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14. ABSTRACT

In the 1st year, we developed immunotherapies using viral vector-based vaccines, as well as peptide-based vaccines. For the first prototypes of these vaccines, Tvax and Tvax 2.0, we have included antigens demonstrated to be highly expressed in human tumors. Unexpectedly, these vaccines resulted ineffective in reducing MM in mice and displayed signs of autoimmunity. We hypothesized that these negative results were caused by a reduced expression in mouse MM of the antigens included in Tvax vaccines. A whole transcriptome analysis to compare protein expression between MM and normal tissues confirmed our assumption. This analysis also allowed us to choose a new set of antigens that presented more effective anti-cancer responses in our experiments, without generating auto-immunity. During the 2nd year of this project, we focused on evaluating the anti-cancer efficacy of Tvax 3.0 vaccines, which include this new set of antigens. We also characterized the antigen-specific immune responses generated by Tvax 3.0 and analyzed immune cell infiltrates in tumors from vaccinated mice. All these data were incorporated in a manuscript that was submitted for publication at the beginning of the 3rd year of this project. During the 3rd year, we performed all the experiments requested by the reviewers and successfully published an article in *Frontiers in Oncology*. During the 3rd and 4th year, we started the development of hTvax for human immunization.

15. SUBJECT TERMS

Malignant Mesothelioma, anti-cancer vaccines, T cell-epitope, multi-epitope vaccines, T regulatory cells, tregitopes.

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TABLE OF CONTENTS

INTRODUCTION:
Malignant Mesothelioma (MM) is an aggressive form of cancer that typically originates in the pleural cavity, but can also occur in the peritoneum, pericardium, and around the testes. Asbestos exposure is the only established risk factor for MM (1).
Even if MM is a rare cancer, a significant number of MM deaths occur among U.S. navy sailors and military personnel that were heavily exposed to asbestos because they worked in shipyards or their proximity (2). Since there are no effective treatments for MM and the median survival is less than 1 year from diagnosis, there is an urgent medical need to develop novel approaches to treat this understudied disease.

Our purpose is to use advanced anti-cancer vaccines to generate effective immune responses against MM cells. In clinical trials, conventional anti-cancer vaccines have shown to be safe and to induce tumor-specific T cell responses, although an increase in survival has been achieved in a limited number of patients. Most of the vaccines utilized in clinical trials, as well in pre-clinical studies usually target a single cancer antigen. Our data obtained with survivin vaccine FP-surv suggest that vaccination with this single antigen was effective against survivin-positive cells (3). However, eradication of survivin-positive cells was followed by an expansion of cancer cell clones with reduced or undetectable survivin expression. These clones elude survivin-specific cytotoxic T cells and maintain the ability to grow uncontrolled. For this project, we developed a novel Tvax vaccine designed to activate antigen-specific T cells against multiple targets and produce stronger anti-cancer responses than single-antigen vaccines.

The scope of this research is to evaluate the therapeutic efficacy of Tvax and its activity on cancer-specific T cells in mouse MM models. We also proposed to develop a clinical version of Tvax (hTvax) and evaluate its efficacy in activating human T cells from healthy donors.

KEYWORDS:
Malignant Mesothelioma, anti-cancer vaccines, T cell-epitope, multi-epitope vaccines, T regulatory cells, tregitopes.

ACCOMPLISHMENTS:
What were the major goals of the project?

Major Tasks for Year 1 as stated in the SOW:
Specific Aim 1, Major Task 1. Obtain regulatory approval for the use of animals.
Milestone Achieved. Regulatory approval was obtained.

Specific Aim 1, Major Task 2. Identify the most efficacious vaccine by comparing different versions of Tvax in combination with OX86 antibodies to induce mesothelioma (MM)-specific T cells in BALB/c mice. Timeline: Previous Timeline: 6-9 months, modified to 6-15 months after change on SOW.
Milestone Achieved: The most effective vaccination to induce MM-specific T cells is identified.

Specific Aim 1, Major Task 3. Evaluate the efficacy of different versions of Tvax vaccines in subcutaneous tumor model of MM progression. Timeline: Previous Timeline: 9-12 months, modified to 9-15 months after change on SOW.

Milestone Achieved: The most effective vaccination to reduce MM subcutaneous tumors is identified.

What was accomplished under these goals? (**Experimental results from Year 1)**
As major activities during this reporting period, we evaluated the efficacy of mTvax vaccines in activating antigen-specific responses and delay cancer progression in mouse MM models. The first version of mTvax, included epitopes to activate antigen-specific T cells against survivin (BIRC5), metastasin (gene S100A4), midkine (gene Mdk), Wilm's Tumor 1 (gene WT1), brachyury (gene T), Fibroblast Activation Protein (gene FAP) and VEGFR2 (gene Kdr). In mTvax, we also included the immunostimulatory molecules CD80, CD54, and CD48 with the purpose of improving T cells responses. The mTvax antigen, comprising of the epitopes for T cell activation and the three immunostimulatory molecules, has been inserted into the DNA of three different vectors: a plasmid DNA vector (p-mTvax) and two viral vectors (MVA-mTvax and FP-mTvax).

Our objective was to compare anti-cancer activities of the different mTvax vectors alone or with OX40-agonist antibodies (OX86). We also tested the combination of the different mTvax vectors in a heterologous prime-boost setting. This immunization strategy has been demonstrated to extend the activation of cancer-specific T cells and can be applied to achieve durable anti-cancer responses.

We performed experiments in which BALB/c mice carrying MM subcutaneous tumors were vaccinated with different immunization schedules. In these mice, we evaluated differences in tumor growth and survival.

The results of these experiments indicated:

a) Activities of viral vaccine vectors as MVA and FP were consistent in repeated experiments. In the other hand, plasmid DNA vaccines showed variable results with significant anti-cancer responses achieved only in few mice for each experiment. To investigate this issue, we analyzed levels of antigen expression after subcutaneous (s.c) injection of p-mTvax in BALB/c mice. As a reporter antigen, we included luciferase in p-mTvax and evaluated its expression using an IVIS imaging system after intraperitoneal (i.p.) injection of luciferin. As showed in Figure 1, some of the mice injected with p-mTvax did not present any luciferase activity. We concluded that s.c. injection of plasmid DNA is not a reliable way to vaccinate mice. Different inoculation methods could be investigated for plasmid DNA vaccines, as Gene gun or *in vivo* electroporation. However, to establish these *in vivo* transfection methods we should acquire instruments that would have a substantial impact on the budget. Viral vector vaccines (MVA and FP) instead demonstrated consisted results and can be produced in our laboratories without additional expenses. For these reasons, we decided to use MVA and FP vectors as leading vaccine platforms for this project.

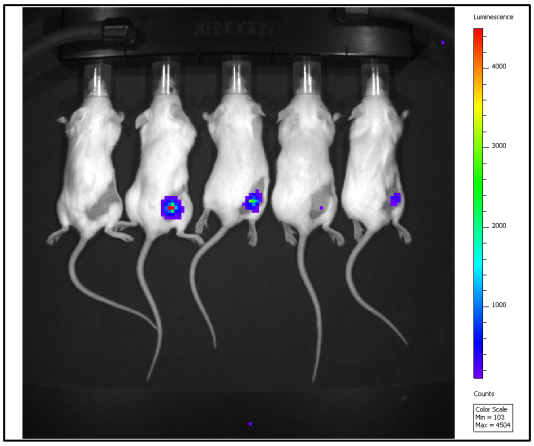

Figure 1. Luciferase activity in mice injected with p-mTvax. Mice were injected s.c. with 5µg of p-mTvax carrying the luciferase gene as reporter antigen. Luciferase expression has been evaluated with an IVIS imaging system after injection of 100µl of luciferin.

b) Vaccination with FP-mTvax as treatment with OX86 antibodies delay tumor growth and extend survival of mice carrying MM tumors (Fig.2).

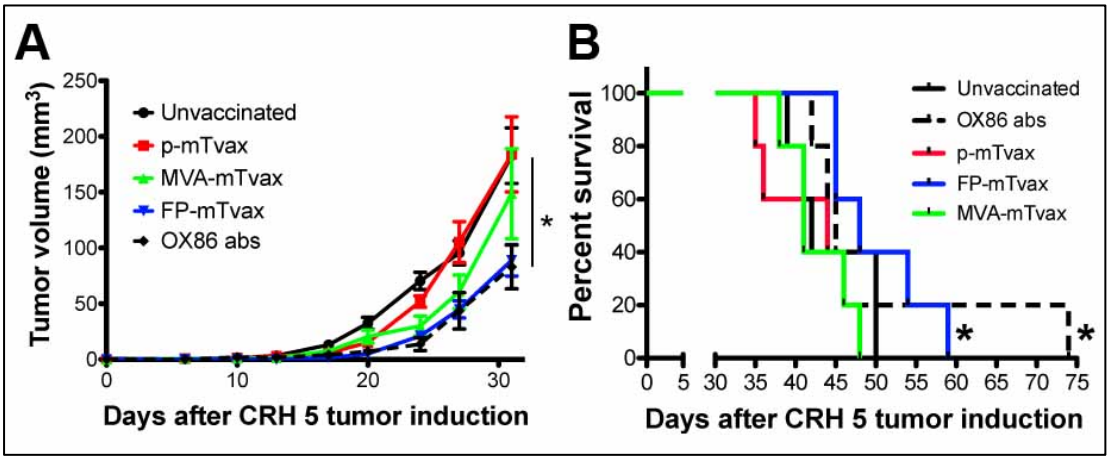

Figure 2. FP-mTvax and OX86 abs delay tumor growth and improve survival of mice carrying MM tumors. BALB/c mice were injected with 100.000 CRH5 MM cells and treated at day 7 and 14 with mTvax vaccines or OX86 abs. Tumor growth was measured with a caliper (A) and survival evaluated with mice euthanized when tumors reached 200mm^3. *P≤0.05

c) Vaccination with FP-mTvax in combination with OX86 antibodies does not reduce tumor growth (Figure 3).

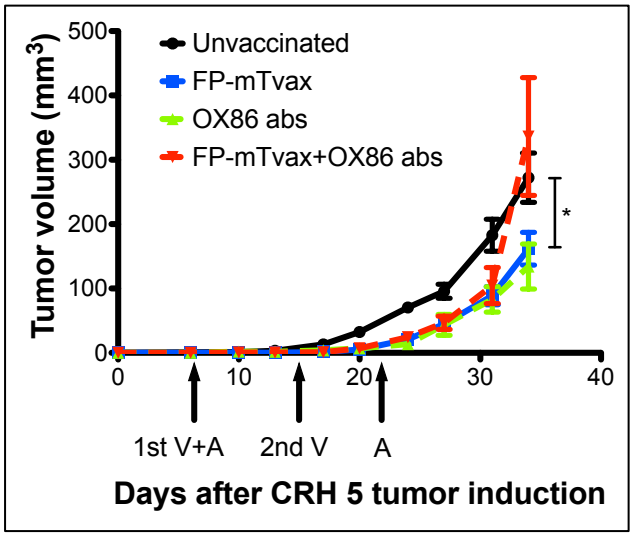

Figure 3. FP-mTvax vaccine and OX86 antibodies delay tumor growth when used as single treatment, but fail when used in combination. BALB/c mice bearing palpable tumors were vaccinated with two i.m. injections of FP-mTvax (days 7 and 14), or with two i.p. injections of OX86 abs (days 7 and 21), or with a combination of the two. Tumor volumes were measured twice a week. *P≤0.01

We hypothesized that the immunostimulatory molecules CD80, CD54, and CD48 included in the mTvax antigen, may contribute to the loss of efficacy showed by FP-mTvax after OX40 engagement with OX86 antibodies. It has been demonstrated that self-full-length protein as CD80, CD54 and CD48 may contain T cell epitopes, called tregitopes, that specifically activate regulatory T cells (Tregs) (4). The activation of Tregs by tregitopes may increase during OX40 engagement and explain the lack of FP-mTvax efficacy observed in Figure 3. The reduced activity of FP-mTvax after OX40 engagement can also be caused by autoimmune responses targeting immune cells expressing CD80, CD54, and CD48.

To investigate our hypothesis that CD80, CD54, and CD48 reduce the efficacy of our vaccines, we produced **mTvax 2.0** that expresses the same cancer antigens of mTvax but lacks the three immune stimulatory molecules. The mTvax 2.0 antigen has been inserted into MVA and FP vectors (MVA-

mTvax 2.0 and FP-mTvax 2.0) and utilized to vaccinate mice carrying subcutaneous MM tumors. In these mice, we evaluated tumor dimension, survival and T cell immune responses.

The results of the experiments performed with mTvax 2.0 indicated:

a) Both MVA-mTvax 2.0 and FP-mTvax 2.0 delayed tumor growth and improved survival of mice carrying MM tumors. The efficacy of both mTvax 2.0 vaccines was significantly improved by treatment with OX86 antibodies (Figure 4).

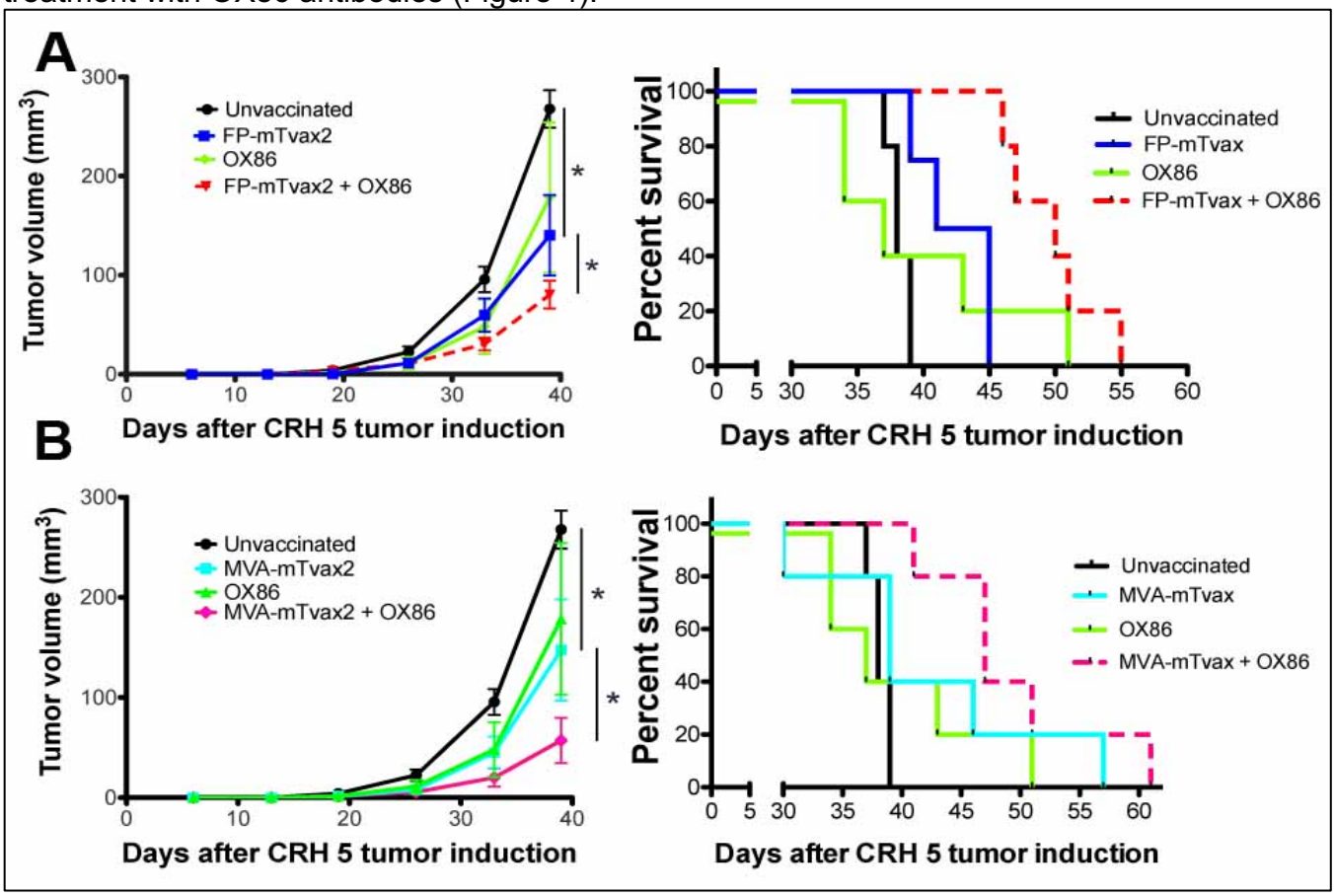

Figure 4. mTvax 2.0 vaccines delay tumor growth and improve survival when used as single treatment or in combination with OX86. BALB/c mice bearing palpable tumors were vaccinated with two i.m. injections of mTvax2 vaccines (MVA or FP in days 7 and 14), or with two i.p. injection of OX86 abs (days 7 and 21), or with a combination of the two. Tumor volumes were measured with a caliper and survival evaluated with mice euthanized when tumors reached 200mm^3. *$P \leq 0.01$*

b) mTvax 2.0 induces epitope-specific T cell immune responses in BALB/c mice. This was evaluated by flow cytometry analysis of T cells from vaccinated mice stimulated with each the 42 epitopes included in mTvax 2.0. In both CD4$^+$ and CD8$^+$ T cells, we measured production of IFNγ as an index of immune activation. To investigate the activation of T regulatory cells, we instead measured production of IL-10 and TGFβ.
The results of this analysis revealed that 13 peptides of those produced by mTvax 2.0 stimulate the production of IFNγ in a significant number of CD4$^+$ and CD8$^+$ T cells. Those peptides were

distributed among all the seven target antigens included in mTvax 2.0. None of the peptides representing the epitopes in mTvax 2.0 induced the production of IL-10 or TGFβ in any of our experiments. A representative image of the flow cytometry analysis of $CD4^+$ T cells after stimulation with the Brachyury peptides is shown in Figure 5. An example of the data obtained for the mTvax 2.0 target-antigen Survivin, is instead shown in Figure 6. The results of the experiments using peptides from the other mTvax 2.0 target-antigens were not shown.

