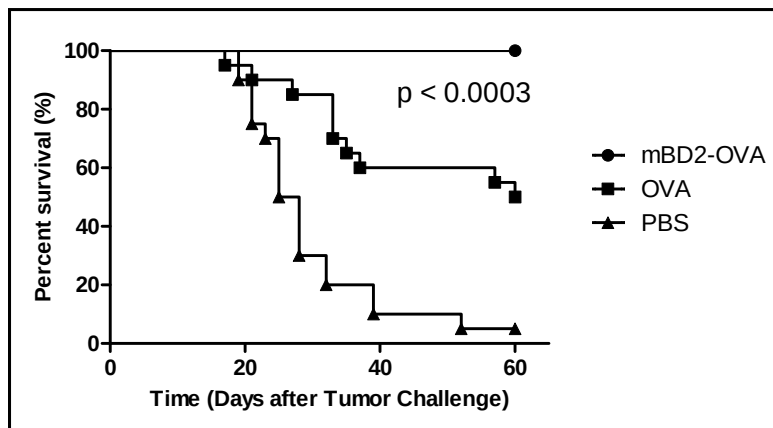


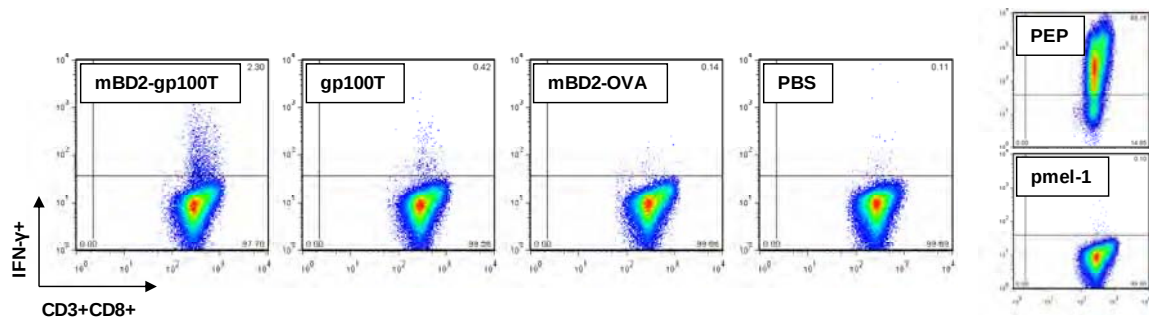
Figure 5. Vaccination with mBD2-OVA fusion protein potentiated protective anti-tumor immunity. Five C57BL/6 mice per group received freshly isolated splenocytes ($\sim 1-2 \times 10^6$ cells) intravenously from OT-I+ transgenic mice, followed by subcutaneous immunization with either mBD2-OVA recombinant fusion protein or OVA protein as in Figure 3A. One week after vaccination, all mice were challenged with a lethal dose of 1×10^5 B16-OVA melanoma cells by subcutaneous injection and were followed for survival for 60 days. Survival differences between groups were analyzed by log-rank test. Data represent pooled data from 4 independent experiments with a total of 20 mice per group.

- **mBD2 fusion enhanced cross-presentation of gp100 and tumor resistance.**

The promising results using OVA as a model antigen provided the rationale to test this approach using the tumor antigen, melanoma gp100. Recombinant mBD2-gp100T fusion and gp100T proteins were generated as described in Figure 1A. Antigen-specific CD8⁺ T cells (pmel-1) stimulated with BM-DC loaded with mBD2-gp100T produced significantly higher levels of IFN- γ compared with BM-DC pulsed with unfused gp100T, mBD2-OVA (irrelevant antigen control) or PBS (Figure 6A). Moreover, compared with unfused gp100T, vaccination of mice with mBD2-gp100T fusion protein significantly improved the protective effect of adoptively transferred pmel-1 T cells against lethal challenge with B16 melanoma ($P < 0.0001$) (Figure 6B).

