The Role of Regulatory T Cells in Mesothelioma

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Abstract Malignant mesothelioma (MM) appears to be responsive to immunotherapy. The lack of complete tumour cure as a result of many immunotherapies tested to date suggests that the immune response to MM is complex and multi-parametric. Regulatory T (Treg) cells are prevalent within murine and human mesotheliomas with their removal shown to result in tumour growth inhibition and the release of anti-tumour effector T cells from immunosuppression. The targeting of immune checkpoints as treatments for various solid tumours has recently shown promise in clinical settings. In addition, synergy between chemotherapy and immunotherapy has been demonstrated for many cancers, including mesothelioma. Here we demonstrate Treg cells as critical mediators of the anti-tumour immune response to MM and potential targets for anti-tumour immunotherapy; though the timing and dosage of Treg cell manipulating immunotherapies need to be optimised.

Keywords Malignant mesothelioma · Regulatory T cells · Immunosuppression · Immunotherapy · Cancer

Introduction

Malignant mesothelioma (MM) is a cancer of the pleura and less commonly of the peritoneum, tunica vaginalis and pericardium [1–3]. Though the relatively recent discovery of a serum marker for MM, mesothelin [4], may improve diagnosis in the future, at present MM remains a universally fatal cancer with median survival from diagnosis being only 9–12 months (reviewed in [5]). Despite MM being a relatively rare cancer, its incidence is increasing steadily and is not expected to peak worldwide until approximately 2020 [6]. The correlation between asbestos exposure and MM is clear [7]. By the late 1990s, governments in many developed countries, including Australia, banned or seriously restricted the use of asbestos. Despite this ban on asbestos sales, the incidence of MM is still expected to rise due to the recent “renovation boom” (particularly in Australia) and the very long lag-times between exposure and diagnosis (30–40 years). In addition, numerous developing countries continue to use asbestos [8]. In a 2005 Lancet review of MM, the management and treatment options for MM were discussed [5]. It was commented that novel therapies, such as immunotherapy and gene therapy, had received much research focus over the preceding 10 years as traditional therapies had proven largely ineffective. MM seems to be responsive to immunotherapy and MM patients are known to mount an anti-tumour immune response with some cases of spontaneous regression reported [9, 10]. The specific research contribution of our laboratory to this field has been the elucidation of the importance of regulatory T (Tregs) cells in the maintenance of an immuno-suppressive microenvironment within and surrounding growing murine mesotheliomas [11–13].

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CD4⁺CD25⁺Foxp3⁺ Treg cells are essential in maintaining peripheral immune tolerance [14]. Dysfunction of this cell population is associated with autoimmune diseases, persistent bacterial and viral infections, and inhibition of the immune response to cancer. Due to the important role of Treg cells in preventing autoimmune disease, homeostasis of this cell population is tightly regulated. Typically, Treg cells account for around 5–10% of the CD4⁺ T cell population [15]. However, in tumour-bearing hosts, Treg cell homeostasis is manipulated by the tumour to assist in the evasion of the immune system (reviewed in [16]).

Our research group was the first to identify CD4⁺ T cells co-expressing both the CD25 cell surface marker and the transcription factor Forkhead box 3 (Foxp3) within murine mesotheliomas [11]. Murine mesothelioma cell lines have been derived for several mouse strains from primary asbestos induced tumours [17, 18]. Intra-tumoural Treg cells in the C57BL/6J model of AE17 mesothelioma were found to increase significantly as a percentage of total CD4⁺ T cells within tumours as they grew [11]. In contrast, the proportion of Treg cells within the CD4⁺ T cell compartment in the periphery of tumour-bearing animals remained relatively constant with increasing tumour size. This constant level of Treg cells in peripheral tumour-draining lymph nodes (TDLNs) and non-tumour draining lymph nodes of mesothelioma bearing mice was independently confirmed by studies performed in a CBA intra-thoracic (AC29) mesothelioma model and a BALB/c subcutaneous (AB1) mesothelioma model as detailed in Table 1. Table 1 also presents confirmatory studies showing that intra-tumoural Treg cell numbers increase as mesotheliomas increase in size. Soon after these reports, CD4⁺CD25⁺Foxp3⁺ Treg cells were found in human mesothelioma pleural effusions, mesothelioma biopsies and the peripheral blood of mesothelioma patients (Table 1). A correlation between Treg cell presence in tumours and survival was proposed as higher levels of intra-tumoural Treg cells correlated with shorter survival in human malignant pleural mesothelioma [19].

Recruitment of Treg Cells to Mesotheliomas

The precise mechanism by which Treg cells are recruited to tumours, including mesotheliomas, and exert their function is unknown. One hypothesis is that Treg cells are generated in the periphery and are selectively recruited from the periphery to the tumour. Recent studies have shown that certain cytokines and chemokines can selectively induce Treg cell migration over other T cell populations [20, 21]. The expression of specific integrins (ie. $\alpha_E\beta_7$ /CD103) has been