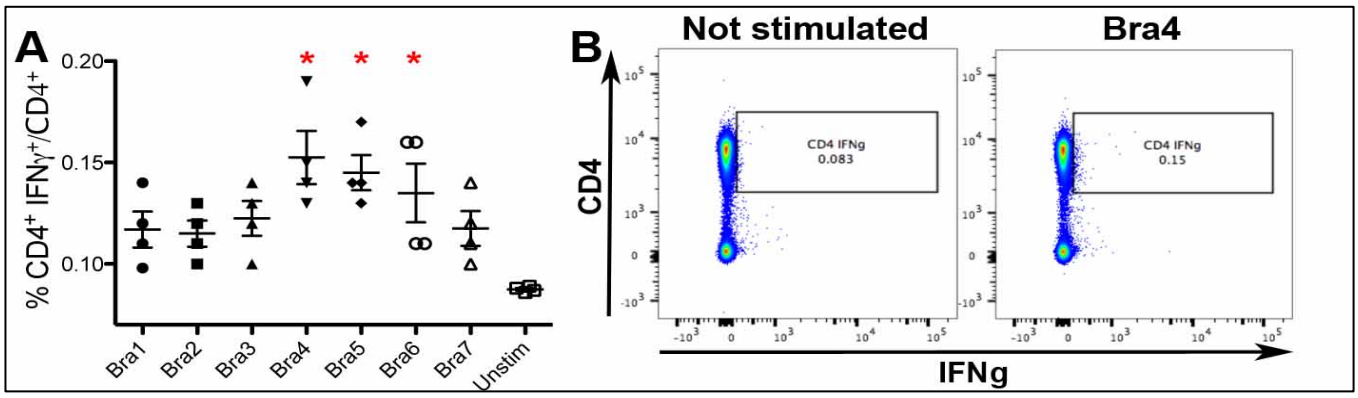

Figure 5. Bracyury peptides induce epitope-specific $CD4^+$ T cell immune responses in BALB/c mice vaccinated with MVA-mTvax 2.0. Groups of 5 mice were vaccinated with MVA-mTvax 2.0 (10e6 pfu) on day 1 and 7. Lymph nodes were harvested on day 17 and immune cells stimulated with 7 different Bracyury peptides representing the same Brachyury epitopes included in mTvax 2.0. After 6 hours of incubation with Brefeldin A, cells were stained for the indicated antibodies and analyzed at the flow cytometer. A) Percentages of CD4+ $IFN\gamma^+$ cells after stimulation with the different Brachyury peptides. Unstimulated cells were used as control. *$P \leq 0.01$ B) Representative image from the flow cytometry analysis.

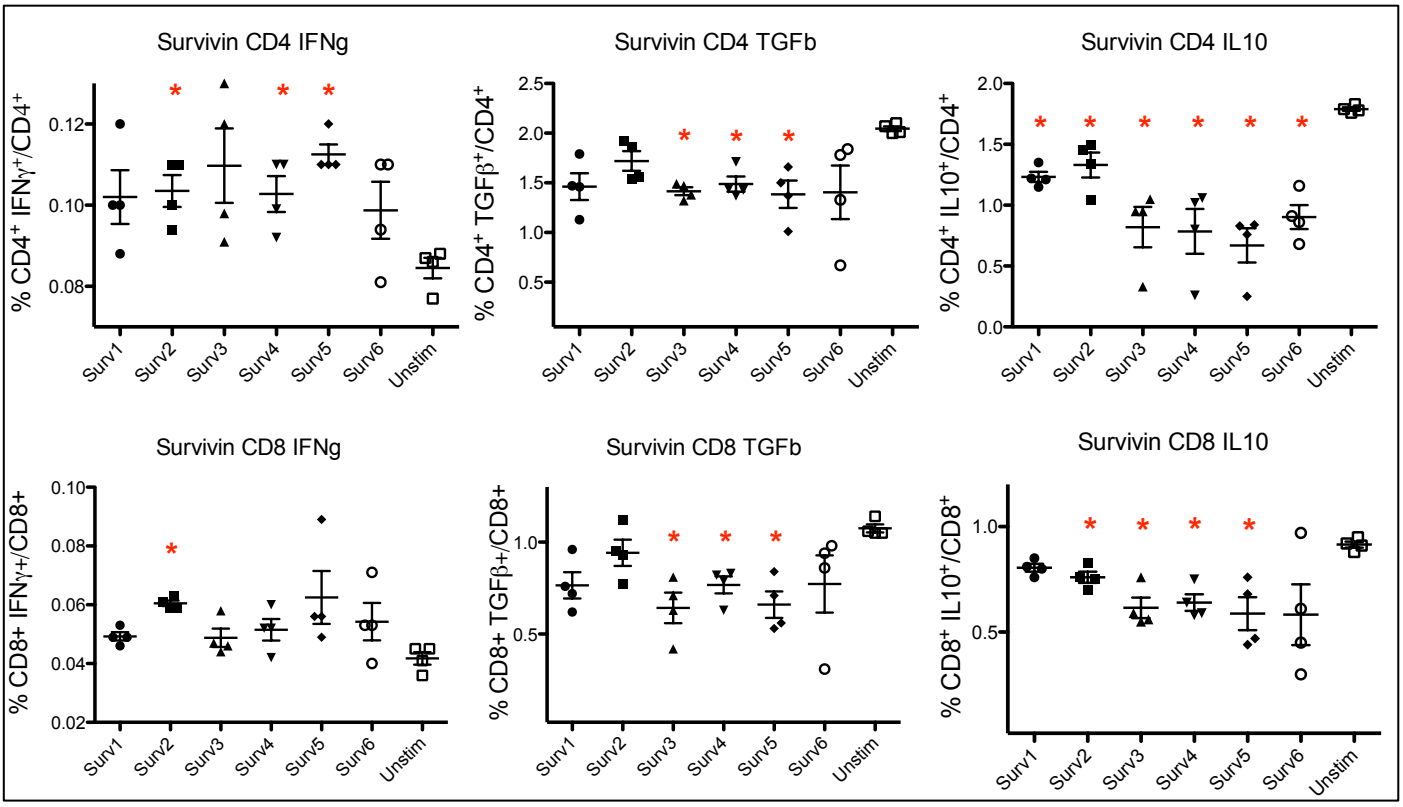

Figure 6. Survivin peptides induce epitope-specific T cell immune responses in mice vaccinated with MVA-mTvax 2.0 without activating T regulatory cells. Groups of BALB/c mice were vaccinated with MVA-mTvax 2.0 and lymph nodes harvested ten days later. Immune cells were isolated and stimulated with 6 different Survivin peptides representing the same Survivin epitopes included in mTvax 2.0. Control cells were not stimulated (Unstim) After 6 hours of incubation with Brefeldin A, cells were stained for the indicated antibodies and analyzed at the flow cytometer. Statistical differences between each Survivin peptide and not stimulated controls were evaluated with ANOVA (*$P \leq 0.01$).

In conclusion, in our experiments we compared different immunization protocols using two mTvax vaccines: mTvax and mTvax 2.0. mTvax demonstrated limited efficacy in terms of tumor reduction and improved survival. FP-mTvax was the only vector that induced statistically significant results, but its effects vanished when combined with OX86 antibodies. In the other hand, mTvax 2.0 showed significant anti-cancer responses with both MVA and FP vectors, and their efficacy was augmented by OX86. mTvax 2.0 also induced epitope-specific T cell responses for all the target antigens, without generating IL-10$^+$ or TGFβ^+ T regulatory cells.

Even if these results obtained with mTvax 2.0 were promising, we expected stronger anti-cancer properties from a vaccine that target multiple antigens. To understand if the reduced efficacy of mTvax 2.0 is due to a limited expression of its target antigens in the tumor tissue, we measured mRNA expression of MM tumors and normal tissues using Clariom S gene array. We evaluated the expression of 40,000 mRNA and together with the Bioinformatics core at our institution we calculated tumor vs.

normal fold changes in expression for each mRNA. As shown in Table 1, only three of the seven antigens included in mTvax 2.0 were expressed higher in CRH5 tumors than in normal tissues. Moreover, when we sorted all the genes based on their tumor vs. normal fold change mRNA expression, we found that in our cancer models there are several other antigens with higher tumor vs. normal fold change than those we included in mTvax 2.0.

Table 1. mRNA expression in CRH5 tumors and normal tissues measured with Clariom S microarray. The first row indicates the position for each gene in a list sorted from the highest tumors vs. normal fold change (Position 1) to the lowest (Position 39,998).

Position	CRH5 vs normal. Fold Change	Normal	CRH5	Gene Symbol
28	25.4712	6.4871	11.1579	S100a4
337	6.5087	3.298	6.0004	Fap
494	4.9774	7.2925	9.6079	Birc5
21682	-1.2182	4.5352	4.2505	T
25740	-1.7431	6.6504	5.8488	Wt1
26857	-2.3683	8.0068	6.7629	Mdk
27946	-4.6569	11.4069	9.1876	Kdr

With the results obtained from the Clariom S assay, we designed mTvax 3.0 that includes an optimal selection of antigens with the highest tumor vs. normal fold change. The epitopes from each target antigen were identified using EpiVax immune-informatics tools, including “Janus Matrix” that has been recently modified for mouse vaccine development and improved selection of highly immunogenic T cell epitopes (4). The epitopes for both $CD4^+$ and $CD8^+$ activation were included in one single transgene using the same structure of mTvax 2.0. Viral vector-based (MVA-mTvax 3.0 and FP-mTvax 3.0) vaccines were developed and their anti-cancer properties evaluated in Year 2 of this project. A peptide-based vaccine has been also developed (p-mTvax 3.0) and evaluated.

In May 2017 we submitted a change in SOW to add experiments with mTvax 2.0 and mTvax 3.0. The change is SOW has been approved and the timelines for Major Tasks 2 and 3 postponed to 6-15 months. Major Tasks 4 and 5 in the next reporting period were conducted using the mTvax vaccine that shows higher anti-cancer properties and induces stronger T cell immune responses.

Major Tasks for Year 2 as stated in the SOW:

Specific Aim 1, Major Task 4. Evaluate the efficacy of the most effective Tvax vaccine in eradicating late-stage tumors. Timeline: Months 15-18

Milestone not Achieved: Tvax vaccines were ineffective in eradicating completely early-stage tumors. Therefore, we did not perform experiments to evaluate the efficacy of Tvax vaccines in eradicating late-stage tumors.

Specific Aim 1, Major Task 5. Evaluate the efficacy of Tvax vaccines in the intraperitoneal tumor model of MM progression. Timeline: Months 15-18
Milestone Achieved: The most effective vaccination to reduce MM intraperitoneal tumors is identified.

Specific Aim 1, Major Task 6. Prepare and submit the manuscript. Timeline: Months 16-18
Milestone Achieved: The article including all the data obtained from the experiments included in Aim1 was submitted for publication in *Frontiers of Oncology*.

What was accomplished under these goals? (**Experimental results from Year 2)**
During the 1st year of this project, we developed different multi-antigen Tvax vaccines to treat MM in mice. The experiments performed to compare specific immune responses and anti-MM efficacy of these vaccines indicated Tvax 3.0 as the best in generating robust immune responses and significant anti-MM efficacy, without showing any sign of autoimmunity. In year 2, we used Tvax 3.0 to complete all the Major Tasks in Specific Aim 1. The results of these experiments were included in a manuscript that was submitted for publication in *Frontiers of Oncology*.

We have chosen the antigens for Tvax 3.0 by analyzing the entire transcriptome of two different MM tumors (CRH5 and EOH6) and two normal tissues (lung and kidneys) using a Clariom S gene array. We compared the expression of 40,000 mRNA and, together with the Bioinformatics core at our institution, we calculated tumor vs. normal fold changes in expression for each mRNA. The antigens that showed higher fold changes for both CRH5 and EOH6 MM tumors were those included in Tvax 3.0 vaccines. The expression levels of these antigens in normal and tumor tissues are showed in Figure 7.

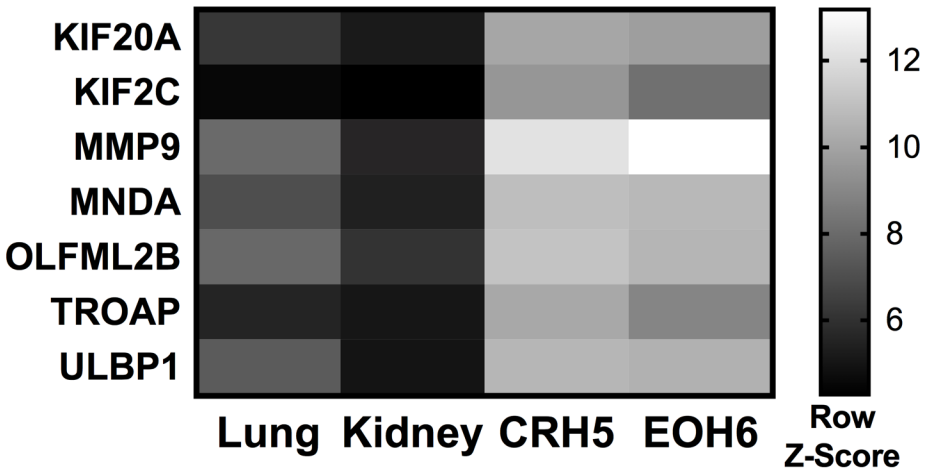

Figure 7. MM tumors overexpress the antigens included in Tvax 3.0. Whole transcriptome analysis was performed on two MM mouse tumors (CRH5 and EOH6) and two normal tissues (kidney and lung). mRNA levels are indicated by Row Z-Score colors in a heat map.

