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Targeting Immunological "Restrainers": Understanding the Immunology Behind Combination Chemoimmunotherapy To Improve the Treatment of Malignant Mesothelioma

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14. ABSTRACT

This FINAL report summarizes all worked completed to date with respect to all Aims listed in the original proposal and highlights key research discoveries. This study investigated the role the body's immune system plays during mesothelioma tumour development with a specific focus on a subset of immune cells called Treg that act to limit anti-tumour immunity. We demonstrate that previously published inh bitors of Treg cells are not effective in our models. However, we employed a new animal model which allows for the selective depletion of Treg cells and observed that targeted removal of Treg, particularly during early tumour development can significantly enhance anti-tumour immunity and delay tumour development. Importantly targeted Treg removal in combination with gemcitabine chemotherapy significantly enhanced overall survival in comparison to chemotherapy alone. .Additionally, we observed that asbestos induced mesothelioma development is slower in mice that lack a functional immune system compared to mice that are immune competent. We have developed a number of cell lines that will enable further investigation into the role of the host immune system during the induction of asbestos induced mesothelioma and disease progression. Data generated from this work underpins our focus on developing immunotherapies for improved treatment of malignant mesothelioma.

15. SUBJECT TERMS

Mesothelioma, immunotherapy, chemotherapy. Combination therapy.

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INTRODUCTION: Malignant mesothelioma is a highly aggressive, incurable asbestos-induced cancer that is increasing in incidence globally. Treatment for mesothelioma is predominantly palliative, with median survival for patients undergoing chemotherapy around 12 months. This poor prognosis highlights the need for improved treatment modalities. A promising new approach for treating cancer has been to combine chemotherapy with immunotherapy. Chemo-immunotherapy has shown survival benefits over chemotherapy alone in the treatment of metastatic melanoma. However, there are few studies that have investigated whether immunotherapy may enhance the outcome of standard first line chemotherapy treatment for malignant mesothelioma.

Our program has focussed on understanding the immunobiology associated with tumour development. Using two unique animal models of mesothelioma, we have demonstrated that the combination of immunostimulatory chemotherapy with an immunotherapy that drives a strong anti-tumour immune response effectively targets and destroys tumour cells. Despite this, not all animals are cured and our data suggests that this is most likely due to the presence of immunological processes that "restrain" an otherwise effective anti-tumour immune response. Regulatory T cells (Tregs) have been identified as playing a critical role in suppressing anti-tumour immunity. While a variety of agents that specifically target Tregs have recently been identified, the role of Tregs in the development of MM has yet to be properly investigated. ***In this grant we aimed to evaluate the efficacy of Treg-specific immunotherapies in combination with immunostimulatory chemotherapy to improve the treatment of MM.***

The data generated by this pre-clinical work will inform and facilitate the development of new clinical trials in mesothelioma. This FINAL report summarises all worked completed to date with respect to all Aims listed in the original proposal and highlights key research discoveries.

KEYWORDS: Mesothelioma, immunotherapy, chemotherapy, combination therapy, Regulatory T cells (Treg), anti-tumour immunity

OVERALL PROJECT SUMMARY:

Aim1. Ablation of restrainers of the anti-tumour immune response, particularly regulatory T cells, will enhance the therapeutic efficacy of chemotherapy. (A) To identify in vivo which Treg-specific immunotherapy (P60, CCR4 antagonist or low dose CY) is best suited for combination chemoimmunotherapy with gemcitabine, pemetrexed, or cisplatin. (B) Determine optimal dose / schedule for best combination treatment identified in aim 1a. (C) Assess optimised treatment parameters in the clinically relevant MexTAg mouse model.

Aim 1a: Identify which Treg-specific immunotherapy (P60, CCR4 antagonist or LDCY) is best suited for combination chemoimmunotherapy with gemcitabine, pemetrexed, or cisplatin.

As reported in our 2013 annual we performed experiments to establish which chemotherapy would be suitable to use in combination with Treg inhibitors. Our data (Figure 1) indicated that gemcitabine (Gem) significantly delayed tumour growth compared to untreated control mice ($p < 0.001$) with 40% of gem treated animals showing complete tumour regression (panels A & B). In contrast to gemcitabine, there was no significant difference observed for AB1-HA tumour growth when mice were treated with cisplatin or pemetrexed (panels 1 C through F).

Based on these results we decided not to pursue any further experiments using pemetrexed or cisplatin and focused on assessing Treg depletion in combination with gemcitabine.