shown important for the homing of Treg cells to inflamed sites [22]. This migration of Treg cells from host tissues into the developing mesotheliomas was demonstrated in a study using a murine model where mesothelioma cells were implanted intra-peritoneally into GFP-expressing mice [23]. CCL24, which recruits resting T cells [24], and interleukin (IL)-16, which has previously been shown in malignant pleural effusions to attract and assist in the differentiation of Treg cells [25], were both found to be upregulated 7 days post tumour implantation. In addition, macrophage derived CCL17 and CCL22, known to recruit and assist in the differentiation of Treg cells, were shown to peak at day 14 post tumour challenge suggesting that macrophages may also play an important role in Treg cell recruitment to mesotheliomas. In our hands, neither the blockade of intra-tumoural CCL22 (0.2 mg anti-CCL22 monoclonal antibody (mAb) (clone 158113, R&D Systems) administered intra-tumourally to established 9 mm² AE17 s.c. tumours in C57BL/6J mice) nor systemic CCL22 (0.5 mg anti-CCL22 mAb administered i.p. to C57BL/6J mice bearing established 9 mm² s.c. AE17 tumours) resulted in any significant tumour growth inhibition. These same experiments showed no depletion of Treg cells within tumours and no activation of effector T cells within tumours [26]. CCL2, CCL12 and CXCL12 secreted or expressed by tumours cells (including mesothelioma cells) plus other tumour-associated cells also have chemotactic properties for Treg cells [27]. CCL2/CCL12 blockade in a murine AE17 model transfected with human mesothelin was shown to marginally inhibit tumour growth [28]. However, the blockade of CCL2/CCL12 combined with a vaccine targeted at the human mesothelin led to complete tumour clearance in two thirds of the animals treated. Follow-up mechanistic studies were not performed in the AE17 model but showed the combination of CCL2/CCL12 blockade with vaccine therapy resulted in a reduction in Treg cells and an accumulation of activated CD8⁺ T cells within tumours. Global gene expression studies performed to phenotype naive from effector Treg cells found that CD25⁺CD103⁺ Treg cells displayed a naïve-like phenotype with high expression of CD62L [29]. CD103⁺ Treg cells appeared to show an activated phenotype as they expressed lower levels of CD62L and higher levels of CD44. Given that integrin $\alpha_E\beta_7$ (CD103) was also important in the homing of Treg cells to inflamed sites, we hypothesised that it may be required for peripheral Treg cell recruitment to mesotheliomas. In our model we examined the expression of CD103 by CD4⁺CD25⁺ Treg cells (clone M290-PE conjugated, BD Pharmingen 557495) by flow cytometry (BD FACS Callibur with CellQuest Pro Software) in peripheral blood, TDLNs and tumours harvested from AE17 s.c. mesothelioma bearing mice as previously described [11, 13]. A total of 10 mice were examined in three independent experiments and the data

Table 1 The identification of Treg cells within or associated with mesotheliomas plus correlation of Treg cell presence with disease state

Species (strain and cell line)		Tissue type	Phenotype of Treg cells and correlation to disease state	Refs
Mouse	C57BL/6J (AE17)	Subcutaneous tumour	The proportion of CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Treg cells in the CD4 ⁺ T cell compartment increased as tumours increased in size	[11]
		Periphery: Blood, spleen and TDLN	Proportion of CD4 ⁺ T cells co-expressing CD25 and Foxp3 remained the same as tumours grew	[11]
	Balb/c (AB1)	Intra-peritoneal tumour	A significant increase in CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Treg cells was found to correlate with tumour burden. Treg cells were found in close proximity to CD8 ⁺ T cells implying direct immunosuppression.	[61]
	Balb/c (ABI-HA)	Subcutaneous tumour	Proportion of Foxp3 ⁺ cells in the CD4 ⁺ T cell compartment increased with tumour growth	[42]
		Periphery: TDLN and non-DLN	Approx 14% of CD4 ⁺ T cells in each of the sites (including tumours) were found to co-express Foxp3	[63]
	CBA (AC29)	Intra-thoracic tumour	Foxp3 gene expression was detected in tumour infiltrating CD4 ⁺ CD25 ⁺ T cells	[38]
		Periphery: pleural effusions and blood	Treg cells also evident in periphery	[38]
Human		Mesothelioma biopsies	CD4 ⁺ T cells identified and correlated with CD25 and Foxp3 expression. High levels of CD4 ⁺ or CD25 ⁺ tumour infiltrating T cells demonstrated a trend towards shorter survival.	[19, 35]
		Mesothelioma pleural effusions	Identification of Treg cells within mesothelioma pleural effusions. Pleural effusions found to contain a high concentration of TGF-β.	[36]
		Peripheral blood of pleural mesothelioma patients	Similar proportion of CD4 ⁺ CD25 ⁺ Treg cells found in both pleural mesothelioma patients and normal healthy controls, this was inferred to be a result of the disease being predominantly localised in nature	[64]