Immune stimulatory epitopes were then identified for each antigen using the informatic tools provided by our collaborator EpiVax Inc., and included in peptide-based vaccines (p-Tvax) or viral vector-based vaccines (MVA-Tvax). The p-Tvax vaccine includes a total of 7 peptides, one for each antigen, and is injected subcutaneously together with CpG as adjuvant. MVA-Tvax codifies multiple epitopes for each

antigen for a total of 56, and is injected intramuscularly. The development of Fowlpox vectors carrying the Tvax 3.0 antigens has been instead abandoned for technical issues. The list of the epitopes included in p-Tvax are showed in Table 2.

Table 2. p-Tvax 3.0 Epitopes

N.	Antigen	Start position	Peptide	Length
1	KIF20A	860	Ac-SSTDSSPYARILRSRHSPLLK-amide	21
2	KIF2C	356	GDLSGKSQNASKGIYAMASRDVFLLKN-amide	27
3	MMP9	594	RVFFFSGRQMWVYTGKTVLGPRSLDKLGL-amide	29
4	MNDA	298	Ac-NETSSVLEAAPKQMIEVPNCITRN -amide	24
5	OLFML2B	50	DNQENVLSQLLGDYDKVKAVSEGSD-amide	25
6	TROAP	156	Ac-KGGTTQRGQSARSSAYLAPRIPTH-amide	24
7	ULBP1	56	Ac-LNRQPLFVYKDKKCHAIGAHRNSMNATKI- -amide	29

When we performed experiments to evaluate the anti-MM efficacy of Tvax 3.0 vaccines, we observed significant delay in tumor growth and improved survival in mice carrying subcutaneous CRH5 tumors treated with p-Tvax alone or in combination with OX86 immune stimulatory antibodies. In intraperitoneal MM models using EOH6 cells, we also observed delay in tumor growth and improved survival following p-Tvax vaccination, but only in combination with OX86. Interestingly, in a different intraperitoneal model with AB12 cells, we observed delay in tumor growth using p-Tvax alone or in combination with OX86, but survival improvement was obtained only using both p-Tvax and OX86 (Figure 8).

It is important to emphasize that we did not know the transcriptome of AB12 MM cells. In these experiments, our purpose was to assess if anti-cancer vaccines designed to express antigens commonly expressed by different MM tumors, can be also used to treat MMs in which the antigen expression profile is unknown, but hypothetically express the common antigens discovered in other MM tumors.

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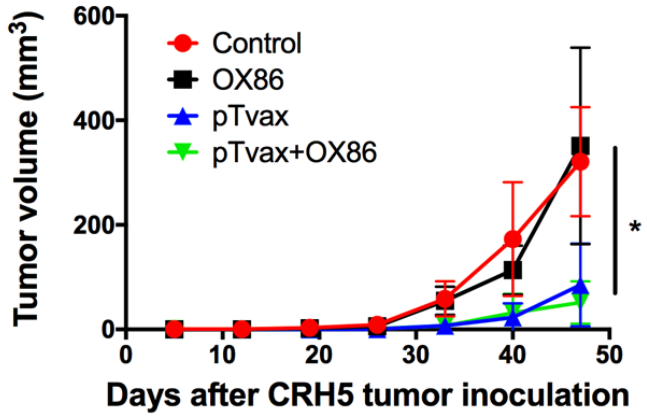
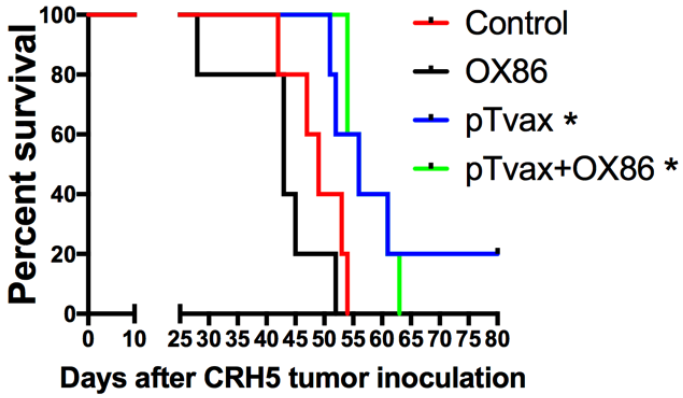
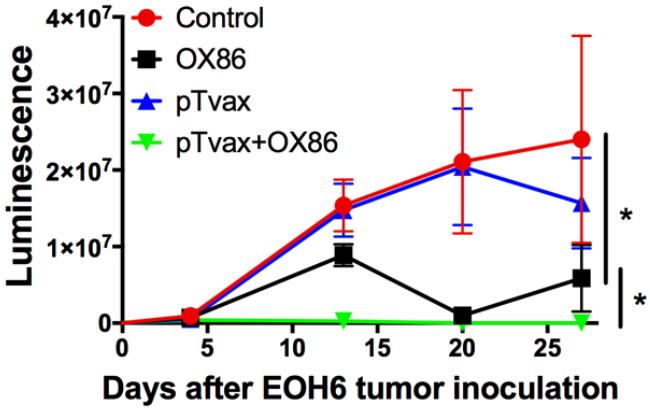

Tumor volume (mm3)
600
400
200
0
Control
OX86
pTvax
pTvax+OX86
*
0 10 20 30 40 50
Days after CRH5 tumor inoculation


Percent survival
100
80
60
40
20
0
Control
OX86
pTvax *
pTvax+OX86 *
0 10 25 30 35 40 45 50 55 60 65 70 75 80
Days after CRH5 tumor inoculation


Luminescence
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OX86
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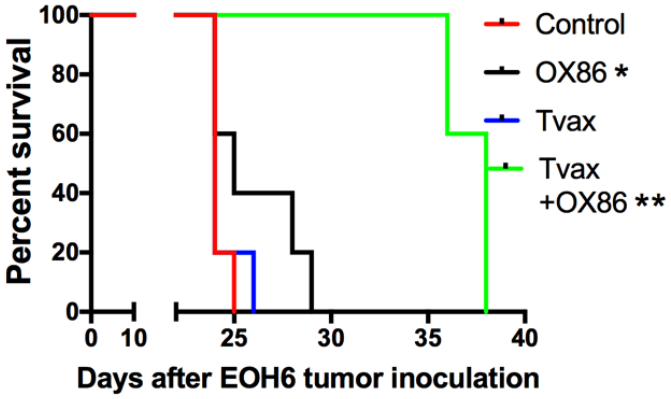
pTvax+OX86
*
*
0 5 10 15 20 25
Days after EOH6 tumor inoculation


Percent survival
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Control
OX86 *
Tvax
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+OX86 **
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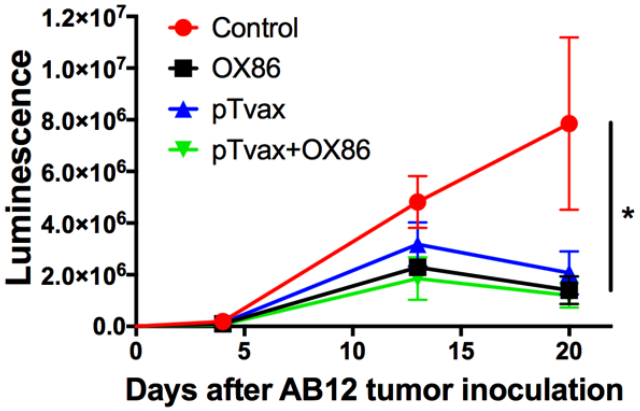
Days after EOH6 tumor inoculation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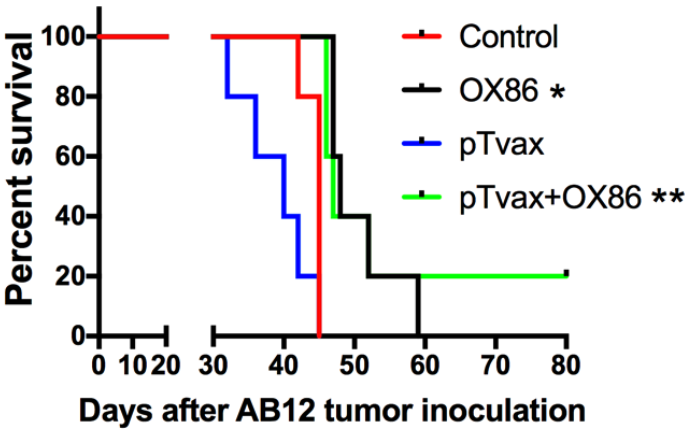
OX86
pTvax
pTvax+OX86
*
0 5 10 15 20
Days after AB12 tumor inoculation


Percent survival
100
80
60
40
20
0
Control
OX86 *
pTvax
pTvax+OX86 **
0 10 20 30 40 50 60 70 80
Days after AB12 tumor inoculation

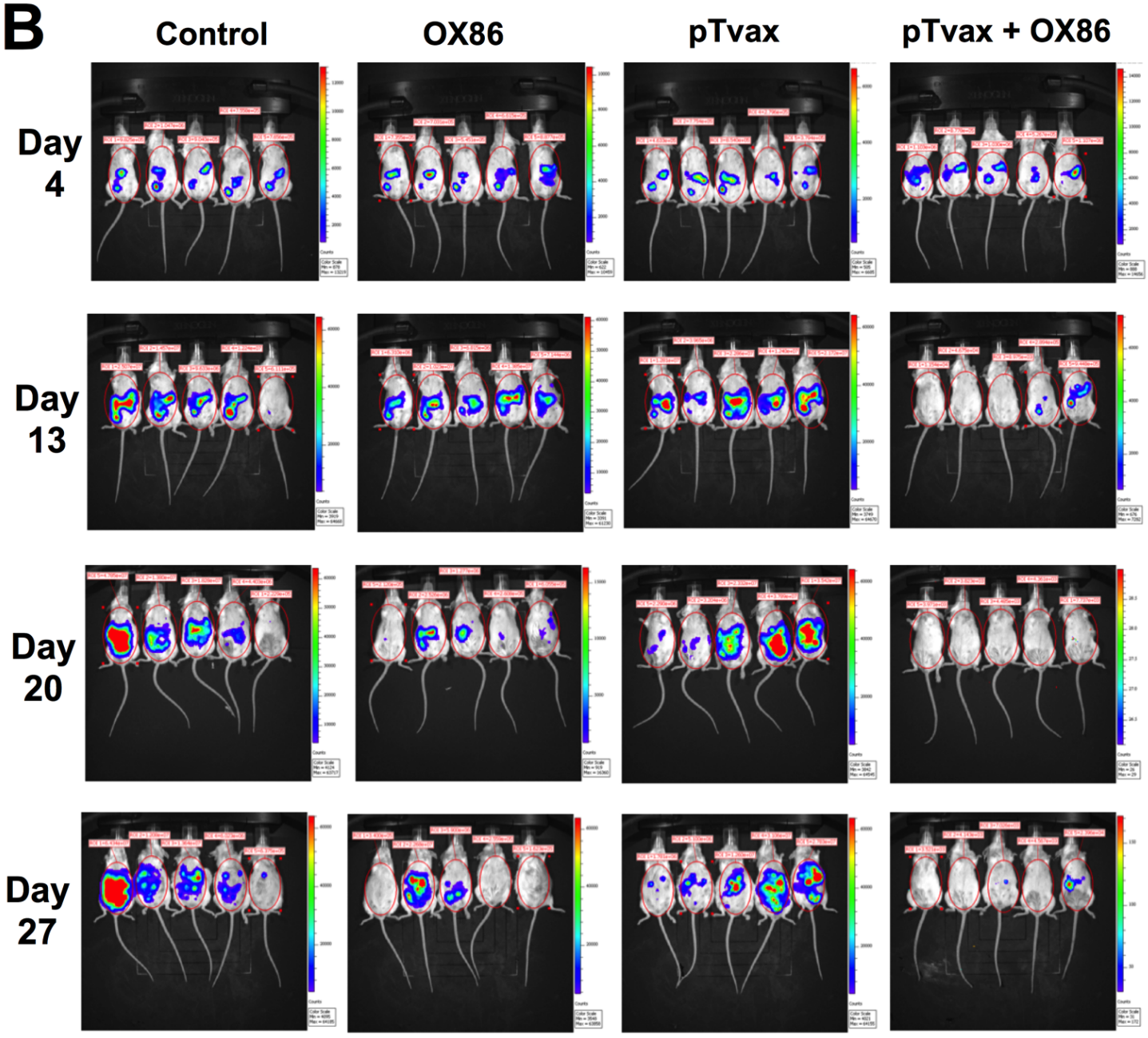

B
Control
OX86
pTvax
pTvax + OX86
Day 4
Day 13
Day 20
Day 27

Figure 8. Vaccination with p-Tvax delays tumor growth and improves survival in subcutaneous and intraperitoneal mouse models of MM. Panels A and B were enlarged to facilitate readability. A) BALB/c mice were injected subcutaneously with 100.000 CRH5 cells, or intraperitoneally with 50.000 of either EOH6 or AB12 cells, both expressing a luciferase gene. 7 and 14 days after tumor injection, mice were vaccinated with a subcutaneous injection of p-Tvax peptides plus CpG adjuvant. 200μg of OX86 were injected at day 9 and 14. Tumor volumes and animal survivals are shown for injection with three different cell lines: CRH5 (Top), EOH6 (Middle) and AB12 (Bottom). Tumors were measured weekly with a caliper for subcutaneous tumor. Intraperitoneal MM dimensions were instead assessed by measuring luciferase activity with IVIS imaging following injection with luciferin substrate. Statistical significance vs. Control or Single treatment was determined by ANOVA followed by Bonferroni test (*$p<0.05$). For survival, mice were followed until subcutaneous CRH5 tumors reached volumes of 300 mm^3 and were then sacrificed. In intraperitoneal models injected with EOH6 or AB12 MM cells, survival was assessed by euthanizing the mice at first sign of morbidity. Log-rank analysis was used to determine significance vs. Control (*) or vs. single treatment (**) ($p<0.05$). B) Representative images from IVIS imaging of mice carrying EOH6 tumors, vaccinated with the different immunotherapies.

Experiments using viral vector-based vaccines as MVA-Tvax, failed in providing MM growth delay or improved survival when used alone or in combination with either OX86 antibodies or with p-Tvax (Figure 9).