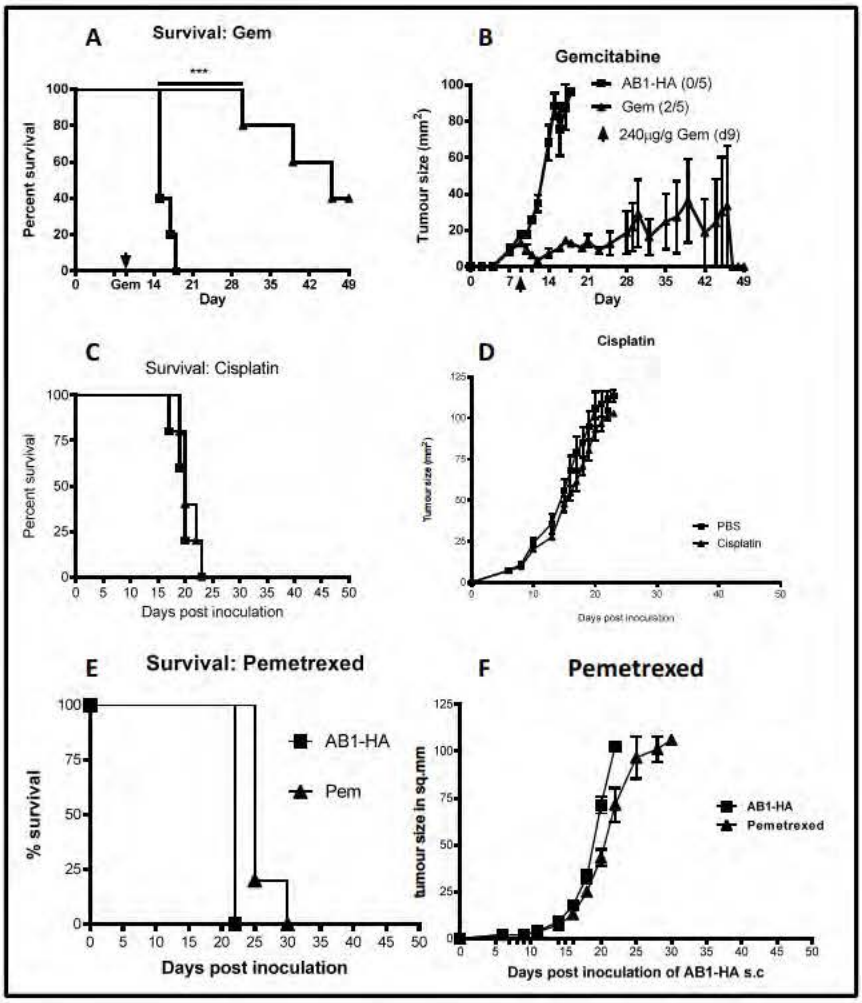

Figure 1: Survival and tumour growth curves depicting the effect of individual chemotherapy on AB1-HA tumour bearing BALB/c mice. (A-B) Gemcitabine: 240 µg/g/mouse d9; (C-D) Cisplatin: 6 µg/g/mouse d9 and (E-F) Pemetrexed: (60 µg/g/mouse d7/8/9 & d14/15/16).

Assessing the role of Treg inhibitors in AB1-HA tumour bearing mice: Before assessing the effect of combining the different Treg inhibitors, P60, CCR4 antagonist AF399 and low dose cyclophosphamide with gemcitabine, we first establish whether these reagents would work in our model at the published dose. Groups of AB1-HA mice were inoculated with subcutaneously $5x10^5$ AB1-HA cells and left untreated or treated with individual Treg inhibitors and overall survival and tumour growth monitored. Mice were culled when tumour reached 100 mm^2 as per UWA animal ethics approvals.

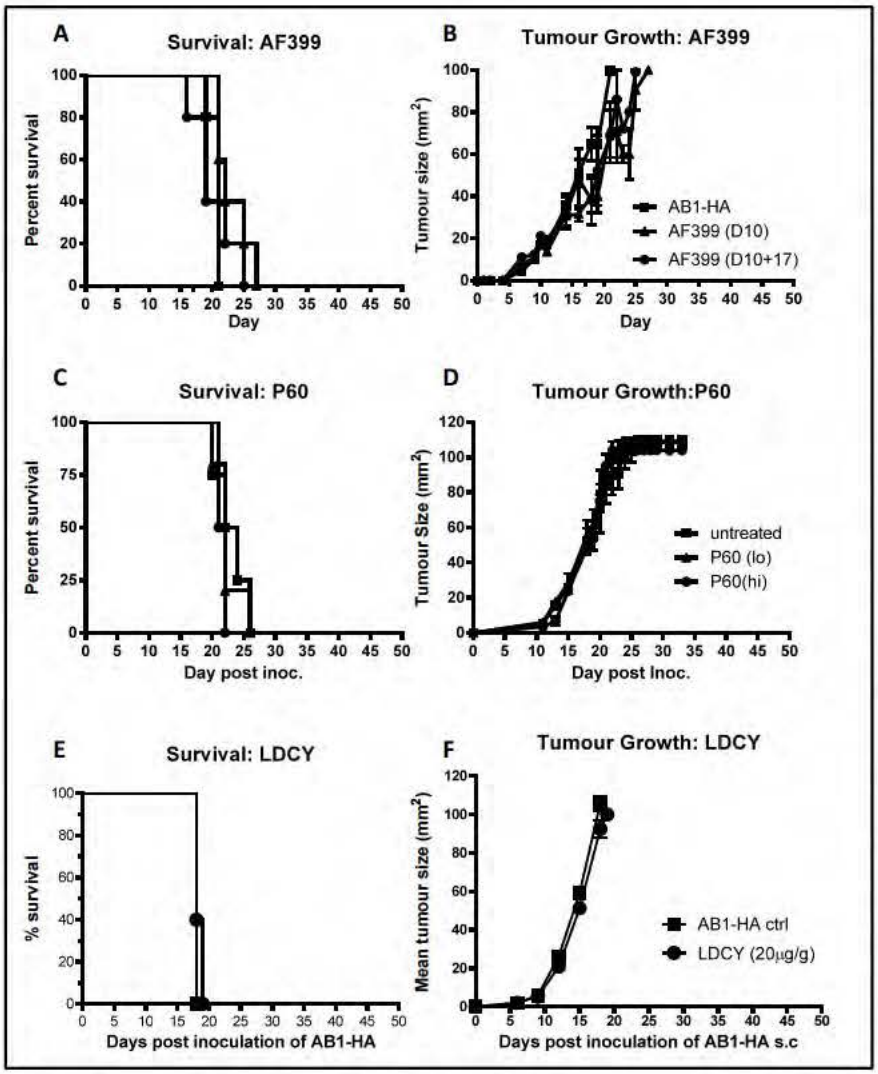

Figure 2: Survival and tumour growth of Ab1-HA bearing BALB/c mice following individual Treg inhibitor treatment. (A-B) CCR4 antagonist AF399 (3 µg/g i.p. on indicated days). (C-D) Small molecule inhibitor P60 low dose (50 µg/dose, q1dx10) and a high dose (100 µg/dose, q3dx3) staring day 9. (E-F) low dose cyclophosphamide (20 µg/g, q3dx5).