pooled to reveal a lower proportion ($20.5 \pm 5.4\%$) of Treg cells expressed CD103 in the blood, whilst a significantly higher percentage of Treg cells expressed this marker in TDLNs ($45.7 \pm 5.3\%$, $p < 0.001$) [26]. A further increase in expression of CD103 by Treg cells was evident within mesotheliomas ($70.4 \pm 18.3\%$, $p < 0.005$). The upregulated expression of CD103 (approximately 3-fold) within tumours was also confirmed by an Affymetrix global gene-chip study comparing the gene expression of activated, tumour-derived Treg cells with naïve, splenic T cells of the same phenotype [26]. These data suggested that movement of Treg cells within murine mesothelioma bearing mice may be mediated by the expression of the surface integrin CD103. The blockade of Treg cell recruitment to tumours using an anti-CD103 mAb to block the interaction of CD103 with its ligand, E-cadherin, did not significantly improve the anti-tumour immune response. Low doses of anti-CD103 mAb ($1 \mu\text{g}$ – $5 \mu\text{g}$ clone 2E7, Ebioscience 14–1031) administered intra-tumourally on a daily basis to established AE17 murine mesotheliomas (9 mm^2) in C57BL/6J mice only transiently inhibited tumour growth. The administration of the anti-CD103 mAb from the time of tumour inoculation significantly increased the time taken for a tumour to become detectable; an increase from 8 ± 1 days in untreated mice to 12 ± 1 days in treated mice. Interestingly, there was little change in the number of Treg cells found within mesotheliomas that had received anti-CD103 mAb treatment

compared to untreated controls (unpublished data, Ireland and Beilharz Laboratory). Thus, although it appeared that Treg cells in murine mesotheliomas expressed CD103, the migration of these cells to the tumour was not solely determined by CD103 expression. A transwell assay to examine migration of Treg cells from within peripheral blood mononuclear cells (PBMCs) towards mesothelioma cells derived from a surgical specimen showed Treg cells migrate in response to both IL-6 and IL-8 produced by the mesothelioma cells [30]. Both IL-6 and IL-8 are detectable in pleural effusions associated with mesotheliomas and have both been demonstrated as growth potentiating factors in mesothelioma [31, 32]. The marked induction of chemokine receptor CXCR1 (IL-8 receptor) expression by Treg cells associated with tumour IL-6 secretion [30] followed by IL-8 induced migration of Treg cells may be a mechanism of tumour escape utilised by mesothelioma tumours. Murine studies have shown that decreasing mesothelioma associated IL-8 levels may inhibit mesothelioma growth [33]. Finally, in further support of tumour induced immunosuppression, Treg cells have been shown to be recruited to tumour sites, including murine mesotheliomas, with kinetics akin to memory T cells responses as opposed to the slow recruitment kinetics of anti-tumour effector T cells seeing antigen for the first time [34]. This swift recruitment of Treg cells establishes a microenvironment of immune tolerance enabling the persistence of mesothelioma cells [23].

Mechanisms of Immunosuppression Exerted by Treg Cells

The immunosuppressive mechanisms of Treg cells are known to be many and varied; depending on the pathology of disease they are associated with. Foxp3 is central to Treg cell development and function and the master regulator of this cell population [14]. The quest for other functional markers of this cell type is however still ongoing. Based on our Treg cell depletion and inactivation studies it was hypothesised that the immunosuppressive cytokine transforming growth factor-beta (TGF- β) was the master regulator of Treg cell mediated immunosuppression in the AE17 murine mesothelioma model [13]. This was supported by the detection of TGF- β in mesothelioma associated pleural effusions; though it was not clear which cell type produced the cytokine [35, 36]. However, although important, TGF- β alone is not solely responsible for maintaining immunosuppression in this model and thus multiple mechanisms of immunosuppression exist [12]. In the s.c. AC29 murine mesothelioma model (CBA mice) granzyme B and perforin, both of which can mediate Treg cell suppression of anti-tumour natural killer (NK) cell and/or CD8⁺ T cell responses, were also found not to be crucial mediators [37]. After Treg cell depletion the levels of granzyme B and perforin were increased and thus associated with effector T cells rather than Treg cells. An analysis of pleural effusions of mice with intra-thoracic AC29 mesothelioma demonstrated that after Treg cell depletion an increase in IL-2 was observed together with a reduction in TGF- β (confirming our own laboratory's findings) and IL-10 [38]. IL-2 activates multiple immune cell subsets including, but not limited to, T cells, NK cells and macrophages but its primary function is considered the generation and survival of Treg cells constitutively expressing CD25 or the IL-2 receptor alpha-chain (reviewed in [39]). Treg cells and activated effector T cells therefore compete for IL-2, particularly when the cells are co-localised [40]. In an in vitro study, exosomes (nanometre sized vesicles) secreted by mesothelioma cells were shown to skew IL-2 responsiveness towards Treg cells with an involvement of tumour cell bound TGF- β [41]. The sequestration of IL-2 by Treg cells away from anti-tumour immune cells is likely an important mechanism of immunosuppression employed by Treg cells in mesothelioma. It potentially inhibits effector T cell accumulation, retention, survival, proliferation or function within tumours [42] but also dendritic cell (DC) activation and maturation [38]. Tumour necrosis factor receptor-2 (TNFR2) and inducible T cell co-stimulator (ICOS) have both been associated, against the accepted norm of Treg cell anergy, with proliferation of Treg cells (Ki-67 expression) in the AB1 murine mesothelioma model [43]. Within this murine mesothelioma

model Treg cells were active suppressors that increased suppressor function in response to tumour antigen. The T cell inhibitory receptor programmed death-1 (PD-1) is a B7 family receptor expressed by activated T cells, including Treg cells, which regulates T cell function through interaction with programmed death-ligand 1 (PD-L1) [44]. A further model for murine mesothelioma immunosuppression has also been proposed where PD-1 acts as the critical mediator [45]. Blockade of the expression of PD-L1 by the same AB1 and AB1-HA mesothelioma cells had a very limited impact on slowing tumour growth but was shown to rescue both CD4⁺PD-1⁺ T cells and CD8⁺PD-1⁺ T cells from immunosuppression. This occurred though only in the absence of Treg cells suggesting that the majority of reactivated CD4 T cells were in fact Treg cells and that they were in part under the control of the tumour. Interestingly, the authors reported unpublished data whereby slower tumour growth was observed when PD-L1 blockade was combined with TGF- β neutralisation.

