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Prognostic and predictive aspects of the tumor immune microenvironment and immune checkpoints in malignant pleural mesothelioma

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List of abbreviations

APC	Antigen presenting cell
FFPE	Formalin-fixed paraffin embedded
IFN-γ	Interferon-gamma
IHC	Immunohistochemistry
IL	Interleukin
LAG-3	Lymphocyte activation gene-3
MPM	Malignant Pleural Mesothelioma
NSCLC	Non-small-cell lung cancer
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
TAM	Tumor associated macrophage
TGF	Transforming growth factor
TILs	Tumor infiltrating lymphocytes
TIM-3	T-cell immunoglobulin mucin-3
TME	Tumor microenvironment
Treg	Regulatory T-cell

Abstract

Malignant pleural mesothelioma (MPM) is an aggressive cancer with a poor prognosis and an increasing incidence, for which novel therapeutic strategies are urgently required. Since the immune system has been described to play a presumed role in protection against MPM, characterization of its tumor immune microenvironment (TME) and immune checkpoints can identify new immunotherapeutic targets and their predictive and/or prognostic value. To characterize the TME and the immune checkpoint expression profile we performed immunohistochemistry (IHC) on formalin-fixed paraffin embedded (FFPE) tissue sections from 54 MPM patients (40 at time of diagnosis, 14 treated with chemotherapy). We stained for PD-1, PD-L1, TIM-3, LAG-3, CD4, CD8, CD45RO, granzyme B, FoxP3 and CD68. Furthermore, we analyzed the relationship between the immunological parameters and survival, as well as response to chemotherapy. We found that TIM-3, PD-1 and PD-L1 were expressed on both immune and tumor cells. Strikingly, PD-1 and PD-L1 expression on tumor cells was only seen in unpretreated samples. No LAG-3 expression was observed. CD45RO expression in the stroma was an independent negative predictive factor for response on chemotherapy, while CD4 and TIM-3 expression in lymphoid aggregates were independent prognostic factors for better outcome. Our data propose TIM-3 as a promising new target in mesothelioma. Chemotherapy influences the expression of immune checkpoints and therefore further research on the best combination treatment schedule is required.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive and fatal cancer that is causally associated with previous asbestos exposure in most afflicted patients. Although a rare disease, MPM incidence has been increasing in recent years and this trend is expected to continue over the next decades. This is mainly due to the ongoing asbestos consumption in developing countries, as well as the long latency period between exposure to asbestos and disease onset. [1] Palliative platinum-antifolate chemotherapy has a significant but moderate impact on patients' outcome, resulting in a median overall survival of about one year compared to the 8-10 months observed for chemotherapy-naïve patients. [2-4] Based on its poor prognosis and increasing incidence, novel therapeutic strategies for MPM are required.

The discovery of immune checkpoints such as cytotoxic T-lymphocyte antigen-4, programmed death-1 (PD-1), T-cell immunoglobulin mucin-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3), introduced a new era in targeted cancer therapy. Several monoclonal blocking antibodies have already shown promising results in different cancer types. [5] Their rationale is to reactivate silenced immune responses by neutralizing the so-called immune checkpoints, which are proteins that induce immune cell exhaustion and tolerance. Characterization of the tumor immune microenvironment (TME) could be of great value to unravel these silenced immune responses. Expression of programmed death-ligand 1 (PD-L1) on tumor cells and TILs has been described in literature. [6-11] However, only one series described PD-1 expression on tumor infiltrating lymphocytes (TILs) [8] and nothing has been reported yet on TIM-3 and LAG-3 in human MPM tissue.

Chemotherapy influences the TME, including the expression of immune checkpoints. An upregulation of PD-1 and/or PD-L1 after chemotherapy for leukemia, thymic epithelial tumors and ovarian cancer has been reported. [12-15] Identification of the effect of chemotherapy on the TME in MPM can guide the rational design of combination strategies of immune checkpoint inhibition with chemotherapeutics. [16-18]

We investigated the expression of TIM-3 and LAG-3 in human MPM tumor tissue using immunohistochemistry (IHC), along with several other immune cell markers of the TME,

and addressed their potential role as targets for immunotherapy. In order to elucidate the effect of chemotherapy on the TME, we compared tissue sections from unpretreated and chemotherapy pretreated patients. We furthermore analyzed the prognostic and predictive value of different immunological parameters.