We were surprised that there was no significant delay in tumour growth between untreated controls and mice receiving any of the three Treg inhibitors as individual monotherapies. This is despite treatments being administered at equivalent, or higher than, the published doses for these therapies [1-3]. From these results we concluded that the P60 small molecule inhibitor of FoxP3 and the CCR4 antagonist were unlikely to be effective in our AB1-HA animal model. Although there may be scope to increase the dose of cyclophosphamide, we have previously demonstrated that doses between 100-200 µg/dose can result in complete tumour regression [3] and therefore care must be taken when using higher doses of cyclophosphamide in combination with gemcitabine.

Targeted depletion of regulatory T cells using FoxP3.dtr.crls transgenic mice.

The above experiments indicated that it would be unlikely that we could investigate the role of regulatory T cells in tumour development using the reagents we had proposed. To overcome this problem, we made use of the BALB/c FoxP3.dtr.crls transgenic mouse model [4], not available to us at the time of initiating Aim 1 experiments. The BALB/c FoxP3.dtr.crls mice (referred to as FoxP3.dtr mice herein) have been genetically modified such that the gene encoding the receptor for Diphtheria Toxin (DTR) has been cloned under the control of the FoxP3 promoter, which results in expression of the DTR on FoxP3 expressing cells, such as Treg. This model enables the specific depletion of FoxP3 expressing cells after the administration of Diphtheria toxin (DTX), without any detrimental effect to other immune cell populations, such as CD4 and CD8 T cells, or to the mouse in general. These experiments did not commenced until mid-late 2013, after approval was granted for the inclusion and use of FoxP3.dtr mice by both the UWA and US Dept. Defence ACURO animal ethics committees.

We first sought to assess the effect of DTX administration on Tregs by administering different doses of DTX to FoxP3.dtr mice. Groups of naive FoxP3.dtr were administered of DTX as sequential intra peritoneal injections on two consecutive days. As shown in Figure 3, DTX administration resulted in a dose depended reduction in CD4+ FoxP3+ Treg (diamonds) without affecting other lymphocytes subsets or the levels of Tregs from normal BALB/c mice. However, DTX mediated Treg depletion was only transient, with the proportion of Treg rebounding four days after DTX administration. Interestingly, this is also the same time point that corresponded with an increase in CD8 T cell activation (data not shown) and the onset of tumour regression in mice receiving > 2ng/g/mouse DTX, suggesting that temporary removal of Treg can affect the developing tumour.