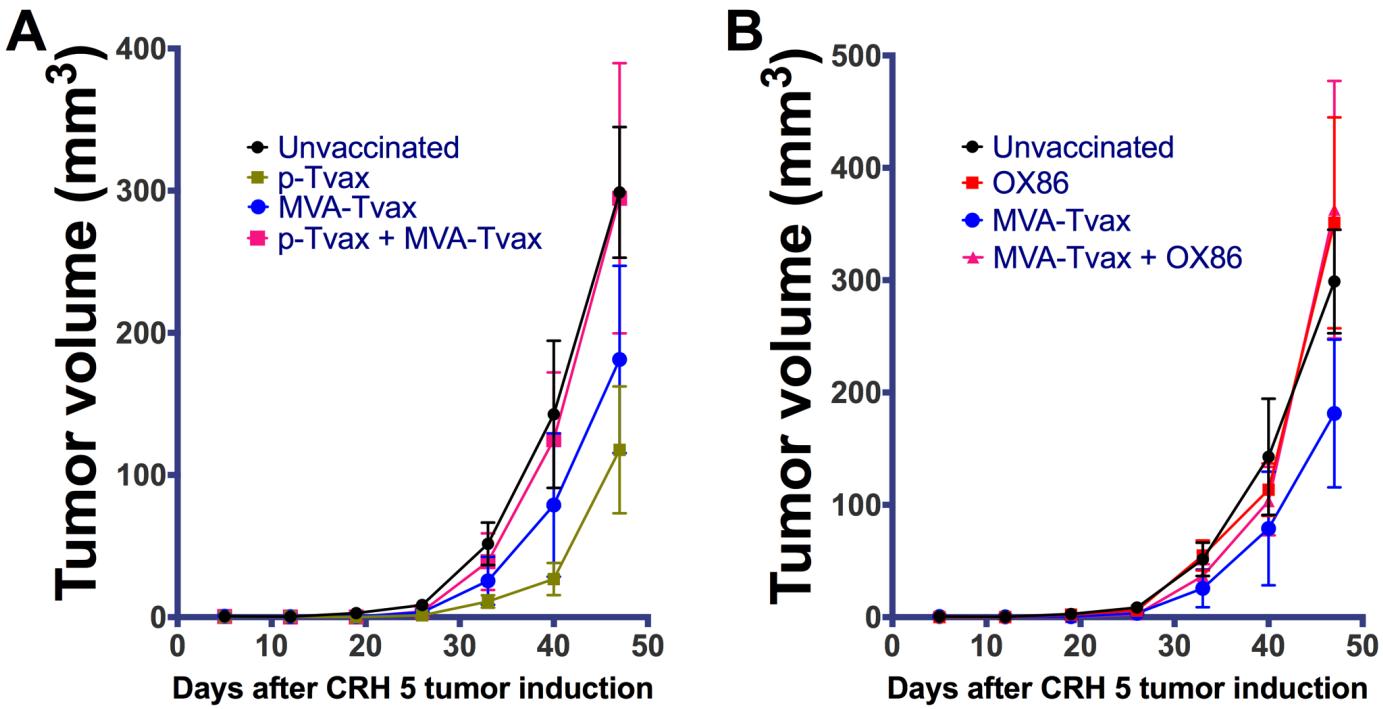
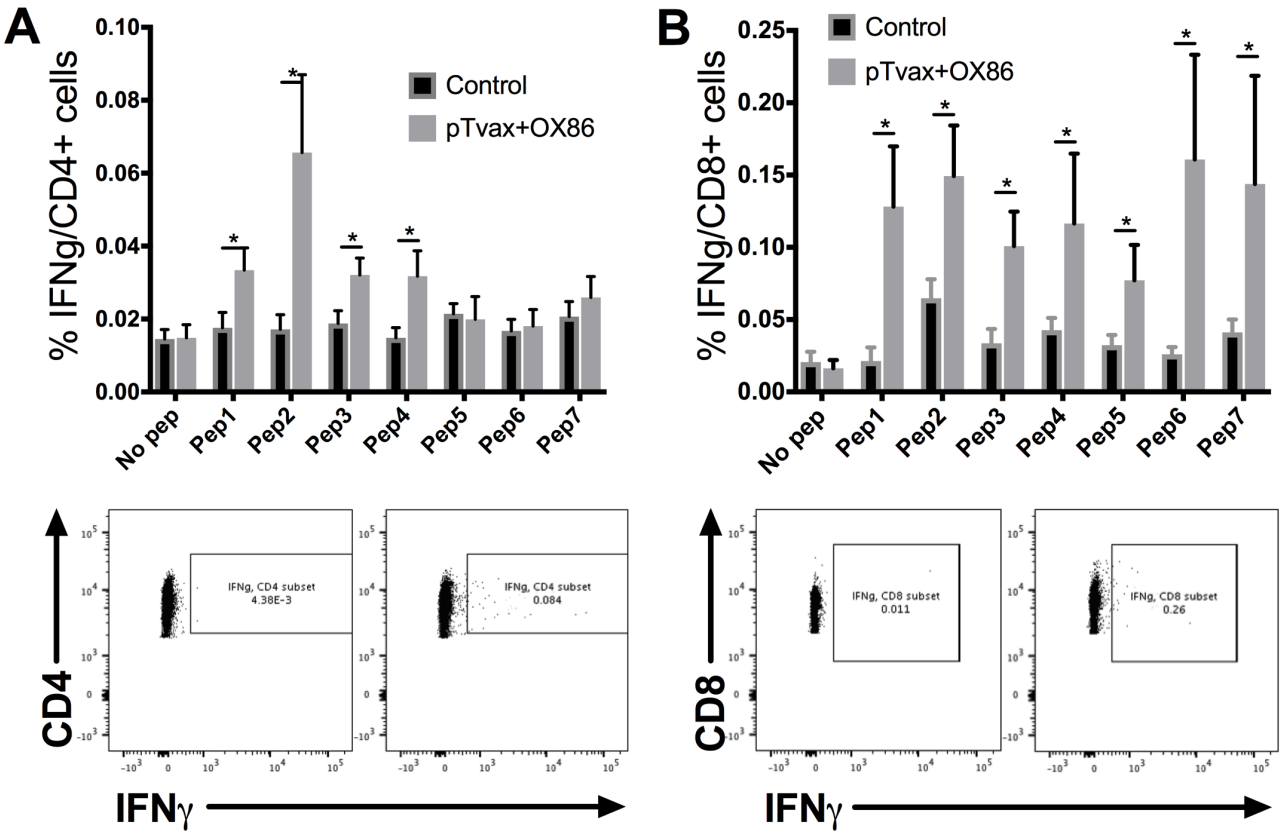


Figure 9. MVA-Tvax did not delay MM growth nor improved survival when used alone or in combination with either OX86 antibodies or with p-Tvax. BALB/c mice were injected subcutaneously with 100.000 CRH5 cells. 7 and 14 days after tumor injection, mice were vaccinated with either subcutaneous injection of p-Tvax peptides plus CpG adjuvant, or with an intramuscular injection of MVA-Tvax. 200μg of OX86 were injected at day 9 and 14. Tumors were measured weekly with a caliper.

Once we identified p-Tvax as the most effective and safe vaccine, we proceeded by analyzing the specific immune responses for each single peptide antigen. In these experiments, we discovered that peptides n.1, 2, 3 and 4 were able to stimulate both antigen-specific $CD4^+$ and $CD8^+$ T cells, while peptides n. 5, 6 and 7 stimulated only $CD8^+$ T cells. In other experiments, we evaluated if splenocytes from vaccinated mice, stimulated with all the Tvax peptides, secrete granzyme B when interact with MM cells. The results demonstrated that T cells from vaccinated mice can recognize and attack all the MM cells involved in this study (AB12, CRH5 and EOH6) as shown in Figure 10.


A
% IFNg/CD4+ cells
0.10
0.08
0.06
0.04
0.02
0.00
Control
pTvax+OX86
No pep
Pep1
Pep2
Pep3
Pep4
Pep5
Pep6
Pep7
B
% IFNg/CD8+ cells
0.25
0.20
0.15
0.10
0.05
0.00
Control
pTvax+OX86
No pep
Pep1
Pep2
Pep3
Pep4
Pep5
Pep6
Pep7
CD4
IFNγ
IFNg, CD4 subset
4.38E-3
IFNg, CD4 subset
0.084
CD8
IFNγ
IFNg, CD8 subset
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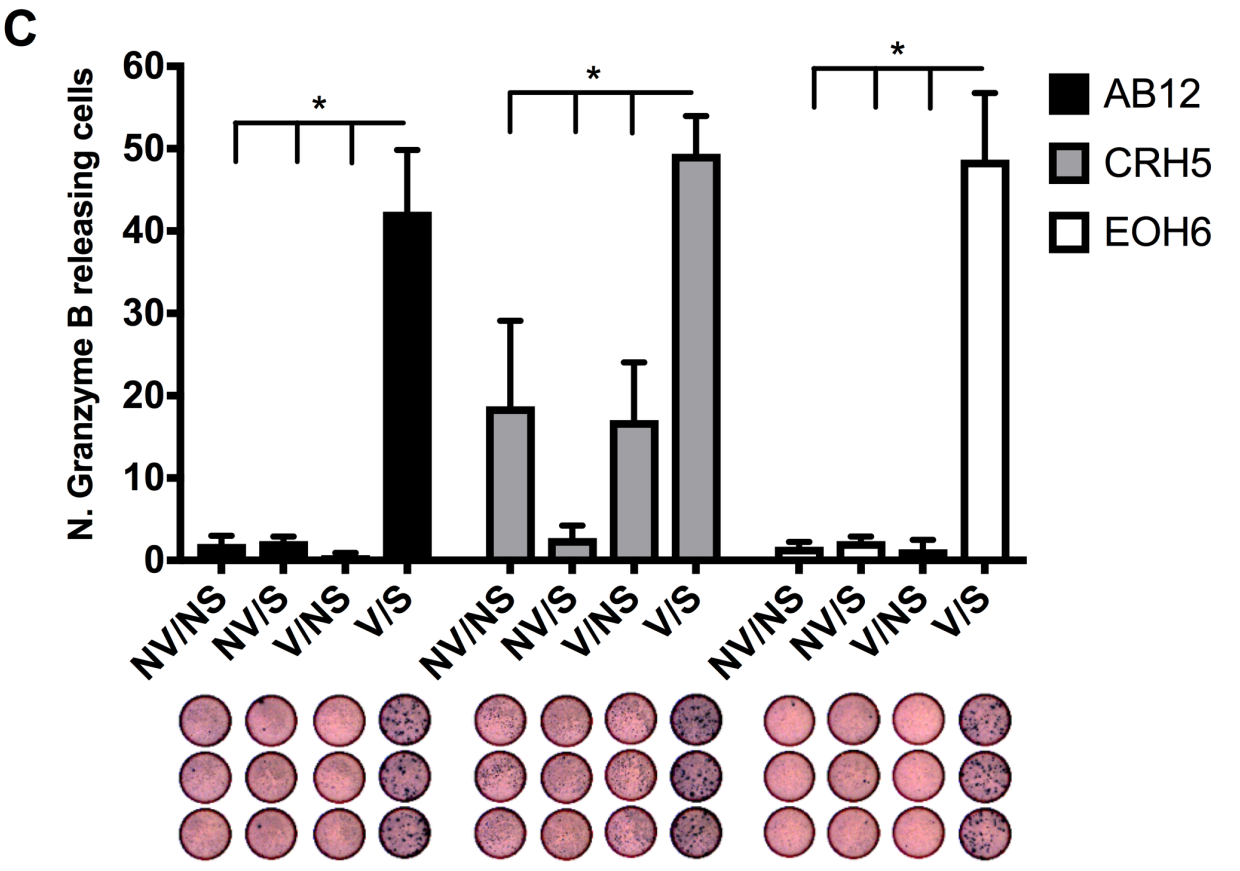
IFNg, CD8 subset
0.26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V/S

Figure 10. Induction of IFN-γ secreting T lymphocytes by p-Tvax vaccination. BALB/c mice were vaccinated with two subcutaneous injections of Tvax peptides +CpG (one week apart). Control mice were left untreated. Five days after the last vaccination spleen cells were isolated and enumerated for assays. A) Intracellular IFN-γ in $CD4^+$ T cells was evaluated by flow cytometric ICC analyses of spleen cells cultured with or without p-Tvax peptides. Percentage of IFN-γ expressing $CD4^+$ T cells are presented as mean ± S.E. Statistical significance vs. Control was determined by ANOVA followed by Bonferroni test (*p<0.05). Bottom Panel: Representative data from flow cytometric analysis of p-Tvax-vaccinated and control mice. Lymphocytes were either not stimulated (NP) or stimulated with peptide n.2. $CD4^+$ T cells were distinguished using a marker gate in the CD3 vs. CD4 dot plot. B) Data are showed for intracellular IFN-γ in $CD8^+$ T cells. C) Secretion of Granzyme B was evaluated by ELISPOT assay. Spleen cells from vaccinated mice (V) or unvaccinated (NV) were activated with p-Tvax peptides in the presence of 5 IU/mL IL-2 for 5 days (S) or incubated with no peptide (NS). Following 4 hours incubation with different MM cells, the number of as spot-forming cells per 10^5 cells was evaluated and results represented as mean ± S.E. Statistical differences between vaccinated mice, stimulated with peptides (V/S) and other conditions were evaluated by ANOVA followed by Bonferroni test (*p<0.05).

We also analyzed the phenotype of tumor-infiltrating lymphocytes in mice vaccinated with p-Tvax peptides alone or in combination with OX86 immune stimulatory antibodies. In these assays, we observed that p-Tvax significantly increases the number of $CD4^+$ T cells, while OX86 increases the number of $CD8^+$ T cells when are both compared with controls. OX86 also reduced the number of $CD4^+$ $CD25^+$ $FoxP3^+$ T regulatory cells in tumors from OX86-treated mice. When p-Tvax and OX86 were used in combination, we observed higher percentages of both $CD4^+$ and $CD8^+$T cells and statistically significant reduction of T regulatory cells (Figure 11). In tumors from mice treated with the different immunotherapy, we also analyzed the level of inflammatory cytokines as TNF-α, IFN-γ, IL-2, IL-4, IL-5, without detecting any difference among conditions.

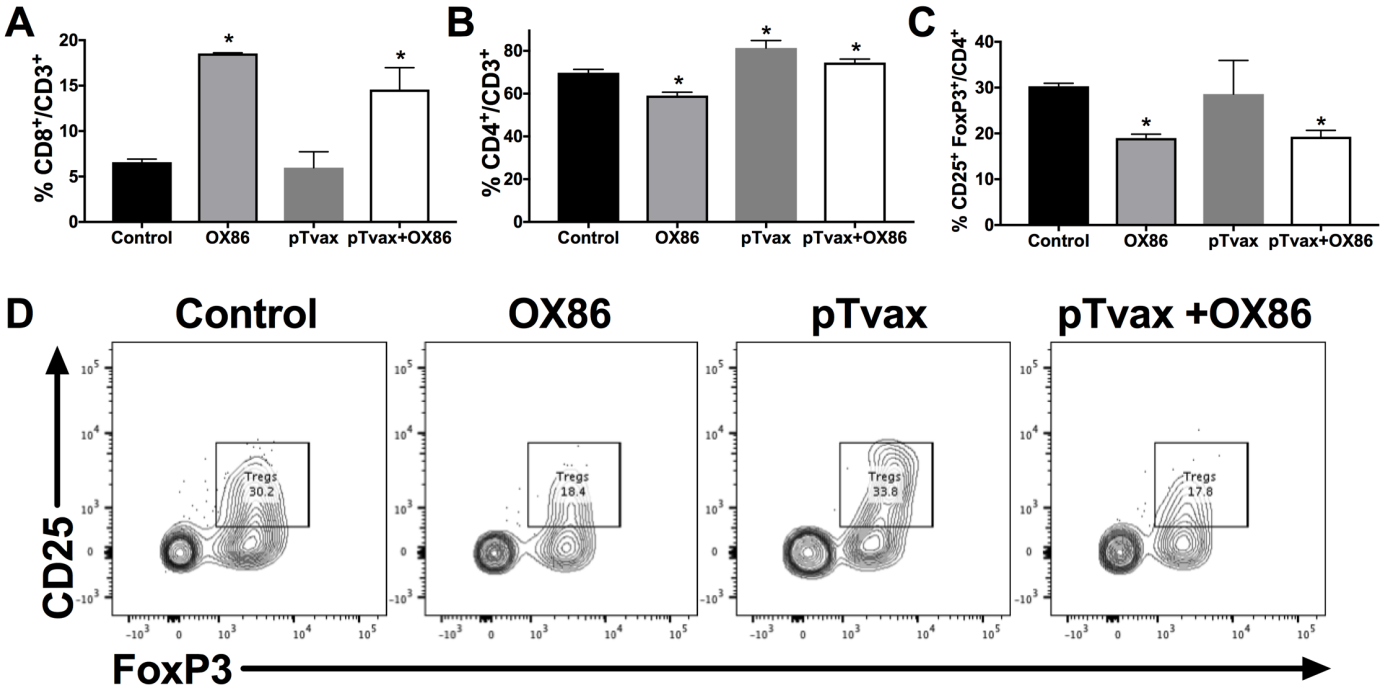

Figure 11. p-Tvax in combination with OX86 antibodies induce $CD8^+$ and $CD4^+$ T cell tumor infiltration while reducing T regulatory cells. Tumors from controls or from mice treated with either p-Tvax or OX86, or with a combination of the two, were collected and enzyme-digested. Staining was performed using anti-mouse CD8, CD4, CD25 and FoxP3 antibodies with live cells distinguished from debris using LIVE/DEAD® cell viability dye. A) Percentage of $CD8^+$ T cells in CRH5 tumors from treated and control mice. Results represent mean ± S.E. with means of each group compared using ANOVA followed by Bonferroni test. Data for $CD4^+$ T cells and T regulatory cells are showed respectively in (B) and (C). D) Representative data from flow cytometric analysis of CRH5 tumors in treated and control mice (*$p<0.05$).