Improved Survival Results from Treg Cell Depletion

The suppression of anti-tumour immune responses by Treg cells in tumour-bearing hosts is now well established. International research focus has largely moved towards the manipulation of these Treg cells to reinstate the anti-tumour immune response (Table 2). We have reported that the intra-tumoural administration of Treg cell depleting anti-CD25 mAb (PC61) is highly effective at inhibiting development of established murine AE17 mesotheliomas of C57BL/6J mice [11]. We found that to successfully inhibit murine mesothelioma growth, the depletion of intra-tumoural Treg cells from tumour-bearing hosts using anti-CD25 mAb requires a titrated dosage for intra-tumoural injection that only just depletes the pool of Tregs, resulting in CD4⁺ helper T cell and CD8⁺ effector T cell activation [13]. In contrast, higher doses of anti-CD25 mAb, often administered systemically, have been shown to lead to accelerated mesothelioma growth caused by excess antibody co-depleting the activated CD8⁺CD25⁺ T cells released from immunosuppression as a result of the initial Treg cell depletion [13, 37, 46]. The positive effect of low dose, intra-tumoural anti-CD25 mAb treatment is however transient with Treg cells returning to tumours by day 10 post depletion. Longer term survival from murine mesothelioma or periods of more complete murine mesothelioma growth inhibition have been reported when treatment with anti-CD25 mAb is administered prior to tumour challenge. In an intra-thoracic model of mesothelioma, systemic anti-CD25 mAb depletion did not inhibit tumour growth at all when administered 4 days after tumour implantation but

Table 2 Mesothelioma survival outcome following Treg cell depletion

Mouse strain and tumour ^a	Treg cell depletion strategy	Efficacy to deplete Treg cells	Survival outcome	Refs
C57BL/6J with s.c. AE17	Low dose i.t. anti-CD25 mAb (PC61) to established tumours	Significant reduction in Treg cells within tumours and some spill over to periphery	10 day period of tumour growth inhibition	[11, 13]
	High dose i.t. anti-CD25 mAb to established tumours	Significant reduction in Treg cells and all CD25-expressing cells within tumours and the periphery	No change in tumour growth compared to non-depleted controls	[46]
	Low dose i.t. anti-CD25 mAb combined with i.t. TGF- β SR to established tumours	Significant reduction in Treg cells within tumours and some spill over to periphery, induction of anti-tumour effector T cells	10 day period of tumour regression	[12, 13]
	Low dose i.t. anti-CD25 mAb combined with i.t. TGF- β SR and i.p. anti-CTLA4 mAb “triple immunotherapy” to established tumours	Long term depletion of Treg cells from within tumours, activation of anti-tumour effector T cells and memory T cells	Complete tumour clearance and protection from tumour rechallenge	[50]
Balb/c with i.p. AB1	Cyclophosphamide in drinking water to established tumours	Significant reduction in CD4 ⁺ Foxp3 ⁺ Treg cells in spleens but not peripheral blood	Approximate 10 day increase in survival	[61]
CBA with i.t. AC29	High dose systemic anti-CD25 mAb prior to tumour challenge	Significant reduction in Treg cell within tumours	Significantly prolonged survival	[38]
	High dose systemic anti-CD25 mAb to established tumours	Significant reduction in Treg cell within tumours	No improvement in survival	[38]
CBA with s.c. AC29	High dose anti-CD25 mAb to established tumours	N/A	A short delay in tumour growth	[37]

^a s.c. subcutaneous; i.p. intra-peritoneal; i.t. intra-thoracic

resulted in a significant prolonging of survival when administered 2 days prior to tumour implantation [38]. Though these pre-treatment studies have shown a clear ability to prevent the development of mesotheliomas in murine models, this data and treatment regime is difficult to translate to the clinic. As such, our team has focussed on developing combined immunotherapies with the ability to regress established tumours in a s.c. model of murine mesothelioma.