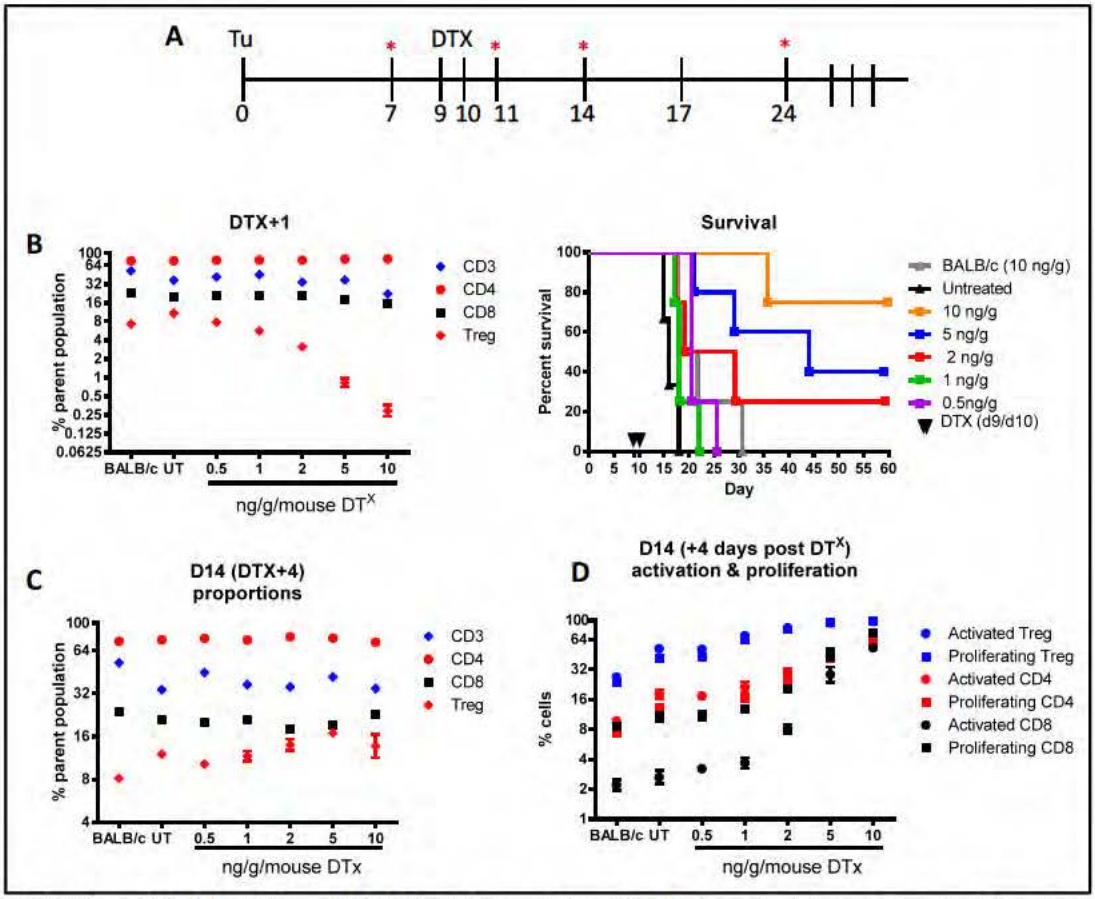

Figure 3: DTX mediated depletion of CD4+ FoxP3+ regulatory T cells. Transient dose dependent Treg depletion. AB1-HA bearing mice (n=5/gp) treated with indicated doses of DTX on days 9+10. Treg-specific depletion in peripheral blood shown one (DTX+1) and four (DTX+4) days after DTX administration. (A) Experimental design. (B) DTX dose dependent depletion of Treg (red diamonds) at DTX+1 and associated survival outcome. (C) Treg proportions (red diamonds) rebound four days after DTX administration. (D) Transient dose Treg depletion correlated with a reciprocal increase in CD8 T cell activation (black circles) at DTX+4.

These data demonstrated the usefulness of the FoxP3.dtr mice for addressing the influence of Treg during tumour development and therefore we continued to use these mice to address the questions outlined in Aims 1a and 1b. We experienced some delays in establishing a breeding colony of FoxP3.dtr which initially produced only small litters, but within a few months were in a position where the quantities needed to perform the experiments was sufficient and continued with our research.
Shown in Figure 4 are data from a set of experiment in which we assessed whether Treg depletion would affect the outcome of chemotherapy with gemcitabine. In these experiments AB1-HA tumour

bearing mice were left untreated or treated with gemcitabine, with or without DTX mediated Treg depletion. Depletion of Treg in combination with gemcitabine was associated with a significantly improved the overall survival in comparison to gemcitabine alone. Furthermore, regression of tumours in DTX+Gem treated group correlated with a 3 to 4-fold increase in CD8 T cell activation relative to Gem only treatment, suggesting that Treg removal enhanced the response to chemotherapy. Interestingly, there was no significant difference observed between Treg depletion alone (DTX only) or when combined with chemotherapy, suggesting that depletion of Treg is important to generating a strong anti-tumour immune response. Similar data were observed in a renal cell carcinoma cell line (Renca), demonstrating that this outcome is not specific to the AB1-HA cell line.