We incorporated all these results obtained with p-Tvax and OX86 in a manuscript that has been submitted for publication in *Frontiers of Oncology* to complete all the tasks included in Specific Aim 1.

Major Tasks for Year 3 as stated in the SOW:

Specific Aim 2, Major Task 7. Determine the immunostimulatory capacities of the iVax-predicted peptides in human DC-T cell co-cultures. Timeline: Months 16-26

Task not achieved. We identified the antigens for the human version of Tvax (hTvax). We also performed the iVax analysis and identified a list of potentially immunogenic peptides. We did not perform the experiments to evaluate the immunostimulatory capacities of these peptides because of lack of funds.

Specific Aim 2, Major Task 8. Generate Tvax vaccines for human immunization. Timeline: Months 26-29

Task 50% achieved. Viral vectors expressing multiple cancer antigens have not been demonstrated by the experiments in Years 1 and 2 to induce significant anti-cancer responses. In Year 2 we demonstrated instead that a peptide vaccine (p-Tvax) induces MM tumor regression and strong anti-cancer immune responses. Therefore, we identified a list of new antigens that can be included in a peptide-based vaccine for human immunization. Unfortunately, we could not synthetize these peptides because of lack of funds. The Tvax vaccine for human immunization would have been a mixture of different peptides similar to p-Tvax that we published in *Frontiers of Oncology.*

Specific Aim 2, Major Task 9. Assess the immunogenicity of Tvax with in vitro immunization experiments using human DC-T cell cocultures. Timeline: Months 29-36

This task is not needed anymore. Viral vectors expressing multiple cancer antigens have not been demonstrated by the experiments in Years 1 and 2 to induce significant anti-tumor responses. In Year 2 we demonstrated instead that a peptide vaccine (p-Tvax) induces MM tumor regression and strong anti-cancer immune responses. The experiments to assess the immunogenicity of a peptide-based vaccine as p-Tvax are those included in **Specific Aim 2, Major Task 7.**

Specific Aim 2, Major Task 10. Prepare and submit manuscript. Timeline: Months 36-38

Milestone not Achieved due to a lack of funds.

What was accomplished under these goals? (Experimental results for Year 3)

During the 1st year of this project, we developed different multi-antigen Tvax vaccines to treat MM in mice and performed experiments to compare their specific immune responses and anti-MM efficacy.

Among these vaccines, Tvax 3.0 generated the most robust immune responses and significant anti-MM efficacy, without showing any sign of autoimmunity. In year 2, we used Tvax 3.0 to complete all the Major Tasks in Specific Aim 1. The results of these experiments were included in a manuscript that was submitted for publication at the beginning of the 3rd year of this project. The paper was reviewed positively for publication in Frontiers of Oncology, but several new experiments were requested to better characterize the immune responses generated by Tvax 3.0. The experiments were performed as requested by the reviewers and the paper was finally accepted for publication in July 2019. This paper includes all the data generated in the tasks included in Aim 1 of this project and is enclosed at the end of this report as an Appendix.

For specific Aim 2, we proposed to develop a clinical version of Tvax (hTvax) and evaluate its efficacy in activating human T cells from healthy donors. In the original proposal, we presented the first version of Tvax that included 7 antigens that were discovered to be overexpressed in the majority of human cancers (Survivin, Metastasin, Midkine, Wilms Tumor-1, Brachyury, Fibroblast Activation Protein and Vascular Endothelial Growth Factor Receptor 2). With the experiments performed during the 1st year of this project, we realized that these antigens may not be the most expressed in human MM tumors. Therefore, we have chosen new MM-specific antigens for the human version of Tvax by analyzing the data from recently published studies in which whole gene expression profiling was performed in MM tumors from human patients (5, 6). These antigens are: Hells, CCNO, CENPF, CHECK1, and KIF23.
With the support of our collaborator Dr. Moise from EpiVax, we analyzed these antigens with the iVax algorithm (4) and identified a list of potential immunogenic T cell epitopes. The experiments to evaluate the immunogenicity of the synthetic peptides representing these epitopes have been temporarily paused due to a lack of funds.

What opportunities for training and professional development has the project provided?
A career development plan for myself, the PI of this project, was included in the proposal for this DoD grant. During this reporting period, I had weekly one-on-one meetings with my mentor, Dr. Hoffmann, to discuss analyses of data and the ongoing planning of this project. I also met once a month with my secondary mentor Dr. Carbone to discuss the future direction of my researches.
I participated in the weekly seminars hosted by the Cancer Center and the Medical School at the University of Hawaii. I also attended some of the webinars organized by Epivax and the Institute of Immunology and Informatics at the University of Rhode Island.
To improve my experience in teaching, I presented lectures for the courses in “Cell and Molecular Biology” (CMB 622) and “Infection and Immunity” (TRMD 610) at the University of Hawaii.

How were the results disseminated to communities of interest?
We published a paper in *Frontiers of Oncology* with the results from the experiments listed in Aim1.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to report

IMPACT:
What was the impact on the development of the principal discipline(s) of the project?
The main goal of this translation proposal is to test innovative vaccines in animal models of MM progression to identify new therapeutic and preventive interventions. In the therapeutic setting, Tvax vaccines may be used to induce tumor regression in patients with MM at the early stages of the disease.

In the case of most late-state tumors, chemotherapy or surgery can be used to reduce tumor burden. In these patients, immunotherapy with Tvax vaccines can be used post-treatment to stimulate the immune system in killing remaining cancer cells and increase post-cancer survival. Our studies would also provide the framework for developing vaccines to prevent cancer development in people exposed to asbestos and/or genetically predisposed who are at high risk for developing MM in the future. Investigators at the University of Hawaii Cancer Center have discovered germline Bap1 mutations in families with a high incidence of MM. Besides, two biomarkers, Soluble Mesothelin Related Peptides (SMRP) and Osteopontin (OPN) have garnered interest in recent years as a means of detecting the recurrence of MM. This progress has enabled the use of PCR analysis to detect Bap1 mutations and the use of ELISA to evaluate SMRP and OPN protein levels as a means of identifying patients at high risk of developing MM for early intervention (7).

What was the impact on other disciplines?
Nothing to report

What was the impact on technology transfer?
A provisional patent describing the peptides included in Tvax 3.0 has been filed by the University of Hawaii.

What was the impact on society beyond science and technology?
Nothing to report

CHANGES/PROBLEMS:
Changes in approach and reasons for change
In our original proposal, we planned to develop and evaluate the anti-cancer efficacy of different vaccine platforms as DNA, peptide, and viral vectors. Among these vaccines, we expected to obtain the best results from viral vector-based vaccines. Unexpectedly, these vaccines lacked in anti-cancer efficacy and displayed signs of auto-immunity in several experiments performed in Year 1 of this project. Therefore, we conducted our studies using only peptide vaccines as they were the most effective in delaying tumor growth, without inducing autoimmunity.

Some of the negative results obtained in Year 1 of this project were also caused by a reduced expression in mouse MM tumors of the antigens included in the first prototypes of the Tvax vaccines. Therefore, we had to perform a whole transcriptome analysis and compare protein expression between MM and normal tissues to identify a new set of antigens. These new targets presented more effective anti-cancer responses than the original antigens, without generating auto-immunity. The results obtained with this new set of antigens are shown in the attached article published in *Frontier of Oncology*.

Actual or anticipated problems or delays and actions or plans to resolve them
For the reasons outlined above, we spent more time than expected to identify the most effective vaccine platform and to select new target antigens for our experiments. Moreover, once the manuscript with all the data from Aim 1 was submitted, the reviewers from *Frontier of Oncology* took several months to provide their criticisms and requested numerous experiments before accepting the paper for publication. These circumstances delayed our work of about one year. Therefore, we requested a no-cost extension

of this project until August 2020. Unfortunately, the lack of funds did not allow us to perform all the experiments listed in Aim 2 and complete the project.

An agreement has been made between the PI and his institution that will provide additional funds to complete this project if the PI will be awarded a new grant. In the last year, the PI of this project submitted grant proposals to both NIH and DoD that received good scores but were not funded. The NIH R01 grant received an impact score of 33 (Percentile 23). The DoD Impact Award received an "excellent" score of 1.6 (1.0 highest merit, 5.0 lowest merit). Both grants have been submitted again by the PI.

Changes that had a significant impact on expenditures
The whole transcriptome analysis performed to identify a new set of MM-specific antigens instead of those included in the original grant proposal consumed a significant part of the funds provided by this grant. Therefore, the funds finished before completing all the experiments listed in Aim 2.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

PRODUCTS:
Publications, conference papers, and presentations

- **Journal publications.**

    - Marciel MP, Khadka VS, Deng Y, Kilicaslan P, Pham A, **Bertino P**, Lee K, Chen S, Glibetic N, Hoffmann FW, Matter ML, Hoffmann PR. Selenoprotein K deficiency inhibits melanoma by reducing calcium flux required for tumor growth and metastasis. Oncotarget. 2018 Feb 3;9(17):13407-13422. doi: 10.18632/oncotarget.24388. eCollection 2018 Mar 2. Erratum in: Oncotarget. 2018 Jul 20;9(56):30937. PMID: 29568366. Status of publication: published. Acknowledgement of federal support: No.

    - Marciel MP, Rose AH, Martinez V, Horio DT, Hashimoto AS, Hoffmann FW, **Bertino P**, Hoffmann PR. Calpain-2 inhibitor treatment preferentially reduces tumor progression for human colon cancer cells expressing highest levels of this enzyme. Cancer Med. 2018 Jan;7(1):175-183. doi: 10.1002/cam4.1260. Epub 2017 Dec 6. PMID: 29210197. Status of publication: published. Acknowledgement of federal support: No.

    - **Bertino P,** Premeaux AT, Fujita T, Haun BK, Marciel MP, Hoffmann FW, Garcia A, Yang H, Pastorino S, Carbone M, Niki T, Berestecky J, Hoffmann PR, and Ndhlovu LC. Targeting the C-terminus of galectin-9 induces mesothelioma apoptosis and M2 macrophage depletion. Oncoimmunology. 2019 Apr;8(8). doi: 10.1080/2162402X.2019.1601482. Status of publication: published. Acknowledgement of federal support: No.

    - Peter R. Hoffmann, Fukun W. Hoffmann, Thomas A. Premeaux, Tsuyoshi Fujita, Elisa Soprana, Maddalena Panigada, Glen M. Chew, Guilhem Richard, Pooja Hindocha, Mark Menor, Vedbar S. Khadka, Youping Deng, Lenny Moise, Lishomwa C. Ndhlovu, Antonio

Siccardi, Andrew D. Weinberg, Anne S. De Groot and **Pietro Bertino**. Multi-antigen Vaccination With Simultaneous Engagement of the OX40 Receptor Delays Malignant Mesothelioma Growth and Increases Survival in Animal Models. Frontiers in Oncology. 2019 Aug; In press. doi: 10.3389/fonc.2019.00720. Status of publication: published. Acknowledgement of federal support: Yes.

- **Books or other non-periodical, one-time publications.**
  Nothing to Report

- **Other publications, conference papers, and presentations.**
  Some of the data from this project were presented during the "Cancer Biology Winter Retreat" and the "Cancer Immunotherapy Symposium" hosted by the Cancer Center of the University of Hawaii the 8th of December 2017 and the 17th of July 2018 respectively.

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
During this reporting period, we produced DNA-based, peptide-based and viral vector-based vaccines expressing different set of cancer antigen. These vaccines will be available for other research projects performed in my lab or Dr. Hoffmann lab. In case of collaboration, this vaccine can be also shared with other laboratories at the Medical School and/or Cancer Center of the University of Hawaii.

Inventions, patent applications, and/or licenses
A provisional patent describing the peptides included in Tvax 3.0 has been filed by the University of Hawaii.

Other Products
The data gathered from the whole transcriptome analysis of mouse MM tumors and normal tissues were deposited in the Gene Expression Omnibus (GEO) database (Accession Number: GSE122004).

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:
What individuals have worked on the project?
Name: Pietro Bertino
Project Role: PI
Research Identifier (ERA Commons ID): pbertino
Nearest person month worked: 9
Contribution to the project: Dr. Bertino had overall responsibility for all aspects of the project. He conducted experiments and analyzed the data. He also was in charge of preparing regulatory approval requests, progress reports and manuscripts.
Funding Support: This award and bridging funds from the University of Hawaii.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

SPECIAL REPORTING REQUIREMENTS: Not applicable

APPENDICES:
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ORIGINAL RESEARCH
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Multi-antigen Vaccination With Simultaneous Engagement of the OX40 Receptor Delays Malignant Mesothelioma Growth and Increases Survival in Animal Models

*Peter R. Hoffmann[1], Fukun W. Hoffmann[1], Thomas A. Premeaux[2], Tsuyoshi Fujita[2], Elisa Soprana[3], Maddalena Panigada[3], Glen M. Chew[2], Guilhem Richard[4], Pooja Hindocha[4], Mark Menor[5], Vedbar S. Khadka[5], Youping Deng[5], Lenny Moise[4,6], Lishomwa C. Ndhlovu[2], Antonio Siccardi[3], Andrew D. Weinberg[7], Anne S. De Groot[4,6] and Pietro Bertino[1]**

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Malignant Mesothelioma (MM) is a rare and highly aggressive cancer that develops from mesothelial cells lining the pleura and other internal cavities, and is often associated with asbestos exposure. To date, no effective treatments have been made available for this pathology. Herein, we propose a novel immunotherapeutic approach based on a unique vaccine targeting a series of antigens that we found expressed in different MM tumors, but largely undetectable in normal tissues. This vaccine, that we term p-Tvax, is comprised of a series of immunogenic peptides presented by both MHC-I and -II to generate robust immune responses. The peptides were designed using *in silico* algorithms that discriminate between highly immunogenic T cell epitopes and other harmful epitopes, such as suppressive regulatory T cell epitopes and autoimmune epitopes. Vaccination of mice with p-Tvax led to antigen-specific immune responses that involved both $CD8^+$ and $CD4^+$ T cells, which exhibited cytolytic activity against MM cells *in vitro*. In mice carrying MM tumors, p-Tvax increased tumor infiltration of $CD4^+$ T cells. Moreover, combining p-Tvax with an OX40 agonist led to decreased tumor growth and increased survival. Mice treated with this combination immunotherapy displayed higher numbers of tumor-infiltrating $CD8^+$ and $CD4^+$ T cells and reduced T regulatory cells in tumors. Collectively, these data suggest that the combination of p-Tvax with an OX40 agonist could be an effective strategy for MM treatment.