Results

Clinicopathological features of the MPM patient cohort

The clinicopathological characteristics of our MPM cohort are summarized in Table 1. All 54 patients were diagnosed between 2000 and 2015. Forty samples were taken at the time of diagnosis and fourteen samples were treated with chemotherapy (detailed information on the pretreated samples see table S1). The unpretreated samples consisted of 9 biphasic, 26 epitheloid and 5 sarcomatoid cases, while the pretreated samples comprised 1 biphasic and 13 epitheloid cases. The median age of the unpretreated patients was 69 years and 63 years for the pretreated patients. In both groups, patients were predominantly male. At the time of last follow-up, 22% of the unpretreated and 36% of the pretreated patients were still alive. Except for age ($p=0.043$), no significant differences were found between the clinicopathological parameters of both groups.

Immune composition of MPM tissue samples and biomarker identification

The tissue sections were analyzed for the presence of lymphocytes, lymphoid aggregates and stroma (Table 2). Lymphocytic infiltration was found in all tissue samples (figure 1). Samples from pretreated patients showed more infiltration than from unpretreated ones. A stromal score of 1 was observed in more than half of the unpretreated samples while the majority of the pretreated samples had a stromal score of 2 or 3. Lymphoid aggregates were present in more than half of the unpretreated and pretreated samples (65% and 71%, respectively). Germinal centers within the aggregates were seen in around one third of the samples (27% unpretreated, 30% pretreated).

Tissue sections were stained for 6 different immune cell markers (Table 2; Fig. 2 A-E). Percentages of TILs in the stroma ranged from 20% to 80%. CD4+ and CD8+ cells showed

a strong intensity in the TILs and in hot spots of the lymphoid aggregates (Fig. 2 A). CD8+ TILs were present in all samples and were the predominant cell type of the immune infiltrate over CD68+ cells, CD45RO+ cells and CD4+ TILs, with 70% of the unpretreated and 57% of the pretreated samples showing CD8+ expression on more than 50% of the lymphocytes.

Although present to a lesser extent than CD8+ TILs, CD68 expression on histiocytes and macrophages was also seen in all samples (Fig. 2 B). The majority of samples had less than 50% CD68+ cells. Similar observations were made for CD45RO, a marker for effector and memory T cells (Fig. 2 C). Both CD68 and CD45RO expression in the stroma were significantly correlated with stromal presence of CD4+FoxP3+ cells ($R=0.41$, $p=0.002$; $R=0.27$, $p=0.046$). Results of a multivariate analysis revealed that an increase in CD45RO expression on stromal lymphocytes was significantly associated with a lower likelihood of partial or complete response to chemotherapy [odds ratio (OR)=0.06, $p=0.008$; Fig. 3 A; Table S2].

CD4+ TILs in the stroma were seen in 75% of the unpretreated and 71% of the pretreated samples. After multivariate analysis the presence of CD4+ lymphocytes in the lymphoid aggregates was a significant good prognostic factor [risk ratio (RR)=0.13, $p=0.014$; Fig. 4 A; Table S3]. For each increase of CD4 expression with one category, there is a lower risk of death. No significant differences were found between the unpretreated and pretreated samples. A subset of the CD4+ cells was also FoxP3+ (Fig. 2 D) with a range from 1% till 50% CD4+FoxP3+ positive cells in the samples (data not shown). Those CD4+FoxP3+ cells were positively correlated with the presence of CD4+ TILs in the stroma [correlation coefficient (R)=0.52, $p<0.001$].

Moderate positivity for granzyme B was observed in the cytoplasm of immune cells in the stroma (mainly plasma cells and mast cells [19,20]) of less than half of the samples (33% unpretreated, 43% pretreated) (Fig. 2 E).