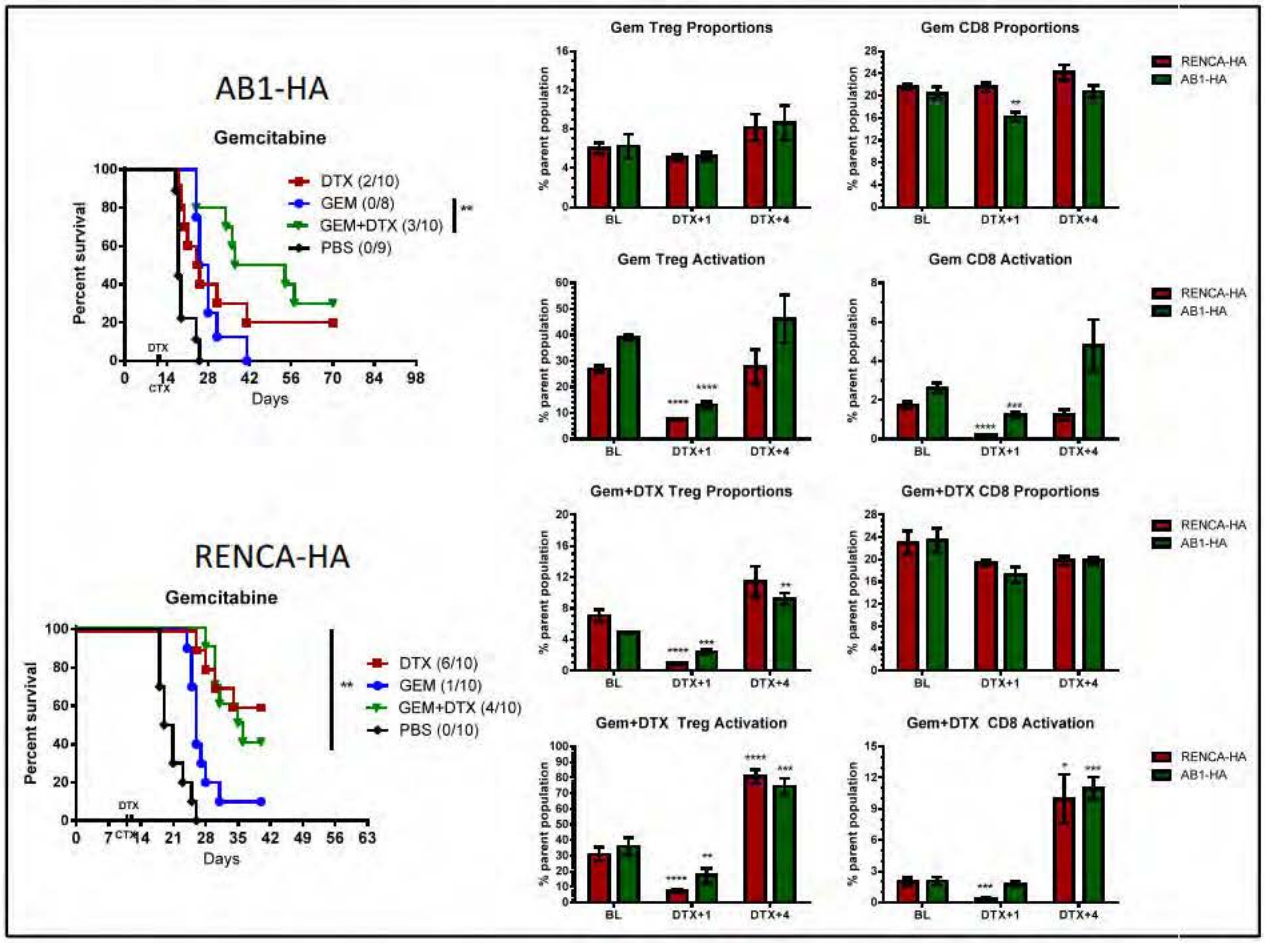

Figure 4: Combination of gemcitabine and Treg depletion on tumour development. Mice were inoculated with tumour s/c on day 0 and treated with 5 ng/g/mouse DTX i.p. (q1dx2) on days 10 and 11. Gemcitabine (240 µg/g/mouse) was administered i.p. on day 10. Kaplan Myer plots showing survival of AB1-HA and Renca-HA bearing FoxP3.dtr mice with different treatments. FACS analysis of Treg and CD8 T cell proportions and activation status for Gem alone or combination treatment for both AB1-HA (green bars) and Renca-HA (red bars). Data ± SEM ** p<0.01, *** p<0.001, p<0.0001.

To summarise our results for Aims 1a and 1b, we demonstrated Gemcitabine to be the most effective chemotherapy a by significantly delaying and in some cases, preventing growth of the mesothelioma cell line AB1-HA. We also demonstrated that this survival benefit could be significantly improved by combining gemcitabine treatment with Treg depletion. Importantly, effective Treg depletion (as achieved in the FoxP3.dtr model, but not with the other tested 'Treg inhibitors') is critical to the success of this combination therapy. Indeed, Treg depletion alone might be the key to the future success for anti-cancer immunotherapy as we did not observed any significant difference between DTX and DTX+Gem groups in two independent tumour models.

This work underpins our current research program focused on developing or improving current immunotherapies, such as immune checkpoint blockade that augment Treg immunosuppressive function.

Aim 1c: Assess optimised treatment parameters in the clinically relevant MexTAg mouse model.

DTX mediated Treg depletion is only feasible in FoxP3.dtr mice and therefore to test whether Treg depletion in combination with gemcitabine chemotherapy would be effective in our asbestos induced mesothelioma mouse model (MexTAg), we chose to deplete Treg with low dose cyclophosphamide. While we were unable to show the efficacy of low dose cyclophosphamide in Aim 1b using the subcutaneous tumour model, we have previously demonstrated its anti-tumour properties in the MexTAg mice (Robinson C, unpublished).

The efficacy of combination gemcitabine chemotherapy and low dose cyclophosphamide was tested in the asbestos-induced mesothelioma mouse model. Dosage was based on our previous experiments in which gemcitabine significantly prolonged survival of asbestos-induced mesothelioma, and cyclophosphamide (50 µg daily) significantly reduced the numbers of Tregs. At this optimised dosage gemcitabine significantly extends survival to about the same extent as found in human mesothelioma patients (allowing for species life time expectancy). As expected, cyclophosphamide as a single treatment did not improve survival. In combination, these treatments significantly prolong survival compared to the gemcitabine single treatment group (Figure 5).

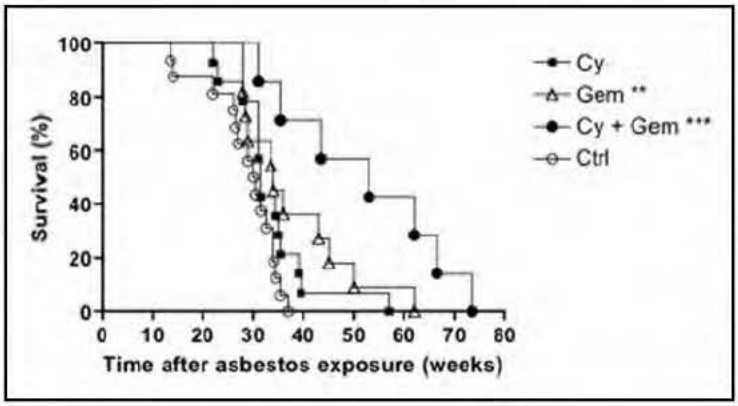

Figure 5. Survival of asbestos-induced mesothelioma in mice treated with chemo and immunotherapy. Disease was induced in MexTAg mice by installation of asbestos, 16 weeks later gemcitabine and low dose cyclophosphamide treatments were commenced. Mice were monitored for mesothelioma development and euthanized at the ethics approved endpoint. **p=0.0093, ***p=0.0005 compared to untreated control (Ctrl) group.

Analysis of T cell subsets showed that Treg cells are depleted in the presence of low dose cyclophosphamide (Figure 6).