Intra-tumoural TGF- β was proposed as an important mediator of Treg cell re-accumulation within tumours and therefore also the loss of anti-tumour immunity observed 10 days post Treg cell depletion [13]. TGF- β located within tumours has been shown to maintain an immunosuppressive environment [47] and to inhibit the cytotoxic capacity of CD8⁺ T cells as well as NK cells [48, 49]. We therefore showed that by combining, and precisely timing, neutralization of TGF- β within mesotheliomas using a TGF- β soluble receptor (SR) together with anti-CD25 mAb depletion, improved transient tumour regression could be achieved. This combined treatment maintained the intra-tumoural TGF- β concentration at a significantly lower level than either the untreated controls or the single anti-CD25 mAb treatment alone and correlated with a significantly higher intra-tumoural concentration of interferon (IFN)- γ ; but tumours eventually relapsed [12]. The presence of Treg cells within TDLNs was also shown to alter the phenotype of DCs in the same location. This suggested that because the anti-tumour immune response is inhibited by multiple mechanisms the development of immunotherapeutic treatment regimes will be more successful if these mechanisms can be simultaneously targeted. This led to our current research where we have demonstrated improved outcome and survival by adding the blockade of CTLA-4 to the anti-CD25 mAb/TGF- β SR regime in the AE17 murine model of s.c. mesothelioma [50]. Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is one of the most potent negative regulators of T cell responses; the blockade of which enhances anti-tumour T cell responses and inhibits the induction of T cell anergy [51]. Data from these experiments has provided evidence that long-term clearance of established mesotheliomas can be achieved as well as resistance to tumour re-challenge. The emergence of resistance to rechallenge in mice cured by our “triple immunotherapy” is of particular note as it denotes a first reversal of dominant tolerance [34]. This “triple immunotherapy” looks particularly promising especially as all components of the “triple immunotherapy” have approved human equivalents or are undergoing clinical trials. Clinical trials have investigated TGF- β blockade [52] and denileukin diftitox (Ontak) to deplete Treg cells in a number of different cancers [53–55] while the use of anti-CTLA4 Ig (Ipilimumab, Bristol Myers Squibb) was approved for the treatment of melanoma based on a recent phase III clinical trial [56].

Clinical Translation of Treg Cell Manipulating Therapies for Mesothelioma

Although many of these Treg cell depletion strategies are showing promise, it is unlikely that they will be used as a single modality to treat an advanced stage mesothelioma patient. Instead, the standard first line chemotherapy for malignant mesothelioma, pemetrexed/cisplatin, is likely to be prescribed in combination with new immunotherapies (personal communication Prof Bruce Robinson, The University of Western Australia [57]). The combination of chemotherapy with immunotherapy is undergoing a revival of interest. It has now been shown that chemotherapy does not always have a negative effect on the immune response by inducing lymphopaenia, but in fact may be compatible or even synergistic with immunotherapy (reviewed in [58]). This may particularly be the case for Treg cell manipulating immunotherapies with the potential to induce autoimmunity if administered either systemically or for a prolonged time and for vaccine approaches such as immunogene therapy where neutralising antibodies are rapidly produced after multiple administrations [59]. Beyond this, many chemotherapies, by nature of their specificity for rapidly dividing cells, have been shown to possess some selectivity for Treg cells. Cyclophosphamide can more specifically target Treg cells, though this appears to be somewhat dependent on dosage and timing [60]. Low-dose cyclophosphamide induces beneficial immunomodulatory effects by preventing the induction of Treg cells, and as a consequence, releases effector T cells from immunosuppression [61]. Pemetrexed/cisplatin is the standard first line chemotherapy for malignant mesothelioma but until recently pemetrexed had not been tested for effect on Treg cells. Using a novel intra-thoracic model of murine mesothelioma in which AC29 cells were implanted into the pleura of mice, pemetrexed was shown to synergise with anti-CD25 mAb depletion to enhance survival of mice with established tumours in a CD8⁺ T cell dependent manner [38]. The mechanisms behind this synergy seem complex with data suggesting it may involve changes in the tumour microenvironment such as increased IL-2 and intra-tumoural IFN- γ ⁺CD8⁺ effector T cells, enhanced DC maturation and a decrease in IL-10. In the subcutaneous AC29 tumour model, the combination of cisplatin chemotherapy with Treg cell depletion was investigated in order to determine the impact of Treg cell depletion on tumour cell repopulation between cycles of chemotherapy [37]. Tumour growth inhibition was most significant (though no regression was achieved) when established tumours (~5 mm in diameter) were treated with 100 μ g anti-CD25 mAb i.p. 1 day post each cisplatin treatment (administered at weekly intervals). This was also correlated with a reduction in tumour cell repopulation. This treatment combination resulted in an almost complete depletion of Treg cells from within tumours and lymphoid organs; whilst CD4⁺ and CD8⁺ T cell

numbers remained unchanged. An increase in intra-tumoural IFN- γ , granzyme-b, perforin and IFN- γ -induced protein (IP)-10 were all observed within tumours. Adenoviral-based immunogene therapy regimens in mice (Balb/c) bearing established AB1 mesothelioma tumours boosted with three weekly administrations of cisplatin/gemcitabine have also been shown to result in improved tumour clearance and an increase in the number of antigen-specific, activated CD8⁺ T cells in the periphery and within tumours [59]. Multiple mechanisms were proposed and studied for this improved outcome including a decrease in Treg cell numbers. Immunogene vaccination almost doubled the number of splenic CD4⁺CD25⁺ Treg cells, chemotherapy alone had little effect on the Treg cells while the combination was shown to reduce the number of Treg cells by about 50% (though note this analysis was not performed in the mesothelioma model) [59].