Immune checkpoint expression in MPM tissue

A cytoplasmic granular staining with a moderate to strong intensity was observed for PD-L1 (Fig. 2 F). Four samples (3 unpretreated and 1 pretreated) also showed membrane staining. PD-L1 was seen on TILs in the stroma, in lymphoid aggregates and in germinal centers of both unpretreated and pretreated samples. Significant differences for PD-L1 expression in the stroma were observed according to the different histological subtypes. Sarcomatoid histology showed more PD-L1 expression than the epitheloid and biphasic subtypes ($p<0.001$, $p=0.008$; data not shown). Strikingly, PD-L1 expression on tumor cells was only detectable in unpretreated tumor samples. 28% of the unpretreated samples with PD-L1+ tumor cells also had PD-L1+ TILs in their stroma (Table 3). Presence of PD-1+ tumor cells and CD8+ TILs was seen in 40% of those samples. CD4+FoxP3+ cells were seen in the majority of samples that had PD-1+ tumor cells and CD4+ TILs (Table 3).

PD-1 expression was localized in the cytoplasm showing membrane accentuation with a moderate intensity (Fig. 2 G). A strong intensity was found on lymphocytes with in the germinal centers of lymphoid aggregates. PD-1 was seen on TILs of both unpretreated and pretreated samples (65% and 71% respectively), while the expression on tumor cells was found only in 10% of the unpretreated samples. In 10% of those samples, expression of PD-1 on both tumor cells and TILs was observed (Table 3). CD8+ TILs together with PD-1+ tumor cells were also seen in 10% of the unpretreated samples (Table 3). The same percentage was found for the expression of CD4 and the co-expression of CD4 and FoxP3 in unpretreated samples (Table 3). According to a univariate analysis the presence of PD-1+ TILs in the stroma was associated with smaller likelihood of response after chemotherapy (OR=0.50, $p=0.0514$; Fig. 3 B; Table S2). PD-1+ TILs were positively correlated with CD4+FoxP3+ and granzyme B+ cells in the stroma ($R=0.36$, $p=0.008$; $R=0.3$, $p=0.014$). 62% of the unpretreated samples with PD-1+ TILs also had PD-L1+ TILs and 50% of those samples showed PD-L1+ tumor cells (data not shown). In contrast, only 30% of the pretreated samples with PD-1+ TILs also had PD-L1+ TILs (data not shown). No PD-L1+ tumor cells were observed in the pretreated samples. After univariate analysis, high expression of PD-1 in the aggregates was a good prognostic factor (RR=0.70,

p=0.029) associated with a lower risk of death than the lower expression category (Fig. 4 B; Table S3). No significant differences were found between the unpretreated and pretreated samples. We observed that PD-1, PD-L1 and TIM-3 were expressed in lymphoid aggregates but only PD-1 and PD-L1 were also expressed in their germinal centers.

TIM-3 scoring was based on the cytoplasmic (Fig. 2 H) and membrane staining of cells. However, often a weak nuclear staining was seen in more than half of the tumor cells in the tissue section, possibly indicating the translocation of TIM-3 proteins from the cytoplasm to the nucleus. TIM-3 expression was found on tumor cells in unpretreated and pretreated samples (40% and 36%, respectively). It was also expressed on TILs and on plasma cells in the stroma however, less often in pretreated samples compared to the unpretreated (29% vs 40%). The presence of TIM-3+ tumor cells in combination with CD8+ TILs was most observed, followed by the expression of PD-1+ TILs in combination with TIM-3+ tumor cells (Table 3). A strong correlation was found between the presence of TIM-3+ TILs and PD-1+ TILs in the stroma (RR=0.48, p<0.001). TIM-3+ lymphocytes were found in lymphoid aggregates of more than half of the unpretreated and pretreated samples (54% and 60%) and were correlated with both TIM-3+ TILs and CD4+ TILs in the stroma (RR=0.64, p<0.001; RR=0.42, p=0.010). Expression of TIM-3 on lymphocytes in the aggregates was found to be an independent good prognostic factor after multivariate adjustment (Fig. 4 C; Table S3). Overall survival was better for patients with high TIM-3 expression in their aggregates (RR=0.47, p=0.002). No significant differences were found between the unpretreated and pretreated samples.

All samples were negative for LAG-3 (Fig. 2 I), in contrast to the control sample showing cytoplasmic staining with strong intensity on lymphocytes (Fig. 2 J).