Keywords: cancer vaccines, OX40, mesothelioma, epimatrix, immunotherapy

INTRODUCTION

Malignant Mesothelioma (MM) is a rare but highly aggressive cancer that develops from mesothelial cells lining the pleura and other internal cavities and is often associated with asbestos exposure. To date, no effective treatments are available for MM (1). Over the past decade, immunotherapeutic approaches have created new opportunities to efficiently combat cancer progression. The introduction of CAR T-cell therapies as well as the successful use of antibody-based inhibitors of immune checkpoints (e.g., Cytotoxic T Lymphocyte Associated protein 4, CTLA-4; Programmed cell Dead protein 1, PD-1; and its ligand PD-L1) have invigorated the field of immunotherapy and benefited an increasing number of cancer patients (2). Anti-tumor therapy using personalized vaccines individually tailored for immune responses against the patient's mutanome have been demonstrated to improve the therapeutic outcome of biologics (3, 4). This approach, however, includes limitations such as potential delays between biopsy, design and production of the vaccine, in addition to high costs for vaccine production. Moreover, MM vaccines containing epitopes that are common to MM may be used as a means to prevent tumor development, for individuals exposed to asbestos and in regions of the world where MM risk is high. MM requires up to 40 years from the time of asbestos exposure to the development of disease, therefore, it is conceivable that preventive vaccination approaches such as the one currently used for HPV-associated cervical cancer might similarly reduce MM incidence.

Herein, we describe the development of a novel immunotherapeutic approach based on a unique vaccine that targets a series of self-antigens that we found to be commonly over-expressed in different MM mouse tumors compared to normal tissues. Efficacious treatment using this vaccine is demonstrated in three different MM animal models, suggesting this approach may be used as a model to develop an off-the-shelf immunotherapy for human patients that overcomes some of the limitations of personalized anti-cancer vaccines described above. Our vaccine, named p-Tvax, is comprised of a series of immunogenic peptides containing both MHC-I and -II restricted T cell epitopes and derived from multiple MM antigens. The antigens included in the vaccine were chosen based on their high expression in MM tumor tissues and low levels in normal tissues in an attempt to minimize the risk of triggering immune responses against healthy tissues. Each peptide was designed using the iVAX platform comprising of state-of-the-art *in silico* algorithms including EpiMatrix and JanusMatrix, which have been previously demonstrated as effective tools to discriminate between highly immunogenic T cell epitopes and other undesirable epitopes such as suppressive regulatory T cell epitopes (Tregitopes) or autoimmune epitopes (autoepitopes) (5–7). JanusMatrix's ability to identify tumor epitopes cross-conserved with autoepitopes is particularly relevant for designing new cancer immunotherapies, as exemplified by previous therapies failing due to off-target cardiac or neurologic toxicities (8, 9).

Even if peptide-based vaccines are found to activate anti-cancer T cells, the efficacy of these immune cells is mitigated within the tumor microenvironment by several mechanisms. Many of these suppressive mechanisms are driven by immunological checkpoint molecules such as PD-1 and CTLA-4, or by immune co-stimulatory proteins such as OX40. To overcome these suppressive mechanisms and generate effective anti-tumor immune responses, we have combined p-Tvax with an OX40 agonist. OX40 is a Tumor Necrosis Factor receptor family member that is expressed by both activated T effector cells and Foxp3$^+$ T regulatory cells (Tregs). Ligands that promote OX40 signaling, as well as agonistic monoclonal antibodies (mAbs) that target this molecule, induce the activation and proliferation of effector T cells, while reducing Treg activity through the inhibition of Foxp3 gene expression (10–12). Humanized versions of OX40 agonists have been positively evaluated in a phase I clinical trial, and are now under investigation in phase II (13). In this study, we present results of this dual therapeutic approach that support the concept that a universal cancer vaccine for MM may offer a safe and potentially curative therapy for this deadly cancer when combined with mAbs that target T cell co-stimulation.

MATERIALS AND METHODS

Mice and Cells

Female 6–8 week-old BALB/c mice were obtained from the Jackson Laboratory. Animal experiments were performed in accordance with institutional guidelines and approved by the University of Hawaii IACUC (#16-2355). Murine AB12 MM cells derived from asbestos-induced tumors in a BALB/c mouse were provided by Dr. B. Robinson (University of Western Australia, Nedlands, Australia) (14). Murine CRH5 and EOH6 MM cells were isolated from peritoneal ascites developed in asbestos- or erionite-injected mice in carcinogenesis experiments as previously described (15). Mesothelial cells were isolated as previously described from naive BALB/c mice (16). All cells were cultured in Ham's F12 medium (Corning) containing 10% fetal bovine serum (FBS) and antibiotics. All MM cells used in this study were provided to our laboratories or purchased between 2004 and 2007.

Transcriptome Microarray Analysis

BALB/c mice were injected subcutaneously (s.c.) with 10^5 of either CRH5 or EOH6 MM cells. When tumors reached 100 mm^3 they were excised and total RNA was extracted. At the same time, RNA was isolated from lungs and kidneys excised from naïve BALB/c mice. RNA expression in the different tissues was evaluated using the Clariom S Mouse Array (Affymetrix). Expression values were normalized and summarized into transcript clusters for analysis using Robust Multi-array Average approach in Array Studio (OmicSoft, Cary, NC). One-way ANOVA was used to look for differential expression between normal and tumor samples, and *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg False Discovery Rate (FDR) method. Only candidates with FDR-adjusted $p < 0.001$ were considered. The data gathered from this analysis were deposited in the Gene Expression Omnibus (GEO) database (Accession Number: GSE122004).

Western Blot Analysis

Frozen tumors and normal tissues were lysed in ice-cold buffer containing 150 mM NaCl, 50 mM Tris, 1% Triton X-100, 1% sodium deoxycholate, and protease inhibitor cocktail (Roche Applied) at 4°C for 1 h. Insoluble material was removed by centrifugation at maximum speed for 5 min, and total protein in the supernatant was determined using a Bradford assay reagent (Bio-Rad). After adjusting to equal protein concentration, lysates were boiled in SDS sample buffer and then separated by SDS-PAGE, followed by transfer of the proteins onto nitrocellulose membranes. Blots were incubated with primary anti-TROAP (Clone 3-11, Novus Biological), anti-OLFML2B (Mybiosurce), anti-KIF20A (Clone D-3 Santa Cruz), or anti-β-actin (Sigma) for 1.5 h, washed, incubated with appropriate HRP-conjugated secondary antibody (1:20,000; Li-Cor), and visualized using the Odyssey Scanner (Li-Cor).

Epitope Selection and Peptide Synthesis

Antigens selected after the transcriptomic analysis were screened for BALB/c MHC-I (H2-Dd, H2-Kd) and MHC-II (H2-IAd, H2-IEd) restricted T cell epitopes using the EpiMatrix algorithm. In addition, putative Tregitopes and autoepitopes were identified using the JanusMatrix algorithm (6). Briefly, each protein sequence was first parsed into overlapping 9-mer frames. Each frame was then evaluated with EpiMatrix and JanusMatrix to determine its likelihood of binding to MHC-I (H2-Dd, H2-Kd) and MHC-II (H2-IAd, H2-IEd) alleles and its potential to induce Tregs, respectively. One peptide sequence enriched in MHC-I and -II epitopes and devoid of putative Tregitopes was derived from each selected antigen. Synthetic peptides were manufactured by twenty-first century Biochemicals (Marlboro, MA) using fluorenylmethoxycarbonyl chemistry and solid-phase synthesis and purified by high-pressure liquid chromatography. The quality of the peptides was assessed by high-performance liquid chromatography analysis. Peptide purity was >90% as ascertained by analytical reversed phase HPLC. Individual peptides were dissolved with 10 μl DMSO, diluted to 1 mg/ml with PBS and used at a final concentration of 10 μg/ml in *ex vivo* assays (DMSO 0.001%). A mixture of all peptides (10 μg/peptide), which we termed p-Tvax, was used to vaccinate mice in 100 μl PBS.

Immunotherapies Schedule and Flow Cytometric Analysis for Intracellular IFN-γ

BALB/c mice were vaccinated with two s.c. injections of p-Tvax peptides (10 μg of each of the seven peptides, diluted in PBS), 1 week apart (day 2 and day 9). Two days before each vaccination, 50 μg CpG ODN 1585 adjuvant (Invitrogen) was injected at day 0 and day 7, while 200 μg of OX40 agonists (clone OX86, kindly provided by Dr. A. Weinberg) were injected at day 4 and day 9. Five days after the last vaccination, spleens were excised from controls, and from mice treated with the different immunotherapies. One million cells were stimulated for 24 h with 10 μg/ml of each p-Tvax peptides. For detection of IFN-γ in the cytoplasm, Brefeldin A was added 6 h before staining. Fixation/permeabilization kits were used in combination with the following fluorochrome conjugated monoclonal antibodies: Anti-CD3-PerCP/Cy5.5 (clone OKT3), anti-CD4-AlexaFluor700 (clone RM4-5), anti-CD8-APC/Cy7 (clone 53-6.7), and IFN-γ-FITC (clone XMG1.2) antibodies. Live cells were distinguished from debris using Aqua LIVE/DEAD® cell viability dye (all from Biolegend). Cells were evaluated using LSRFortessa Flow Cytometer (BD Biosciences) and the data were analyzed with FlowJo software.

Granzyme B ELISpot Assay

To measure T cell cytotoxicity, granzyme B secretion was analyzed using the Mouse Granzyme B ELISpot Kit (R&D system). Splenocytes were cultured with each single p-Tvax peptide for 6 d, with peptides replenished every 2 d. At day 3, cultures were supplemented with 5 IU/ml IL-2. At day 6, splenocytes stimulated with the different peptides were pooled together and dead cells removed with Lympholyte M (Cedarlane). Splenocytes not stimulated with the peptides were processed similarly. Stimulated and control splenocytes (10^4 effector cells) were then co-cultured with different MM cells (5 × 10^3 target cells) or with mesothelial cells in 200 μL media. Negative controls consisted of effector cells in the absence of target cells, target cells in the absence of effector cells and media only. After 4 h of incubation, detection of granzyme B spots was performed following the manufacturer's directions. Spots were then analyzed and counted with an ImmunoSpot analyzer (CTL), with the instrument sensitivity kept low to reduce background.

Murine Therapeutic Experiments

To evaluate the efficacy of the different immunotherapies on tumor dimensions and mouse survival, s.c., and intraperitoneal (i.p.) mouse models of MM were employed. In the s.c. model, 5 × 10^4 CRH5 cells were injected in the hind flank in cohorts of five BALB/c mice. When tumors became palpable on day 7 (3–4 mm in maximal diameter), mice were vaccinated as described above. Tumor size was measured weekly using digital calipers until the first death was recorded. Survival was then followed until tumors reached volumes >300 mm^3. For the i.p. model of MM, 2 × 10^5 EOH6 cells or 5 × 10^4 AB12 cells were injected i.p. in cohorts of 5 BALB/c mice. Both MM cells were previously transduced with the lentiviral vector hPGK.lu2.WPRE.mhCMV.dNGFR.SV40PA, which encoded the bioluminescent genetic marker luciferase (provided by Dr. Naldini, San Raffaele University and Research Institute, Milano, Italy). Immunotherapies were performed as scheduled for the s.c. model. To assess tumor dimension and localization of luminescent cells, mice were injected i.p. with 15 mg/ml d-luciferin, bioluminescence signals of MM inoculated mice were monitored using the IVIS system (PerkinElmer). Regions of interest were identified around the tumor sites and were quantified as total photon counts using Living Image software (PerkinElmer). For survival, animals were monitored weekly and euthanized when they appeared moribund according to IACUC guidelines.

Isolation and Analysis of Tumor-Infiltrating Immune Cells

CRH5 or EOH6 MM cells were injected s.c. in cohorts of five BALB/c mice. When tumors reached 50 mm in maximal

diameter, mice received the same immunotherapies regimens indicated above. Five days after the last vaccination, tumors were excised, washed with PBS, minced and incubated for 1 h at 37°C in digestion buffer consisting of 1 mg/ml collagenase IV, 100 μl/ml hyalurodinase and 15 mg/ml DNAse I (all from Roche Applied Sciences) in PBS. After digestion, tumors were forced through a 40 μm cell strainer. A total of 10^6 cells were stained for flow cytometer analysis to characterize tumor-infiltrating T lymphocytes. The same fluorochrome conjugated monoclonal antibodies used for the analysis of T cell responses were utilized plus anti-CD25-PE/Cy7 clone PC61 and anti-FoxP3-FITC clone MF-14 for Tregs analysis, or plus anti-PD-1-PE/Cy7 clone RMP1-30 and anti-OX40-PE clone OX86 for T cell marker analysis (all from Biolegend). Cells were analyzed using LSRFortessa Flow Cytometer (BD Biosciences) and data organized with FlowJo software.

Statistical Methods

All statistical tests were performed using GraphPad Prism 7.0. Means were compared using two-way ANOVA followed by the Bonferroni multiple comparison test. For survival, differences were evaluated using Kaplan-Meier curves with log-rank test. Data are represented as mean ± S.E. with statistical significance values indicated in the figure legends together with the n values used to calculate the statistics. All *in vitro* experiments with MM cells have been repeated at least three times using samples from the same source as technical replicates. *In vivo* studies as well as experiments with primary cells were also repeated at least three times using different sources as biological replicates.

RESULTS

Transcriptome Analysis and Antigen Selection

With the goal of designing a universal vaccine that can be used to target MM as tested in mice, we analyzed expression of all the mRNA produced by different MM tumors and normal tissues. Our hypothesis was that antigens highly expressed in MM tumors can be targeted for vaccination, as long the expression of those antigens is extremely low in all normal mouse tissues. mRNA was analyzed in two tumors originating from two different MM cell lines injected in BALB/c mice, CRH5, and EOH6. These cells were previously generated by injecting asbestos or erionite in the same mouse strain (15). For the normal tissues, we analyzed antigen expression in lungs and kidneys isolated from naïve BALB/c mice. Selected antigens had the highest expression in both MM tumors and the lowest in both normal tissues. Among these, only antigens that were commonly overexpressed by both MM tumors were included in the multi-antigen universal vaccine. Since we analyzed only two normal mouse tissues, we also considered transcriptome studies performed in other studies to evaluate the expression of the selected antigens in normal tissues (17, 18). At the end of this process, we selected the following seven antigens: KIF20A, KIF2C, MMP9, MNDA, OLFML2B, TROAP, and ULBP1. The mRNA expression levels for these antigens in tumor and normal tissues are shown in **Figure 1**. We also tried to evaluate protein expression of these antigens in CRH5 and EOH6 MM tumors with western blot assays. In these experiments, among the numerous antibodies tested, only those for OLFML2B, TOAP, and KIF20A specifically detected the proteins targeted by the universal vaccine (**Supplementary Figure 1**).

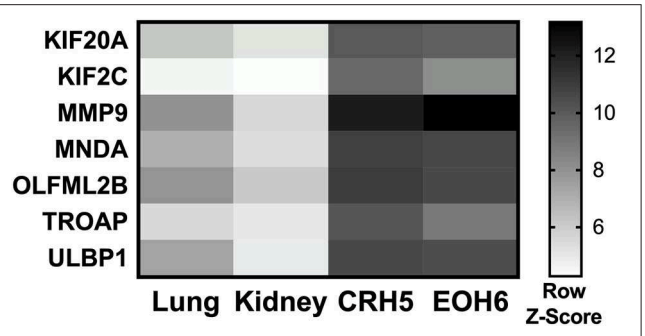

FIGURE 1 | p-Tvax antigens are overexpressed in MM tumors. Transcriptome analysis using mRNA-microarrays was performed on two MM mouse tumors (grown from injected CRH5 or EOH6 cells) and two normal tissues (kidney and lung). mRNA levels are indicated by Row Z-Score colors in a heat map. Antigens with the higher mRNA level for both tumors, compared with both normal tissues, were selected for inclusion in the p-Tvax vaccine. Differences in mRNA levels between each MM tumor and each normal tissue were statistically significant for all antigens ($p < 0.001$).

TABLE 1 | p-Tvax epitopes.