Conclusion

It is becoming increasingly clear that MM tumours are responsive to immunotherapy. The lack of complete tumour cure as a result of many immunotherapies tested so far suggests that the immune response to MM is complex and multi-parametric. The removal of immunosuppressive Treg cells alone has been shown on numerous occasions to result in tumour growth inhibition and the induction or release of anti-tumour effector T cells from immunosuppression. It is likely however that to achieve complete tumour cure or long-term survival that Treg cell depletion will need to be combined with effector T cell boosting and likely also applied as an adjunct to conventional therapies. Treatments aimed at targeting immune checkpoints of cancer have recently shown promise in clinical settings. These include CTLA-4 blockade which is approved for treatment of human metastatic melanoma [56] and PD-1 blockade which is undergoing early clinical trials in humans and appears to be better tolerated than anti-CTLA4 whilst still showing an anti-cancer effect [62]. These studies indicate that targeting mechanisms of immunosuppression is a viable strategy for the treatment of human cancers. For mesothelioma, the prevalence of Treg cells within human tumours, together with the results of animal studies, suggests these cells are an intelligent target for immunotherapy. Finally, the combination of chemotherapy and immunotherapy has been shown to be synergistic in many settings including mesothelioma.

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References

- Berry G, de Klerk NH, Reid A, Ambrosini GL, Fritschi L, Olsen NJ, Merler E, Musk AW (2004) Malignant pleural and peritoneal mesotheliomas in former miners and millers of crocidolite at Wittenoom, Western Australia. *Occup Environ Med* 61(4):e14
- Hirano H, Maeda T, Tsuji M, Ito Y, Kizaki T, Yoshii Y, Sashikata T (2002) Malignant mesothelioma of the pericardium: case reports and immunohistochemical studies including Ki-67 expression. *Pathol Int* 52(10):669–676
- Abe K, Kato N, Miki K, Nimura S, Suzuki M, Kiyota H, Onodera S, Oishi Y (2002) Malignant mesothelioma of testicular tunica vaginalis. *Int J Urol* 9(10):602–603
- Creaney J, Francis RJ, Dick IM, Musk AW, Robinson BW, Byrne MJ, Nowak AK (2011) Serum soluble mesothelin concentrations in malignant pleural mesothelioma: relationship to tumor volume, clinical stage and changes in tumor burden. *Clin Cancer Res* 17(5):1181–1189
- Robinson BW, Musk AW, Lake RA (2005) Malignant mesothelioma. *Lancet* 366(9483):397–408
- Ismail-Khan R, Robinson LA, Williams CC Jr, Garrett CR, Bepler G, Simon GR (2006) Malignant pleural mesothelioma: a comprehensive review. *Cancer Control* 13(4):255–263
- Fingerhut M, Nelson DI, Driscoll T, Concha-Barrientos M, Steenland K, Punnett L, Pruss-Ustun A, Leigh J, Corvalan C, Eijkemans G, Takala J (2006) The contribution of occupational risks to the global burden of disease: summary and next steps. *Med Lav* 97(2):313–321
- Bianchi C, Bianchi T (2007) Malignant mesothelioma: global incidence and relationship with asbestos. *Ind Health* 45(3):379–387
- Robinson BW, Robinson C, Lake RA (2001) Localised spontaneous regression in mesothelioma—possible immunological mechanism. *Lung Cancer* 32(2):197–201
- Robinson C, Callow M, Stevenson S, Scott B, Robinson BW, Lake RA (2000) Serologic responses in patients with malignant mesothelioma: evidence for both public and private specificities. *Am J Respir Cell Mol Biol* 22(5):550–556
- Needham DJ, Lee JX, Beilharz MW (2006) Intra-tumoural regulatory T cells: a potential new target in cancer immunotherapy. *Biochem Biophys Res Commun* 343(3):684–691