Discussion

In this series, we report a comprehensive description of the TME in MPM. In summary, we are the first to describe the presence of TIM-3 and absence of LAG-3 expression in MPM tissue, as well as PD-1 expression on MPM tumor cells. PD-1 and CD45RO expression in the stroma were associated with worse response to chemotherapy. After multivariate

analysis stromal CD45RO expression remained a negative predictive factor for response to chemotherapy. Expression of PD-1, CD4 and TIM-3 in lymphoid aggregates were good prognostic factors after univariate analysis. CD4 and TIM-3 expression in lymphoid aggregates remained independent good prognostic factors after multivariate adjustment.

PD-L1 expression was observed in 68% of the unpretreated samples using a cut off value of ≥1%. Differences with other series results [7,9-11,21] might be due to the use of different antibody clones, sample sizes or cut off values. Teng et al. [22] described a classification of tumors into 4 groups based on their pretreatment PD-L1 expression status and the presence of TILs, that might be used to predict a patient's response to anti PD-1/PD-L1 blockade. In our own MPM cohort TILs were present in all unpretreated samples. 40% of the tumor samples can be classified as type I (PD-L1+TILs+), while the others are type IV (PD-L1-TILs+). Type I tumors with adaptive immune resistance have been described to be the most likely type to benefit from anti PD-1/PD-L1 therapy [23], suggesting that 40% of our unpretreated MPM patients would respond to this checkpoint blockade. It is suggested that other suppressors might be present in the type IV TME leading to immune tolerance, thus targeting other suppressive pathways might offer an alternative treatment approach for these types of cancer. We saw that PD-L1 on tumor cells was not always expressed simultaneously with PD-L1 on TILs and other stromal components, which is in concordance with the findings in other tumor types. [23] Presence of both PD-1+ and PD-L1+ TILs was observed in unpretreated and pretreated samples. This might reflect a potential immunosuppressive microenvironment created by the interaction between PD-1 and PD-L1.

PD-1 was expressed to the same extent on immune cells in unpretreated and pretreated samples. We found PD-1 expression on TILs in 65% of the unpretreated samples, which is in line with the 62% reported by of Combaz-Lair et al. in MPM. [8] Our data are the first to report PD-1 expression on TILs in the stroma as a negative predictive factor associated with worse response on chemotherapy in MPM. This is in line with the finding of Zhang et al. that large B-cell lymphoma patients with low PD-1 expression on T cells are more likely to respond to chemotherapy. [24] Results derived from an immunodeficient mesothelioma

mouse model suggest that the effect of pemetrexed is mediated through activation of CD8+ T cells, rather than direct killing of tumor cells. [25] Since more PD-1 expression can point at exhausted CD8+ T cells, this might decrease the antitumor efficiency of pemetrexed. PD-1 on lymphocytes in aggregates on the other hand was a prognostic factor for better overall survival. Percentages of positive lymphocytes in the aggregates ranged from 1% to 50%, stained with a weak to moderate intensity suggesting these are activated PD-1+^{LOW} cells. It has been described that these cells are still functional, able to secrete IFNγ, resulting in activation of other immune cells that play a role in the antitumor response. [26] We observed PD-1 expression on tumor cells in unpretreated but not in pretreated samples, which has not been described in other cancer types so far. Since a rather low number of pretreated samples was used in our series, future studies including larger validation cohorts are needed to draw any meaningful conclusions.

Although until now TIM-3 expression has been predominantly shown on T-cells, our data demonstrate TIM-3 expression also on MPM tumor cells which is consistent with findings in melanoma, NSCLC and renal cell carcinoma. [27-29] While PD-1 was only expressed on tumor cells in 10% of the unpretreated samples, TIM-3 expression was observed on tumor cells in both unpretreated and pretreated samples. More unpretreated samples had TIM-3+ TILs compared to the pretreated, taken into consideration that our number of pretreated samples is rather low. TIM-3 blockade shows promising results in vitro and in vivo in several cancer types [30,31], but nothing has been described for mesothelioma so far. We are the first to report TIM-3 expression in lymphoid aggregates as an independent prognostic factor associated with better overall survival in MPM. Our data support further research on TIM-3 as a target of new treatment strategies of MPM and advocate to prioritize clinical translation of TIM-3 above LAG-3, which has not been detected in our tumor samples. In this context, patients are currently being recruited for a phase 1 trial of an anti-TIM-3 blocking antibody in patients with solid tumors (NCT02817633, *ClinicalTrials.gov*).