N.	Antigen	Start position	Peptide	Length
1	KIF20A	860	Ac-SSTDSSPYARILRSRHSPLLK-amide	21
2	KIF2C	356	GDLSGKSQNASKGIYAMASRDVFLLKN-amide	27
3	MMP9	594	RVFFFSGRQMWVYTGKTVLGPRSLDKLGL-amide	29
4	MNDA	298	Ac-NETSSVLEAAPKQMIEVPNCITRN -amide	24
5	OLFML2B	50	DNQENVLSQLLGDYDKVKAVSEGSD-amide	25
6	TROAP	156	Ac-KGGTTQRGQSARSSAYLAPRIPTH-amide	24
7	ULBP1	56	Ac-LNRQPLFVYKDKKCHAIGAHRNSMNATKI-amide	29

Amino acid sequences of each antigen were screened with EpiMatrix to identify epitopes with potential immune stimulatory properties in BALB/c mice. Putative regulatory T cells epitopes (Tregitopes), which promote suppression of vaccine-induced T cell responses, and autoimmune epitopes (autoepitopes) that have potential of inducing autoimmune reactions were selected against using JanusMatrix algorithm. For each antigen, a peptide that contained both MHC-I and -II epitopes was synthetized as listed and used for vaccination, or for detection of antigen-specific immune responses.

In silico Epitope Mapping and Peptide Selection

Each of the antigens selected for vaccination was screened for MHC-I and MHC-II T cell epitopes using the EpiMatrix algorithm. Using the JanusMatrix algorithm, we also identified putative regulatory T cell epitopes (Tregitopes), which can promote suppression of vaccine-induced T cell responses, as well as autoimmune epitopes (autoepitopes). For each of the seven antigens, we selected a sequence that contained multiple predicted MHC-I and MHC-II restricted T cell epitopes, and no Tregitopes nor autoepitopes (**Table 1**). A

mixture of all these peptides was used as multi-antigen vaccine named p-Tvax.

p-Tvax Vaccination Induces Antigen-Specific T Cell Responses

To determine if p-Tvax induces antigen-specific $CD4^+$ and $CD8^+$ T cells, we vaccinated BALB/c mice and evaluated T cell responses using intracellular cytokine staining (ICS). Cells from spleens of immunized mice were re-stimulated with each peptide listed in **Table 1** and production of IFN-γ investigated with flow cytometry. In these assays, each of the p-Tvax peptides induced higher levels of IFN-γ^+ $CD8^+$ T cells compared to unstimulated $CD8^+$ T cells, but only peptides 1, 2, 3, and 4 induced significant numbers of IFN-γ^+ $CD4^+$ T cells (**Figures 2A,B**).

We also confirmed that p-Tvax-induced T cells are able to recognize the antigen-specific epitopes on the surface of MM cells and initiate a Granzyme B-based cytolytic response. For these assays, we pooled the T cells stimulated with each peptide and evaluated their Granzyme B secretion by ELISPOT, following incubation with MM cells. Three different MM cell lines were used as target cells for these analyses. This included CRH5 and EOH6 cells, which are known to express the antigens included in p-Tvax, and AB12 cells, in which antigen expression was unknown at the time of the vaccine design. In these assays, we used effector T cells from unvaccinated mice as controls that, along with T cells from vaccinated mice, were unstimulated or stimulated with all the p-Tvax peptides. T cells from vaccinated mice stimulated with the p-Tvax peptides produced high numbers of Granzyme B spots when co-cultured with all 3 MM cell lines (**Figure 2C**). Importantly, even though the p-Tvax design was specific for the antigens expressed in CRH5 and EOH6 MM cells, this vaccine was able to elicit T cells that also recognized AB12 MM cells. This suggests that the antigens included in p-Tvax represent a group of commonly expressed proteins across mouse MM tumors. In contrast, Granzyme B ELISPOT experiments performed using non-tumor mesothelial cells did not show differences between unstimulated and stimulated T cells in both vaccinated and control mice (data not shown).

Vaccination With p-Tvax and Engagement of the OX40 Receptor Elicits Potent Anti-tumor Responses in Subcutaneous and Intraperitoneal Mouse Models of MM

Multi-antigen cancer vaccines such as p-Tvax may serve as an effective approach to stimulate multiple populations of cancer-specific T cells, but the activity of these immune cells is hindered within the tumor microenvironment by several mechanisms, including the suppressive action by T regulatory cells (Tregs). Since OX40 agonists have been demonstrated to reduce the number of Tregs in the tumor microenvironment (10, 19), we evaluated the combination of p-Tvax with these agonistic antibodies as a therapeutic approach for MM in animal models.

For the immunization protocol, we chose to administer two vaccinations with p-Tvax separated by 1 week. CpG adjuvant injections were performed 2 d before each p-Tvax immunization. This protocol elicits potent immune responses in mice (20). To engage the OX40 receptor on T cells, we performed two injections of the OX86 mAb, with the first dose injected 5 d prior to the last p-Tvax vaccination, and the second on the same day as the last p-Tvax injection (**Figure 3**). This protocol was chosen to minimize the clearance of the OX86 mAb by the immune system since these antibodies were developed in a species different from mice (i.e., rat). In s.c. models of MM, we used CRH5 MM cells and initiated vaccinations when tumors reached 3–4 mm in diameter. In these mice, we observed reduced tumor growth with p-Tvax alone or in combination with the OX86 mAb compared to unvaccinated controls or OX86 mAb alone. Survival analyses revealed that mice vaccinated with p-Tvax or with p-Tvax and OX86 mAb exhibited prolonged median survival compared with the controls or with OX86 mAb alone (**Figure 4A**). Since MM usually develops from mesothelial cells lining both thoracic and peritoneal cavities, we developed a clinically relevant MM model to test the therapeutic efficacy of p-Tvax and OX86 mAbs. For this model, we i.p.injected EOH6 cells previously transduced with a lentiviral vector encoding the luciferase enzyme, which enabled the localization and evaluation of tumor dimensions in live mice using an IVIS imaging system. In these mice, p-Tvax was less efficacious as indicated by reduced tumor dimensions closer to the end of the protocol (day 27) and differences over time were not statistically significant when compared with controls. In contrast, the OX86 mAb alone exerted statistically significant anti-cancer activity that delayed tumor growth for the entire protocol. These results were dramatically improved by the combination of p-Tvax and OX86 mAb, with mice showing no signs of tumor at day 20. In these mice, however, tumor cells were not completely eliminated and became visible with the IVIS machine the following week. Survival analysis of these mice showed that vaccination with p-Tvax together with the OX86 mAb resulted in prolonged survival compared with OX86 mAb alone. The p-Tvax vaccine alone did not lead to increased survival (**Figure 4**).

We also evaluated the anti-cancer effects of p-Tvax and the OX86 mAb in mice injected with AB12 MM cells. Results showed that p-Tvax reduced tumor growth, but did not improve survival. The OX86 mAb alone delayed tumors and improved survival compared to controls. The combination of p-Tvax plus the OX86 mAb was effective in delaying tumor growth equivalent to the monotherapy treatments. In survival experiments, p-Tvax in combination with OX86 mAbs dramatically improved overall survival, with 20% of vaccinated mice experiencing complete tumor regression. These results were statistically significant when compared with controls or each immunotherapy alone (**Supplementary Figure 2**). Importantly, no adverse events were observed for any treatment groups such as acute effects, distress, or weight loss, and gross tissue examination failed to uncover any toxicity in the organs (kidney, brain, spleen, liver, and lungs). It is important to mention that the expression of p-Tvax antigens was unknown in AB12 MM cells when these experiments were performed, but subsequently confirmed before the publication of this article (**Supplementary Figure 2**).

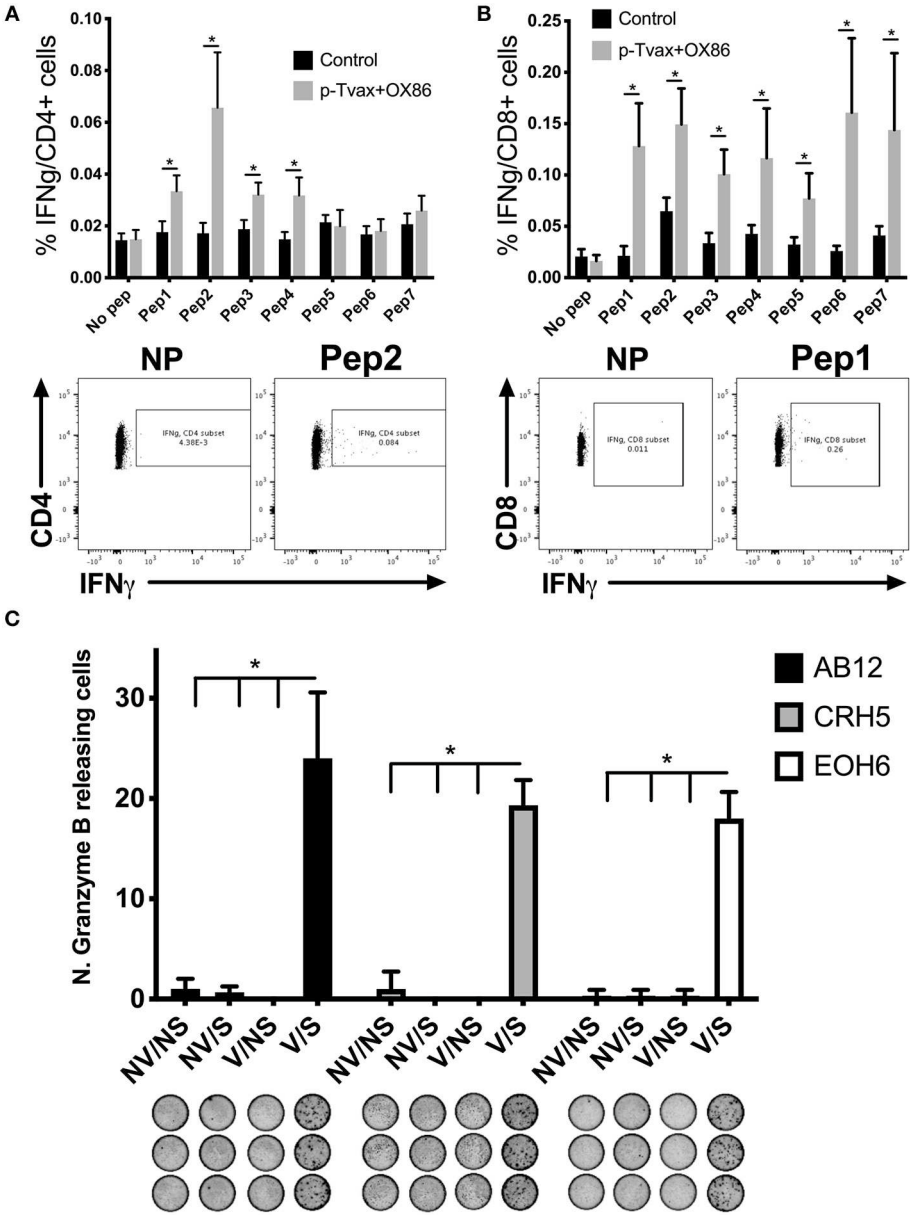

FIGURE 2 | p-Tvax stimulates antigen-specific IFN-γ secreting T lymphocytes that produce Granzyme B when co-cultured with MM cells. BALB/c mice were vaccinated with two s.c. injections of p-Tvax peptides at days 2 and 9. CpG 1585 was used as adjuvant and injected at the same site of the p-Tvax peptides 2 days before vaccination, at days 0 and 7. OX40 agonist mAbs (OX86) were i.p. injected at days 4 and 9. Control mice were left untreated. Five days after the last vaccination, spleen cells were isolated and enumerated for assays. **(A)** Intracellular IFN-γ in $CD4^+$ T cells was evaluated by flow cytometric ICS analyses of spleen cells cultured with or without p-Tvax peptides. Percentage of IFN-γ expressing $CD4^+$ T cells are presented as mean ± S.E. Bottom Panel: Representative data from flow cytometric analysis of vaccinated and control mice. Lymphocytes were either unstimulated (no peptide = NP) or stimulated with peptide 2. $CD4^+$ T cells were distinguished using a marker gate in the CD3 vs. CD4 dot plot. **(B)** IFN-γ in $CD8^+$ T cells was evaluated in spleen cells cultured with or without p-Tvax peptides. Percentage of IFN-γ expressing $CD8^+$ T cells are presented as mean ± S.E. Bottom Panel: Representative data from flow cytometric analysis of vaccinated and control mice. Lymphocytes were either not stimulated (NP) or stimulated with peptide 1. $CD8^+$ T cells were distinguished using a marker gate in the CD3 vs. CD8 dot plot. For A and B, statistical significance between treated and control groups was determined by ANOVA followed by Bonferroni test (*$p < 0.05$, $n = 5$). **(C)** Secretion of Granzyme B was evaluated by ELISPOT assay. Spleen cells from vaccinated mice (V) or unvaccinated (no vaccine = NV) were activated with p-Tvax peptides in the presence of 5 IU/ml IL-2 for 6 d (stimulation = S) or incubated with no peptide (no stimulation = NS). Following 4 h of incubation with different MM cells (AB12, CRH5, EOH6), the number of spot-forming cells per 10^5 cells was evaluated and results represented as mean ± S.E. Statistical differences between vaccinated mice, stimulated with peptides (V/S), and the other control conditions were evaluated by ANOVA followed by Bonferroni test (*$p < 0.05$, $n = 3$).

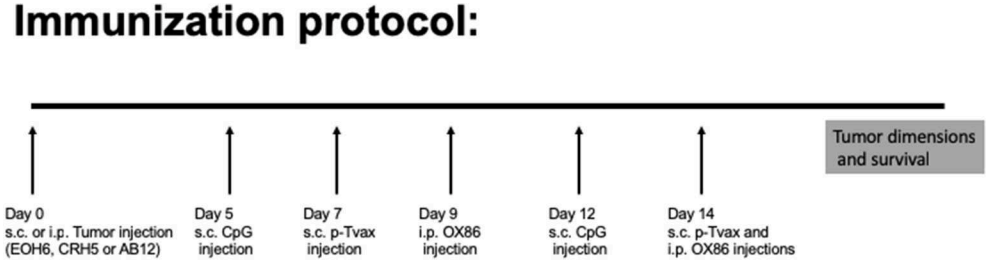

FIGURE 3 | Experimental approach to evaluate p-Tvax and OX40 agonists anti-cancer properties. Two s.c. vaccinations were administered with p-Tvax separated by 1 week. Two days before each p-Tvax immunization, CpG adjuvant injections were performed. To engage the OX40 receptor on T cells, we performed two injections of the OX86 mAb, with the first dose injected 5 d prior to the last p-Tvax vaccination, and the second on the same day as the last p-Tvax injection. Untreated controls and mice treated with the different immunotherapies were followed for tumor dimension and survival.

Immunotherapy With p-Tvax Plus the OX86 mAb Induces an Immunostimulatory Tumor Microenvironment

We next evaluated the effects of the different immunotherapies in MM models in terms of leukocyte cell populations in tumors using multicolor flow cytometry. This enabled the simultaneous identification of several different cell types in one sample, including $CD8^+$ and $CD4^+$ T cells, and Tregs. In CRH5 tumors, a higher percentage of $CD4^+$ T helper cells was detected in p-Tvax vaccinated mice vs. controls, while abundance of $CD8^+$ T cells and Tregs was not different from controls. The OX86 mAb increased abundance of $CD8^+$ cytotoxic T cells, but reduced numbers of $CD4^+$ T cells and Tregs. The combination of p-Tvax and the OX86 mAb modified the tumor microenvironment in a manner that led to more optimal anti-cancer immune conditions as supported by higher abundance of both $CD8^+$ and $CD4^+$ T cells, and reduced percentages of Tregs (**Figure 5**). In EOH6 tumors, we observed similar results with the only difference found with OX86 mAb that did not reduce significantly the number of $CD4^+$ Tregs when used as single treatment (**Supplementary Figure 3**). To further characterize the tumor infiltrate, we also measured the expression of PD-1 in $CD4^+$ and $CD8^+$ T cells, as well as the expression of OX40 in Tregs. PD-1 expression was significantly reduced in $CD4^+$ T cells following treatment with both p-Tvax and OX86 mAb, while its expression did not change in $CD8^+$ T cells. Interestingly, the expression of OX40 in $CD4^+$ $CD25^+$ Tregs, which represent their activation status, was reduced following treatment with either OX86 mAb or with the combination of p-Tvax plus OX86 (**Supplementary Figure 4**).