Figure 6

A



B

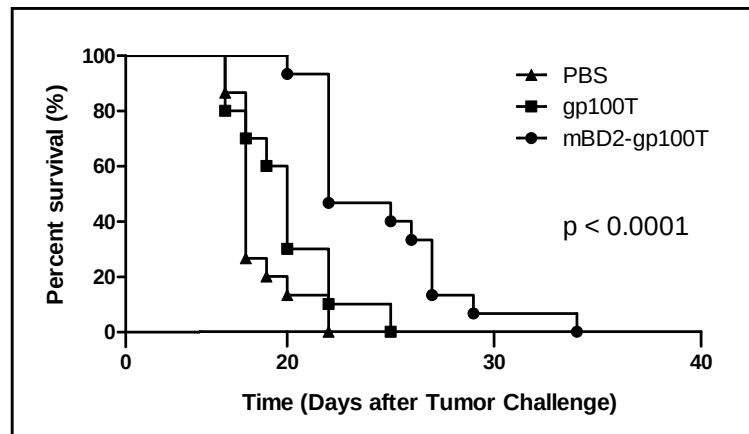


Figure 6. mBD2 fusion tumor associated self antigen gp100 also is associated with enhanced antigen cross-presentation and induction of anti-tumor resistance. (A) gp100-specific T cells were enriched by culturing RBC lysed splenocytes from pmel-1 transgenic mice in the presence of gp100₂₅₋₃₃ peptide and IL-2 for 8 days. T cells were then stimulated with BM-DC previously loaded with mBD2-gp100T, gp100T, mBD2-OVA (mBD2 with irrelevant antigen), or PBS (no protein), respectively. BM-DC were loaded with respective protein for 24 hours then washed prior to co-culture with T cells at a 1:5 ratio for 5.5. IFN- γ produced by activated pmel-1 T cells was assessed with intracellular staining of IFN- γ as in Figure 2. pmel-1 T cells stimulated by gp100₂₅₋₃₃ peptide (PEP) served as a positive control and untreated T cells as a negative control. Data are representative of 4 independent experiments with 3 replicates per group for each experiment. (B) Five C57BL/6 mice per group received freshly isolated splenocytes ($\sim 3 \times 10^6$ cells) intravenously from pmel-1 transgenic followed by subcutaneous immunization with 1.5 mg mBD2-gp100T fusion or gp100T recombinant protein, or PBS. One week later, all mice were challenged with a lethal dose of 1×10^5 B16 melanoma cells by subcutaneous injection and were followed for survival. Survival differences between groups were analyzed by log-rank test. Data represents pooled data from 2 combined independent experiments with a total of 10-15 mice per group.

3. Key Research Accomplishments

a. DNA vaccine Construction

Cloning and construction for mBD2, MCP-3, MIP-3 α , and RANTES chemokine vaccine plasmid for gp100 antigen was previously described (5, 14). RANTES was cloned using primers Forward - 5'-TTGGATCCTCGACATGGCCTCACCATATGGCTCGGA- 3' and Reverse - 5'-TTGAATCCGCTCATCTCCAAATAGTTGAT-3'. OVA DNA fragment was cloned by RT-PCR using the total RNA extracted from B16-OVA cells.

b. Baculovirus Generation, Protein Expression, and recombinant Protein vaccine Purification

mBD2-gp100T, mBD2-OVA, MIP-3 α -gp100T, MIP-3 α -OVA, gp100T, and OVA targets were cloned into pENTR™/D-TOPO® Gateway vector as Invitrogen guidelines. To increase the solubility of the recombinant gp100 protein, we generated a truncated protein (gp100 Residues 22-236 or gp100T) by removing the hydrophobic regions. The immune epitopes were retained in the truncated gp100. Target specific baculovirus were generated as directed in Invitrogen's BaculoDirect C-Term Expression Kit instructions. Baculovirus titrating and viral stock production were done by the Baculovirus/Monoclonal Antibody Core Facility (Baylor College of Medicine, Houston, TX). All large scale *Spodoptera frugiperda* SF9 insect cell protein expression was done in 5L oxygenated bioreactors by the Baculovirus/Monoclonal Antibody Core Facility (Baylor College of Medicine, Houston, TX). Correct protein expression was validated by Coomassie staining, MALDI-TOF by the Proteomics Facility (MD Anderson Cancer Center, Houston, TX), and Western blotting. Protein purification of 6xHis tagged proteins was done using Qiagen Ni-NTA Superflow and Bio-Rad's Econo-Pac Chromatography Columns following an optimized version of The QIAexpressionist's Protocol 16 (Purification of 6xHis-tagged proteins from baculovirus-infected cells under native conditions). PBS buffer exchange and concentration of proteins was done using Millipore's Amicon® Ultra Centrifugal Filter Units. The purified recombinant proteins were filtered through an endotoxin removing column (Pierce's Detoxi-Gel Endotoxin Removal Gel) and verified for endotoxin contamination.