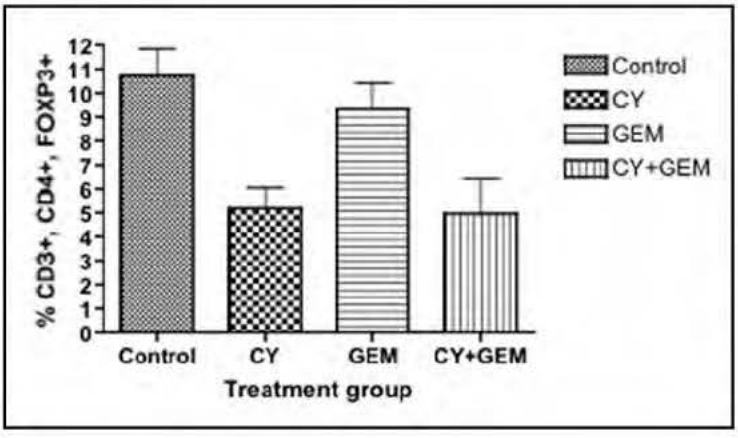

Figure 6: Cyclophosphamide significantly reduces regulatory T cells. Treg cells were identified as staining positive for antibodies to CD3, CD4 and FOXP3 using flow-cytometry. The percentage of Tregs out of total lymphocyte population, 6 weeks after start of treatment, confirmed that Tregs numbers had been reduced compared to untreated controls. P=0.0084.

These data demonstrate that chemoimmunotherapy using gemcitabine and low dose cyclophosphamide is effective at delaying tumour development in an asbestos induce mesothelioma model. The ability of low dose cyclophosphamide to reduce the level of Treg, together with our other studies demonstrating that gemcitabine increases tumour antigen cross-presentation [5,6] suggests that the increase in overall survival of the treatment group might result from an enhanced anti-tumour immune response. Therefore, understanding how the immune system recognises and responds to a developing tumour would be extremely useful in guiding the development of new treatment regimens that induce an anti-tumour immune response.

Aim 2a: To assess the role the adaptive immune system plays during early stages of mesothelial cell transformation.

The MexTAg model is extremely useful for assessing the induction and development of asbestos induced mesothelioma. To assess the role the adaptive immune system plays during early stages of mesothelioma development we compared asbestos induced mesothelioma development in immune competent (MexTAg) and immune deficient (MexTAg Rag-/-) mice. Based on the principle of immunosurveillance and immunoediting (Refs), in which the immune system actively keeps tumour growth and development in check, we had initially hypothesised that the host immune system played an important role in controlling tumour development, while tumour growth would occur unchecked in immune deficient MexTAgRAG mice. To our surprise the results from this experiment clearly indicated the opposite; after mesothelioma induction using asbestos fibres, disease induction was slower in the MexTAgRAG mice that lacked a functional immune system (p=0.0002, Figure 7).

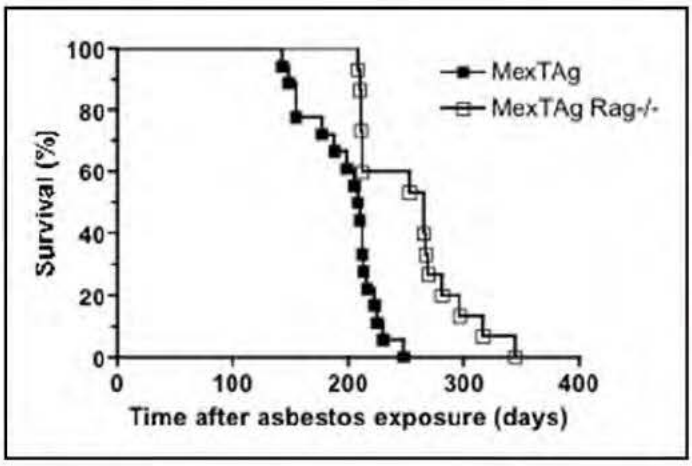

Figure 7. Survival of asbestos induced mesothelioma in transgenic mice with (MexTAg) and without (MexTAgRAG-/-) a functional immune system.

To confirm our initial results repeat mesothelioma induction experiments were performed and with similar results to the first experiment (Figure 7). We then assessed whether the increase in overall survival observed in immunodeficient MexTAgRAG mice compared to immune competent MexTAg mice was related to disease induction (i.e. the time taken from asbestos instillation to first signs of disease-the latency period) or whether immune deficient mice survived longer once disease was established. There was a significant ($p<0.01$) delay in the latency period in asbestos exposed immunodeficient MexTAgRAG mice compared to normal MexTAg, suggesting that tumour induction takes longer in the absence of a functional immune system. However, there was no difference in survival between groups once disease was established. These data are intriguing and suggest that the tumour induction might require factors associated with the presence of a functional immune system. It would be easy to speculate that inflammatory cytokines induced after installation of asbestos fibres could play an important role in the induction or mesothelioma development, but further studies are required to test this hypothesis.