DISCUSSION

In this study, we developed a novel immunotherapy combination comprised of a multi-antigen MM-specific vaccine combined with an OX40 agonist mAb leading to the reduction of tumor growth. The vaccine design started with the analysis of the MM tumor transcriptome performed in neoplastic tissues generated from two different MM cell lines, CRH5 and EOH6. Antigens that were highly expressed in both cells were chosen as candidates for inclusion in our vaccine. It has been established that the high expression of certain proteins within tumor cells leads to increased antigenic processing and presentation in the context of MHC-I and -II (21, 22). These fragments, referred to as tumor epitopes, represent targets for T cells that can specifically recognize the tumor cell for immune-mediated elimination. To minimize off-target effects that could lead to the development of autoimmunity, it is important to choose antigens that are not expressed or minimally detectable in normal tissues (23). In this regard, we also evaluated antigen expression in normal tissues in our laboratory as well as expression data from the literature (17, 18). This led to the selection of seven antigens found to be overexpressed in both mouse MM tumor lines under investigation and that were nearly absent in all other mouse normal tissues: KIF20A, KIF2C, MMP9, MNDA, OLFML2B, TROAP, and ULBP1. It is important to mention that we also tried to confirm the protein expression of these antigens in MM tumors using western blot. In these experiments, we evaluated several antibodies and specifically detected high levels of KIF20A, OLFML2B, and TROAP in MM tumors. Regarding the other proteins, the antibodies tested could not specifically recognize the antigens that we included in the vaccine. These negative results may be due to the rapid degradation of the proteins, or to their improper folding in tumor cells. However, it has been demonstrated that pre-maturely truncated or abnormally folded proteins are exported to the cytosol, processed by the proteasome and resultant peptides loaded on MHC-I and -II to trigger specific immune responses (24–26).

For each of the seven chosen antigen, we designed a peptide that contained both MHC-I and -II epitopes with the capacity to stimulate both $CD8^+$ and $CD4^+$ T cells. Epitope screening was performed using the iVAX platform which contains advanced *in silico* tools designed to identify highly immunogenic epitopes with EpiMatrix, while selecting against epitopes that may stimulate Treg or autoimmune responses with JanusMatrix. By including these optimized epitopes in our vaccine, we reduced the potential for suppressive or off-target effects. The development of these innovative *in silico* tools will increasingly provide the capacity for safe and effective vaccines (5–7).

Mice vaccinated with the multi-antigen vaccine (p-Tvax), mounted antigen-specific immune responses that involved both

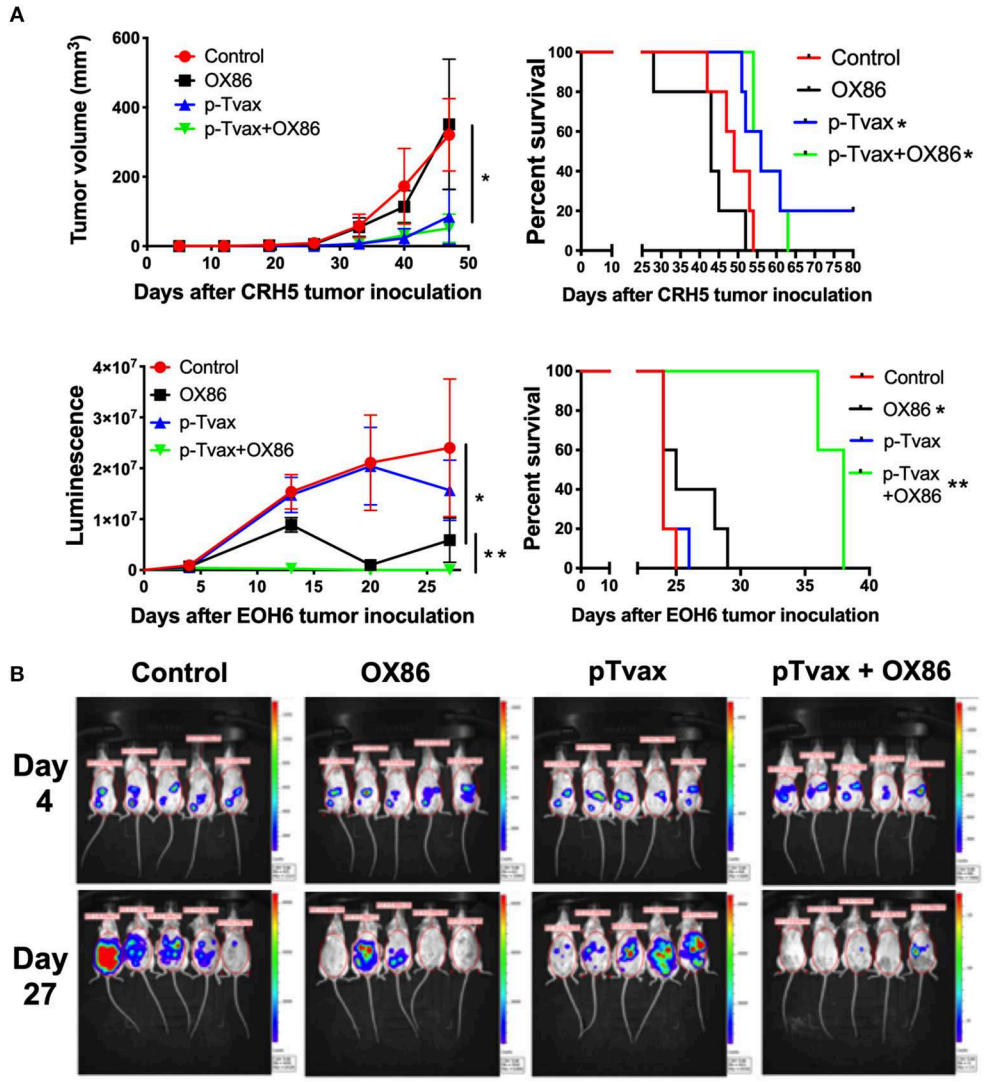

FIGURE 4 | Combination of p-Tvax vaccine and OX40 agonists delays tumor growth and improves survival in subcutaneous and intraperitoneal mouse models of MM. **(A)** BALB/c mice were injected s.c. with 5×10^4 CRH5 MM cells, or i.p. with 2×10^5 EOH6 MM cells expressing luciferase. Seven and 14 d after tumor injection, mice were vaccinated with a s.c. injection of p-Tvax peptides. CpG adjuvant was injected at days 5 and 12, while 200 μg of OX86 was injected at day 9 and 14. Tumor volumes are showed on the left and animal survivals on the right for mice injected with CRH5 (Top) and EOH6 (Bottom). Tumor volumes were measured weekly with a caliper for s.c. tumors. I.p. MM dimensions were assessed by measuring luciferase activity with IVIS imaging following injection with luciferin substrate. Statistical significance between unvaccinated controls and single treatment (*) as well as between single and combination treatments (**), was determined by ANOVA followed by Bonferroni test (*$p < 0.05$, $n = 5$). For survival, mice were followed until s.c. CRH5 tumors reached volumes of 300 mm^3 and were then sacrificed. In i.p. models with EOH6 MM cells, survival was assessed by euthanizing mice at first sign of morbidity. Log-rank analysis was used to determine significance between control and single treatment (*), and between single and combination treatments (**) ($p < 0.05$, $n = 5$). **(B)** Representative images from IVIS tumor dimension analysis of mice carrying EOH6 tumors, vaccinated with the different immunotherapies.

$CD8^+$ and $CD4^+$ T cells. These data were obtained using intracellular staining of peptide-pulsed T cells followed by flow cytometry analysis. A different approach to confirm these data, and to provide more quantitative results, would have been the characterization of antigen-specific T cells using MHC tetramers. Unfortunately, these reagents are not commercially available for the epitopes involved in this study. T cells from p-Tvax vaccinated mice were also demonstrated to recognize and attack tumor cells by secreting Granzyme B in ELISPOT assays performed with different MM cell lines. Among these MM cells recognized by vaccine-induced T cells was the AB12 cell line. AB12 cells were not included in the initial transcriptomic analyses for antigen selection, and in this manner served as an important comparison group in our studies. These data demonstrate that, by selecting multiple antigens commonly expressed in several MM cases, it is possible to construct an off-the-shelf vaccine that may be efficacious for a wide variety of MM. This approach to cancer vaccine development may complement or be used in

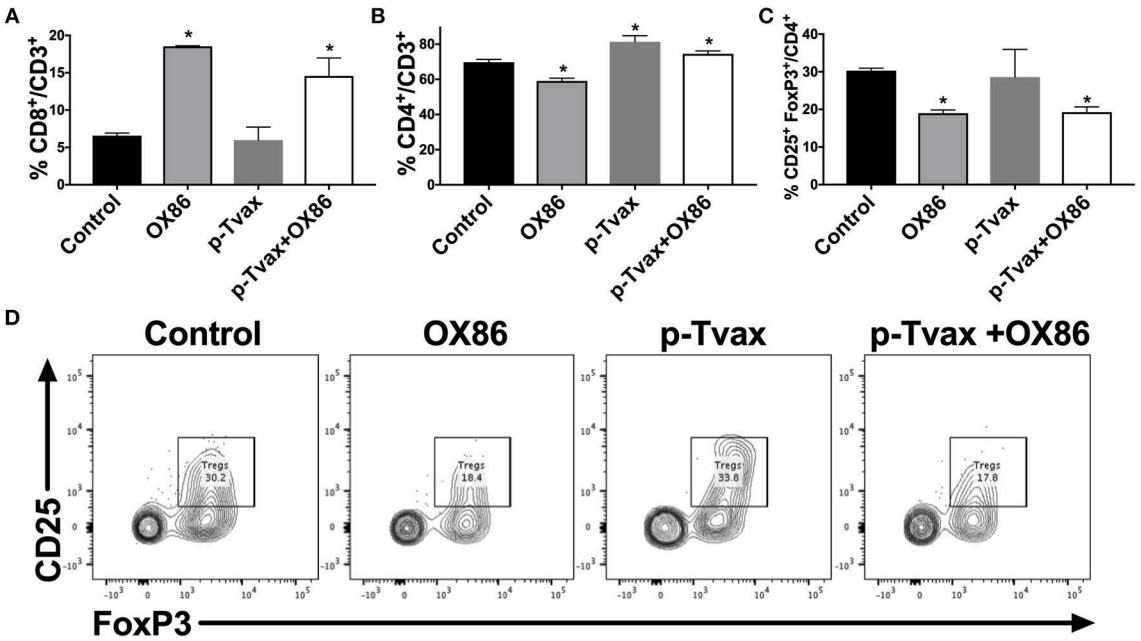

FIGURE 5 | p-Tvax in combination with OX40 agonists induce $CD8^+$ and $CD4^+$ T cell tumor infiltration while reducing T regulatory cells. CRH5 tumors from controls or from mice treated with p-Tvax, OX86 mAbs, or with a combination of the two, were collected and enzyme-digested. Staining was performed using anti-mouse CD3, CD8, CD4, CD25, and FoxP3 antibodies with live cells distinguished from debris using LIVE/DEAD® cell viability dye. **(A)** Percentage of $CD8^+$ T cells in CRH5 tumors from treated and control mice. Results represent mean ± S.E. with means of each group compared using ANOVA followed by Bonferroni test ($^*p < 0.05$, $n = 5$, vs. Control). Data for $CD4^+$ T cells and Tregs cells are showed, respectively in **(B,C)**. **(D)** Representative data from flow cytometric analysis of CRH5 tumors in treated and control mice using CD25 and FoxP3 markers for Tregs, including Tregs that were previously gated in the CD3 vs. CD4 dot plot.

place of personalized cancer vaccine approaches. In addition, off-the-shelf cancer vaccines bring the possibility of prophylactic immunization of individuals, which cannot be achieved with therapeutic personalized vaccines. Personalized immunizations are considerably more expensive than off-the-shelf vaccines due to of the cost of peptide synthesis (small vs. large production under GMP conditions). In addition, personalized vaccine epitopes tailored for each cancer patient's mutanome requiring sophisticated techniques such as whole-exome sequencing, RNA sequencing, and *in silico* immunogenicity prediction algorithms that are not as yet widely available. The safety profile of "off the shelf" vaccines such as the one proposed here can be ascertained well in advance of treatment, whereas the e safety profile of personalized anti-cancer vaccines remains to be fully evaluated. For example, a recent study showing that the number of somatic mutations in normal cells in cancer-related genes may be several times higher than in the cancer counterparts (27). Alternatively, vaccines such as the one described in this research study could be used in conjunction with personalized vaccines, to augment the number of epitopes to which the patients may respond.

In mouse models, p-Tvax as a stand-alone treatment showed limited efficacy in delaying tumor growth or in improving survival in mice carrying certain MM tumors. This was an expected outcome given the likelihood of immune suppression within the tumor environment. This is why efficacy of cancer vaccine can be enhanced by the inclusion of immune stimulatory Abs such as the OX40 agonist we chose for our study. In fact, the combination therapy using p-Tvax plus the OX40 agonist showed both decreased tumor volumes and increased survival for all three MM mouse models. Interestingly, the OX40 agonist alone was not effective in one animal model carrying CRH5 MM cells when used as a single agent. This is consistent with data obtained in clinical trials involving such immune checkpoint mAbs, in which clinical responses are often limited to a subset of patients (28, 29).

Analyses of tumor tissues in mice treated with the different immunotherapies produced results that may be best explained when considering the combination treatment as a sum of all the benefits induced by each therapeutic approach, namely, the combined increase in $CD4^+$ T cells induced by p-Tvax and the increase in $CD8^+$ T cells induced by OX40 agonists. Further analysis of the tumor infiltrate also showed that the combination of p-Tvax and OX40 agonists produced other effects that may favor tumor clearance such as the reduced expression of the T cell exhaustion marker PD-1 in $CD4^+$ T cells and the decrease in number of Tregs. It is important to point out that mice treated with the combination of p-Tvax and OX40 agonists also showed reduced expression of OX40 in intra-tumor Tregs, which indicates a lower regulatory activity of these cells and a more favorable prognosis (10, 30, 31). Our data collectively suggest that this combination of immunotherapies could be an attractive therapeutic strategy for MM patients. Also, considering that humanized OX40 agonists have already been developed and successfully passed the phase I clinical trials

(13), this immunotherapeutic mAb approach may serve as an effective adjuvant to include along with cancer vaccines. We have demonstrated proof-of-concept supporting p-Tvax in human immunization, in which target antigens can be chosen from transcriptome studies performed in MM patients (32, 33).This may provide the framework for a new approach to combine this broadly acting vaccine with immunotherapy and/or personalized cancer vaccines, for treating not only MM, but other types of cancers as well.

ETHICS STATEMENT

Animal experiments were performed in accordance with institutional guidelines and approved by the University of Hawaii IACUC (#16-2355).

AUTHOR CONTRIBUTIONS

PRH, LM, AD, and PB conceived of the idea. PRH, LN, AS, AW, and PB designed the methods with feedback from the others. PB implemented the methods and collected the results. ES, MP, and AS prepared the vaccines included in this study. TP, GC, TF, FH, and PB performed animal studies and T cell analyses. GR, PH, LM, and AD identified T cell epitopes using JanusMatrix algorithm. MM, VK, and YD analyzed the transcriptome data and performed statistical analyses. PRH, AD, and PB wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00720/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.