The effect of chemotherapy on tumoral PD-L1 expression has previously been investigated in several cancer types. Still, data about the influence of chemotherapy on the TME remain contradictory. For this series, samples of only 14 pretreated patients were at our disposal

and thus no strong conclusions can be drawn based on our results. In our hands, PD-L1 expression on tumor cells was observed only in unpretreated samples, which is in contrast with recent studies that have been presented at the AACR and ASCO annual meetings. Preliminary data from the KEYNOTE-028 [6] and the JAVELIN [32] trial in mesothelioma indicate that PD-L1 expression can be seen irrespective of prior chemotherapy treatment. However, similar to our own observation a downregulation of PD-L1 following chemotherapy treatment has also been noted in other cancer types, such as non-small cell lung cancer (NSCLC) and breast cancer. [33,34] Expression of PD-L1 on the cell surface has been associated with the activation of the PI3K/Akt signaling pathway and PD-L1 has been described to be a downstream target of Akt. [35] Ghebeh et al. [34] reported a significant downregulation of PD-L1 on the surface of breast cancer cell lines after doxorubicin treatment which was accompanied by an upregulation of PD-L1 in the nucleus. They saw that the redistribution of PD-L1 from the cell surface to the nucleus was associated with a translocation of phosphorylated Akt from the membrane to the nucleus. They also reported that inhibition of Akt partially decreased PD-L1 expression on the surface, findings that are supported by Latswika et al. [35] Further research on the effect of chemotherapeutics on Akt signalling is warranted in order to unravel the underlying mechanisms that might be responsible for PD-L1 downregulation.

Compared to the unpretreated, fewer pretreated patients had TIM-3+ TILs in their stroma, which is in line with findings from Zhang et al. in diffuse large B-cell lymphoma. [24] A stromal immune cell score of 3 was more often found for pretreated samples, suggesting that chemotherapy causes an increase in immune infiltration. This is in concordance with findings in other tumor types, showing that cisplatin promotes recruitment and proliferation of effector immune cells. [36] Future studies with a larger number of samples are required to unravel the best treatment schedule to combine chemotherapy with immunotherapy.

Although data about the prognostic role of TILs in MPM are controversial [37-40], it is clear that these cells are important for antitumor immunity. Like TIM-3 after multivariate adjustments CD4 expression on lymphocytes in lymphoid aggregates was demonstrated to be a good independent prognostic factor, with better overall survival observed for patients

with more CD4+ lymphocytes in their aggregates, confirming the findings of Yamada et al. in mesothelioma. [39] Also in NSCLC CD4+ TILs have been described as a positive prognostic factor. [41-43]

CD4+ TILs play an important role in antitumor immunity. Via secretion of several immunoregulatory cytokines, such as interferon-gamma (IFN-γ) and interleukin-2, they provide help for priming and proliferation of CD8+ TILs and activate natural killer cells. CD4+ TILs also express CD40-ligand on their surface which binds to CD40 expressed by antigen presenting cells (APC). [44-46] This interaction between causes activation of APC, that also contribute in priming of CD8+ TILs. Taken together, CD4+ TILs have both a direct and indirect impact on the generation of a T cell-mediated antitumor response.