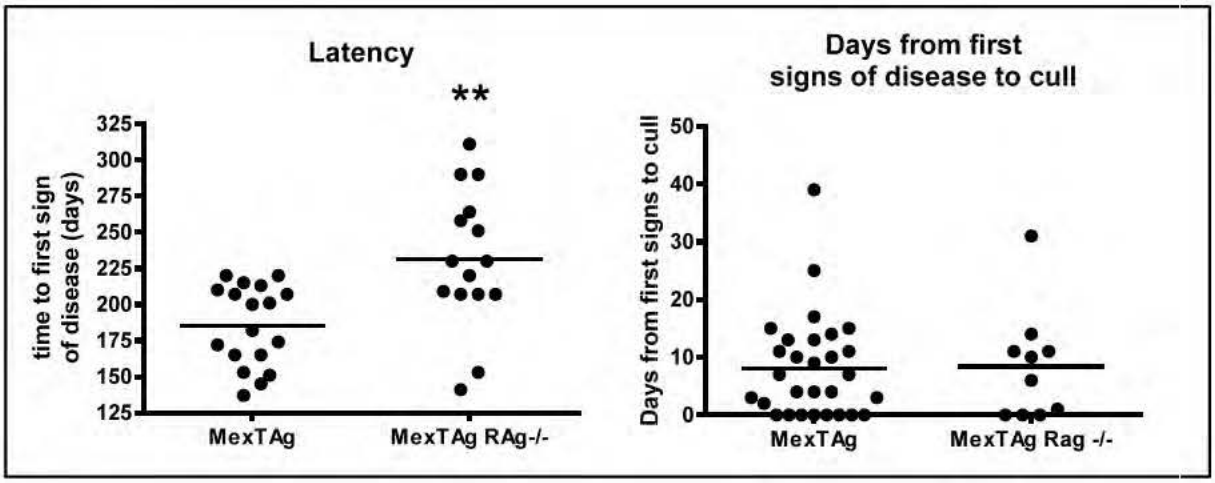

Figure 8: Latency vs. Disease. The contribution of disease induction (latency) and onset to the overall survival of asbestos exposed immune competent (MexTAg) and immune deficient (MexTAgRAG) mice were compared. Induction of asbestos induced mesothelioma was significantly delayed (p=0.0018) in that absence of a functional immune system. However, there was no significant difference in survival between groups once disease had developed.

Aim 2b Identify the key components of the anti-tumour adaptive immune response.

The unexpected results from Aim 2a in which asbestos induced mesothelioma development was delayed in immune deficient mice in comparison to immune competent mice precluded us from performing Aims 2b and 2c. However, while we confirmed the results from Aim 2a in subsequent experiments (Figures 7 and 8) we also chose to characterise the tumours that arose from the immune deficient MexTAgRAG mice by generating cell lines from ascites fluid. To date we have successfully established 7 cells lines derived from ascites fluid harvested from asbestos exposed MexTAgRAG mice. These cell lines were tested for malignancy by injection into the flank of syngeneic mice and assessed for tumour growth. We have observed variable growth characteristics between different cell lines. Some are consistent with the principle of immunosurveillance, in which 'unselected' tumours derived from immunodeficient MexTAgRAG mice can grow in immunodeficient mice, but are delayed when injected into immunocompetent mice, while others grow equally well in both MexTAg and MexTAgRAG mice. Data from two of the seven ascites derived cell lines representing the variable growth characteristics are shown in Figure 9.

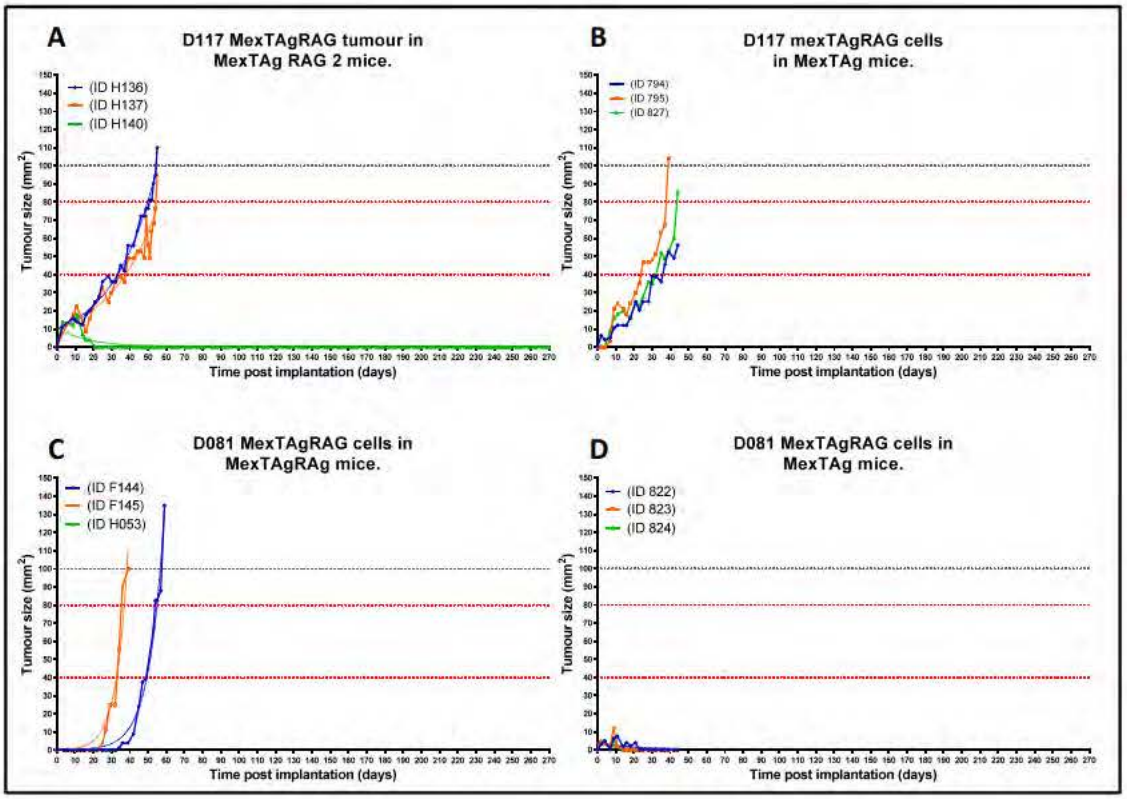

Figure 9: Growth of 'unselected' ascites derived mesothelioma cell lines. Immune competent MexTAg of immune deficient MexTAgRAG mice were subcutaneously inoculated with $1x10^6$ ascites derived mesothelioma cell lines. Experiments are representative of at least two independent experiments per cell line and show individual tumour growth curves from3 mice per group. Data showing two of seven cell lines representing variability of tumour growth over 270 days are shown. Two of three (2/3) D117 cells grew equally well in both immune deficient MexTAgRAG (A), and immunocompetent MexTAg mice (B). D081 cells show adhere to the principle of immune surveillance, growing in MexTAgRAg mice (C), but not in the presence of a functional immune system (D).