c. Publication of results:

During this past year our comparable work in the lymphoma model system was published in a premier peer-reviewed journal:

Qin H, Cha SC, Neelapu SS, Lou Y, Wei J, Liu YJ, Kwak LW. Vaccine site inflammation potentiates idiotype DNA vaccine-induced therapeutic T cell-, and not B cell-, dependent antilymphoma immunity. Blood. 2009 Nov 5;114(19):4142-9. Epub 2009 Sep 11. PMID: 19749091

4. Conclusions and Future Plans

These studies extend our original results focused on mBD2 as the genetic fusion partner for a lymphoma antigen. Specifically, we have extended the principle of mBD2 fusion to two additional model antigens; namely, OVA and melanoma gp100. In addition to the critical role of TLR4 on mBD2-induced DC maturation, we have now demonstrated that mBD2-fusion vaccine-induced T-cell priming is also primarily dependent on TLR4, rather than CCR6. Finally, we have demonstrated that mBD2 fusion results in the antigen gaining access to the MHC class I, rather than class II, processing pathway. Taken together, our studies demonstrate the **Induction of TLR4-dependent CD8+ T cell immunity by Murine β -defensin2 fusion Protein Vaccines.**

DNA vaccines have been of substantial interest because of the relative low cost and ease of production advantages. However, DNA vaccines are intrinsically weak immunogens. Thus, we focused our studies on recombinant proteins corresponding to mBD2-antigen DNA

fusions, which generated limited protective and anti-tumor immunity (Table 1). Expressing the fusion proteins in a baculovirus/insect cell system also had the potential to further increase the immunogenicity of proteins.

We observed that antigen-specific CD8⁺ T cells (OT-I and pmel-1) generated significantly more IFN- γ after stimulation with DC loaded with mBD2 fused target antigen (gp100 or OVA, respectively) compared with antigen alone or mBD2 fused to irrelevant antigen (Fig. 2 and 6). We speculate that this enhanced cross-presentation is partly attributed to mBD2's ability to induce DC maturation, CD40/B7.2 expression, and expression of other co-stimulatory molecules.

We verified these results by testing the expansion of transferred OT-I and OT-II cells *in vivo* following vaccination with mBD2 fused OVA proteins. We observed an approximately 2-4 fold higher expansion of OT-I cells if immunized with mBD2-OVA over OVA alone (Fig. 3A). In contrast, this difference in expansion was not noted with OT-II cells, suggesting that mBD2 induces only MHC class I T cell directed expansion.

Mechanistically, we tested if mBD2-enhanced cross-priming required CCR6 or TLR4 (Fig. 4). The results from these experiments using genetically deficient mouse strains suggested a dependence on TLR4, but not CCR6. A link to CCR6 had been previously suggested by studies showing β -defensin interaction with CCR6 present on immature DC. However, our data does not rule out chemokine receptor mediated endocytosis of antigen by APC for cross-presentation. It is also possible that receptor-mediated endocytosis of mBD2 fusion proteins could occur through another, as yet unidentified receptor. Finally, adding TLR4 agonist (MPL) to unfused antigen also stimulated T-cell expansion, suggesting an additional role of TLR4 in cross-presentation (Fig 4C).

It is also tempting to speculate that mBD2-fused antigen proteins may be internalized by TLR4 and enter the correct subcellular compartment to mediate cross-presentation to CD8⁺ T cells. One candidate subcellular compartment is the early endocytic compartment (EEC), described previously. Previous studies have found that the MyD88 pathway controls the efficiency of cross-presentation and further shown that a TLR4-MyD88 dependent recruitment of the essential MHC class I antigen processing and loading component, transporter associated with antigen processing (TAP), to the EEC occurs (26). Our previous results clearly suggested targeting of chemokine receptors by chemokine-antigen fusions, as simple mixing of free chemokine and antigen did not trigger immunity.

Our plans for the next year of funding are to continue to follow the specific leads above to further elucidate the mechanism by which chemokine-antigen fusion vaccines elicit CD8⁺ T-cell immunity, with the goal of optimization, and to move one or more candidate vaccines toward clinical translation in an eventual first-in-human clinical trial.