- Kissick HT, Ireland DJ, Greay SJ, Beilharz MW (2010) Mechanisms of immune suppression exerted by regulatory T-cells in subcutaneous AE17 murine mesothelioma. *J Interferon Cytokine Res* 30(11):829–834
- Kissick HT, Ireland DJ, Beilharz MW (2009) Combined intratumoral regulatory T-cell depletion and transforming growth factor-beta neutralization induces regression of established AE17 murine mesothelioma tumors. *J Interferon Cytokine Res* 29(4):209–215
- Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4(4):330–336
- Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155(3):1151–1164
- Nishikawa H, Sakaguchi S (2010) Regulatory T cells in tumor immunity. *Int J Cancer* 127(4):759–767
- Davis MR, Manning LS, Whitaker D, Garlepp MJ, Robinson BW (1992) Establishment of a murine model of malignant mesothelioma. *Int J Cancer* 52(6):881–886
- Jackaman C, Bundell CS, Kinnear BF, Smith AM, Filion P, van Hagen D, Robinson BW, Nelson DJ (2003) IL-2 intratumoral immunotherapy enhances CD8+ T cells that mediate destruction of tumor cells and tumor-associated vasculature: a novel mechanism for IL-2. *J Immunol* 171(10):5051–5063
- Anraku M, Cunningham KS, Yun Z, Tsao MS, Zhang L, Keshavjee S, Johnston MR, de Perrot M (2008) Impact of tumor-infiltrating T cells on survival in patients with malignant pleural mesothelioma. *J Thorac Cardiovasc Surg* 135(4):823–829
- Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10(9):942–949
- McFadden C, Morgan R, Rahangdale S, Green D, Yamasaki H, Center D, Cruikshank W (2007) Preferential migration of T regulatory cells induced by IL-16. *J Immunol* 179(10):6439–6445
- Suffia I, Reckling SK, Salay G, Belkaid Y (2005) A role for CD103 in the retention of CD4+CD25+ Treg and control of Leishmania major infection. *J Immunol* 174(9):5444–5455
- Miselis NR, Lau BW, Wu Z, Kane AB (2010) Kinetics of host cell recruitment during dissemination of diffuse malignant peritoneal mesothelioma. *Cancer Microenviron* 4(1):39–50
- Cheadle EJ, Riyad K, Subar D, Rothwell DG, Ashton G, Batha H, Sherlock DJ, Hawkins RE, Gilham DE (2007) Eotaxin-2 and colorectal cancer: a potential target for immune therapy. *Clin Cancer Res* 13(19):5719–5728
- Qin XJ, Shi HZ, Huang ZX, Kang LF, Mo WN, Wu C (2005) Interleukin-16 in tuberculous and malignant pleural effusions. *Eur Respir J* 25(4):605–611
- Ireland DJ (2006) Intra-tumoural regulatory T cells: a potential new target for anti-cancer immunotherapy (PhD Thesis). The University of Western Australia, Crawley, WA, 6009, Australia
- Shimizu Y, Dobashi K, Imai H, Sunaga N, Ono A, Sano T, Hikino T, Shimizu K, Tanaka S, Ishizuka T, Utsugi M, Mori M (2009) CXCR4+FOXP3+CD25+ lymphocytes accumulate in CXCL12-expressing malignant pleural mesothelioma. *Int J Immunopathol Pharmacol* 22(1):43–51
- Fridlender ZG, Buchlis G, Kapoor V, Cheng G, Sun J, Singhal S, Crisanti MC, Wang LC, Heitjan D, Snyder LA, Albelda SM (2010) CCL2 blockade augments cancer immunotherapy. *Cancer Res* 70(1):109–118
- Huehn J, Siegmund K, Lehmann JC, Siewert C, Haubold U, Feuerer M, Debes GF, Lauber J, Frey O, Przybylski GK, Niesner U, de la Rosa M, Schmidt CA, Brauer R, Buer J, Scheffold A, Hamann A (2004) Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J Exp Med* 199(3):303–313
- Eikawa S, Ohue Y, Kitaoka K, Aji T, Uenaka A, Oka M, Nakayama E (2010) Enrichment of Foxp3+ CD4 regulatory T cells in migrated T cells to IL-6- and IL-8-expressing tumors through predominant induction of CXCR1 by IL-6. *J Immunol* 185(11):6734–6740
- Galfy G, Mohammed KA, Dowling PA, Nasreen N, Ward MJ, Antony VB (1999) Interleukin 8: an autocrine growth factor for malignant mesothelioma. *Cancer Res* 59(2):367–371
- Fujino S, Yokoyama A, Kohno N, Hiwada K (1996) Interleukin 6 is an autocrine growth factor for normal human pleural mesothelial cells. *Am J Respir Cell Mol Biol* 14(6):508–515