Due to the slow growth of some of these cell lines, these experiments remain ongoing (for the remaining 5 cell lines) and we will continue this important work as these cell lines represent a unique and irreplaceable resource for our future research program looking at using the genetic differences between asbestos induced mesotheliomas to inform the development of new treatment protocols.

KEY RESEARCH ACCOMPLISHMENTS:

- Established gemcitabine as chemotherapy agent with best efficacy at delaying mesothelioma tumour growth in murine tumour model.
- Response to gemcitabine chemotherapy is significantly improved when treatment is combined with the targeted depletion of regulatory T cells (Treg). *Demonstrates the importance of Treg removal in generating effective anti-tumour immunity.*
- Combination of chemo-immunotherapy was proven successful in a murine model of asbestos induced mesothelioma that mimics human disease; *demonstrating the translational potential of this approach.*

- Generation of unique ascites derived mesothelioma cell lines as an invaluable resource for future studies investigating the interplay between the host immune system and induction of asbestos induced mesothelioma.

CONCLUSION:

Malignant mesothelioma is an aggressive, incurable tumor caused by exposure to asbestos. Over 25 million people have served with the US military and many of them have been exposed to asbestos with up to a third of Veterans making up the estimated 2,000 and 3,000 cases of mesothelioma per year in the US. Although chemotherapy with an antimetabolite and a platinum based agent is the only strategy demonstrated to improve survival in randomised clinical trials; this treatment is predominantly palliative with MM patients undergoing chemotherapy having a median survival of only around 12 months (2). This poor prognosis highlights the need for improved treatment modalities. Combining chemotherapy with immunotherapy has shown promise in other solid cancers. However, there are few studies that have investigated whether immunotherapy may enhance the outcome of standard first line chemotherapy treatment for mesothelioma.

Our data demonstrate that immunotherapy has the potential to significantly impact on the current treatment of mesothelioma, particularly if used in combination with current first-lint therapies. Data from Aim 1 clearly demonstrates the potential of removing Treg to improve the overall response to chemotherapy. Importantly, the difficulty in doing this with currently available ‘Treg inhibitors’ was highlighted by the fact we needed to utilise the BALB/c.FoxP3.dtr mouse model to demonstrate this. However, while the FoxP3.dtr mouse model is not directly translatable into the clinic, the recent breakthrough in the use of monoclonal antibodies to specifically target and augment different immune cell subsets, including Treg (referred to as immune checkpoint blockade [7]) will no doubt bring the use of immunotherapy for mesothelioma into clinical reality. This has become one of the key research focus for our lab and the data generated by this grant has and will be used to further our research into the use of immune checkpoint blockade, alone or in combination with chemotherapy for the treatment of malignant mesothelioma.

Another key research focus is to extent the intriguing results obtained from Aim 2 experiments to further investigate the role of the immune system in the induction of asbestos induced mesothelioma and subsequent disease progression. While the results of Aim 2 were contrary to our initial hypothesis, the outcome resulted in the generation of a variety of unique cell lines that will be an invaluable resource. We will apply newly developed next generations genome sequencing technology to identify genetic mutations unique and common to these cell lines in comparison to

‘normal’ tissue that will help advance our knowledge of the genetic basis of mesothelioma as well as identify potential neo-antigens that could be used as targets for tumour specific immunotherapies such as cancer vaccines which could be used in conjunction with other newly developed immunotherapies such as immune checkpoint blockade. Taken together, the data from this grant has advanced our understanding of Treg immunology with respect to mesothelioma and will underpin the next 3-5 yrs of research in our lab.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

At least two manuscripts are currently being drafted for publication. Both require additional experiments to be performed in 2015. Manuscripts will be submitted in 2015 and we will notify the US department of defence when they have been published.

Abstracts: Aspects of the work covered by this grant have been presented at a variety of local, national and international conferences during the full period of this grant. They are listed below.

2013:

2013: Poster-Australian Society of Medical Research (Fisher)-Poster

2013: Poster- Australian Society of Immunology (Fisher) - Poster

2013: Poster-15th International Congress of Immunology. Milan, Italy. (Fisher)

2014: Invited Speaker -Combined Biological Science Meeting. Perth, Australia. (Fisher)

2014: invited Speaker – UWA School of Pathology and Laboratory Medicine Seminar series (Fisher)

2014: Speaker and Poster-12th International Mesothelioma Interest Group Conference. Cape Town. South Africa (Robinson and Fisher).

INVENTIONS, PATENTS AND LICENSES:

Nothing to report

REPORTABLE OUTCOMES:

Nothing to report

OTHER ACHIEVEMENTS:

- Generation of ascites derived mesothelioma cell line.
- Cell lines form basis of PhD project starting in 2015.

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APPENDICES:

No appendices