33. Galfy G, Mohammed KA, Nasreen N, Ward MJ, Antony VB (1999) Inhibition of interleukin-8 reduces human malignant pleural mesothelioma propagation in nude mouse model. *Oncol Res* 11 (4):187–194
34. Darrasse-Jeze G, Bergot AS, Durgeau A, Billiard F, Salomon BL, Cohen JL, Bellier B, Podsypanina K, Klatzmann D (2009) Tumor emergence is sensed by self-specific CD44hi memory Tregs that create a dominant tolerogenic environment for tumors in mice. *J Clin Invest* 119(9):2648–2662
35. Hegmans JP, Hemmes A, Hammad H, Boon L, Hoogsteden HC, Lambrecht BN (2006) Mesothelioma environment comprises cytokines and T-regulatory cells that suppress immune responses. *Eur Respir J* 27(6):1086–1095
36. Delong P, Carroll RG, Henry AC, Tanaka T, Ahmad S, Leibowitz MS, Sterman DH, June CH, Albelda SM, Vonderheide RH (2005) Regulatory T cells and cytokines in malignant pleural effusions secondary to mesothelioma and carcinoma. *Cancer Biol Ther* 4(3)
37. Wu L, Yun Z, Tagawa T, Rey-McIntyre K, Anraku M, de Perrot M (2011) Tumor cell repopulation between cycles of chemotherapy is inhibited by regulatory T-Cell depletion in a murine mesothelioma model. *J Thorac Oncol*
38. Anraku M, Tagawa T, Wu L, Yun Z, Keshavjee S, Zhang L, Johnston MR, de Perrot M (2010) Synergistic antitumor effects of regulatory T cell blockade combined with pemetrexed in murine malignant mesothelioma. *J Immunol* 185(2):956–966
39. Bluestone JA (2011) The yin and yang of interleukin-2-mediated immunotherapy. *N Engl J Med* 365(22):2129–2131
40. Busse D, de la Rosa M, Hobiger K, Thurley K, Flossdorf M, Scheffold A, Hofer T (2010) Competing feedback loops shape IL-2 signaling between helper and regulatory T lymphocytes in cellular microenvironments. *Proc Natl Acad Sci U S A* 107(7):3058–3063
41. Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z (2007) Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. *Cancer Res* 67(15):7458–7466
42. Rudge G, Barrett SP, Scott B, van Driel IR (2007) Infiltration of a mesothelioma by IFN-gamma-producing cells and tumor rejection after depletion of regulatory T cells. *J Immunol* 178(7):4089–4096
43. van der Most RG, Currie AJ, Mahendran S, Prosser A, Darabi A, Robinson BW, Nowak AK, Lake RA (2009) Tumor eradication after cyclophosphamide depends on concurrent depletion of regulatory T cells: a role for cycling TNFR2-expressing effector-suppressor T cells in limiting effective chemotherapy. *Cancer Immunol Immunother* 58(8):1219–1228
44. Latchman YE, Liang SC, Wu Y, Chernova T, Sobel RA, Klemm M, Kuchroo VK, Freeman GJ, Sharpe AH (2004) PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci U S A* 101(29):10691–10696
45. Currie AJ, Prosser A, McDonnell A, Cleaver AL, Robinson BW, Freeman GJ, van der Most RG (2009) Dual control of antitumor CD8 T cells through the programmed death-1/programmed death-ligand 1 pathway and immunosuppressive CD4 T cells: regulation and counterregulation. *J Immunol* 183(12):7898–7908
46. Jackaman C, Cornwall S, Lew AM, Zhan Y, Robinson BW, Nelson DJ (2009) Local effector failure in mesothelioma is not mediated by CD4+ CD25+ T-regulator cells. *Eur Respir J* 34(1):162–175
47. Rabinovich GA, Gabrilovich D, Sotomayor EM (2007) Immunosuppressive strategies that are mediated by tumor cells. *Annu Rev Immunol* 25:267–296
48. Smyth MJ, Teng MW, Swann J, Kyriassoudis K, Godfrey DI, Hayakawa Y (2006) CD4+CD25+ T Regulatory Cells Suppress NK Cell-Mediated Immunotherapy of Cancer. *J Immunol* 176 (3):1582–1587
49. Chen ML, Pittet MJ, Gorelik L, Flavell RA, Weissleder R, von Boehmer H, Khazaie K (2005) Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A* 102(2):419–424
50. Kissick HT, Ireland DJ, Krishnan S, Madondo M, Beilharz MW (2012) Tumour eradication and induction of memory against murine mesothelioma by combined immunotherapy. *Immunol Cell Biol* in press
51. Pedicord VA, Montalvo W, Leiner IM, Allison JP (2011) Single dose of anti-CTLA-4 enhances CD8+ T-cell memory formation, function, and maintenance. *Proc Natl Acad Sci U S A* 108(1):266–271
52. Saunier EF, Akhurst RJ (2006) TGF beta inhibition for cancer therapy. *Curr Cancer Drug Targets* 6(7):565–578
53. Callahan MK, Wolchok JD, Allison JP (2010) Anti-CTLA-4 antibody therapy: immune monitoring during clinical development of a novel immunotherapy. *Semin Oncol* 37(5):473–484
54. Ku GY, Yuan J, Page DB, Schroeder SE, Panageas KS, Carvajal RD, Chapman PB, Schwartz GK, Allison JP, Wolchok JD (2010) Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival. *Cancer* 116 (7):1767–1775
55. Small EJ, Tchekmedyian NS, Rini BI, Fong L, Lowy I, Allison JP (2007) A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer. *Clin Cancer Res* 13(6):1810–1815
56. Hodi FS (2010) Overcoming immunological tolerance to melanoma: targeting CTLA-4. *Asia Pac J Clin Oncol* 6(Suppl 1): S16–S23
57. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, Gatzemeier U, Boyer M, Emri S, Manegold C, Niyikiza C, Paoletti P (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J Clin Oncol* 21(14):2636–2644
58. McCoy MJ, Nowak AK, Lake RA (2009) Chemoimmunotherapy: an emerging strategy for the treatment of malignant mesothelioma. *Tissue Antigens* 74(1):1–10
59. Fridlender ZG, Sun J, Singhal S, Kapoor V, Cheng G, Suzuki E, Albelda SM (2010) Chemotherapy delivered after viral immunogene therapy augments antitumor efficacy via multiple immune-mediated mechanisms. *Mol Ther* 18(11):1947–1959
60. Nowak AK, Lake RA, Robinson BWS (2006) Combined chemoimmunotherapy of solid tumours: improving vaccines? *Adv Drug Deliv Rev Hum Cancer Vaccines* 58(8):975–990
61. Veltman JD, Lambers ME, van Nimwegen M, de Jong S, Hendriks RW, Hoogsteden HC, Aerts JG, Hegmans JP (2010) Low-dose cyclophosphamide synergizes with dendritic cell-based immunotherapy in antitumor activity. *J Biomed Biotechnol* 2010:798467
62. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I, Topalian SL (2010) Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 28(19):3167–3175
63. van der Most RG, Currie AJ, Cleaver AL, Salmons J, Nowak AK, Mahendran S, Larra I, Prosser A, Robinson BW, Smyth MJ, Scalzo AA, Degli-Esposti MA, Lake RA (2009) Cyclophosphamide chemotherapy sensitizes tumor cells to TRAIL-dependent CD8 T cell-mediated immune attack resulting in suppression of tumor growth. *PLoS One* 4(9):e6982
64. Meloni F, Morosini M, Solari N, Passadore I, Nascimbene C, Novo M, Ferrari M, Cosentino M, Marino F, Pozzi E, Fietta AM (2006) Foxp3 expressing CD4+ CD25+ and CD8+CD28- T regulatory cells in the peripheral blood of patients with lung cancer and pleural mesothelioma. *Hum Immunol* 67(1–2):1–12