In our series, the two immune-related parameters with prognostic significance after multivariate adjustments (CD4+ TILs, PD-1, TIM-3) are all situated in the lymphoid aggregates, suggesting these are important structures influencing a patient's outcome. We observed CD4, CD8 and CD45RO expression in lymphoid aggregates, suggesting that these structures might function as a site for the generation of antitumor adaptive immune responses, as also suggested by Pagès et al. [47] This would imply that reactivation of antitumor T-cell responses might occur in lymphoid aggregates, resulting in a favorable prognosis, and that these aggregates show functional similarity with tertiary lymphoid structures, in which T and B lymphocytes are segregated into two adjacent regions surrounded by high endothelial venules. [48,49]

A subset of the CD4+ cells in the tissue sections was also FoxP3+. These double positive cells were found in 80% of the unpretreated and 56% of the pretreated samples with CD4+ TILs in the stroma, suggesting that cisplatin and/or pemetrexed have a negative effect on the number of CD4+FoxP3+ cells, which might be regulatory T cells (Treg). This idea is supported by data from Wu et al. [50] who reported a decreased number of Tregs in a mesothelioma mouse model after treatment with cisplatin. We found a positive correlation between Tregs and PD-1+, PD-L1+ and CD4+ TILs in the stroma. The more activation of the PD-1/PD-L1 pathway, the less FoxP3 transcription is controlled, eventually resulting in an increased amount of CD4+FoxP3+ cells. [30] However, FoxP3 expression is also described

in activated non-suppressive T cell populations, so it is not a 100% specific Treg marker. [51] More CD4+FoxP3+ cells in the stroma were also associated with more CD68+ macrophages in the stroma, suggesting that the latter affect the adaptive immune response by secreting several molecules that lead to recruitment and stimulation of CD4+ T cells, as previously described by Solinas et al. [52] CD4+FoxP3+ cells were negatively correlated with CD8+ TILs in the stroma, as also found for CD4+ and CD8+ TILs: the more CD4+ TILs, the more CD4+FoxP3+ cells and the less CD8+ TILs. As observed for CD8+ TILs, CD45RO+ memory T cells were also seen in the stroma of all samples. The latter were significantly associated with response to chemotherapy. More specifically, our data show CD45RO expression in the stroma to be an independent negative predictive factor. MPM patients with many CD45RO+ T cells had a higher likelihood of non-responding to chemotherapy compared to those with few CD45RO+ T cells, suggesting that CD45RO is an interesting predictive marker for response to chemotherapy in MPM patients.

Stromal expression of CD45RO was significantly correlated with the presence of CD4+FoxP3+ cells in the stroma. Under the assumption that the latter are Tregs, derived from CD45RO+ memory T cells, the negative predictive value of CD45RO expression might be explained by a Treg-mediated suppression of the immune cells that are recruited after cisplatin and pemetrexed treatment. [25,53] In addition, a significant correlation was also found for CD4+FoxP3+ cells and CD68+ macrophages in the stroma (R=0.410, p=0.002), as stated at page 6. Tumor associated macrophages (TAMs) in mesothelioma have been described to be of the tumor promoting M2-phenotype (data presented by L. Coussens at the 13th International Mesothelioma Interest Group meeting in May 2016, Birmingham UK). [54,55] M2 macrophages secrete several molecules, such as IL-10 and transforming growth factor-bèta (TGF-β) that results in down-regulation of adaptive immunity for example via stimulation and recruitment of Tregs. [52] The latter explains our correlation observed for Treg and macrophages in the stroma.

Conclusion

In conclusion, we report that the immune composition of the TME and expression of immune checkpoints and their ligands is strongly patient dependent. In future research it would be interesting to investigate the within patient dynamics of the TME in a prospective study. Our results point to TIM-3 as a promising new target in mesothelioma. Regarding combination of chemotherapy with immunotherapy, larger validation cohorts are needed to determine the best treatment schedule.

Materials & Methods

Patient selection and samples

Formalin fixed paraffin embedded (FFPE) tissue samples from 54 MPM patients were retrieved from the tumor biobank at the Antwerp University Hospital. Of those, 40 patients were unpretreated and 14 were pretreated with standard chemotherapy (platinum and pemetrexed). Exclusion criteria for the untreated group were: prior surgery or chemotherapy treatment and tissue samples older than 10 years. The latter exclusion criterion was also used for the pretreated group. Response was assessed with modified RECIST criteria and confirmed after at least 4 weeks. [56] All the tissue samples had been obtained through surgical biopsy, fixed in 4% formaldehyde for 6-18h and paraffin embedded on a routine basis. This retrospective study has been approved by the Ethics Committee of the Antwerp University Hospital/University of Antwerp.

Immunohistochemistry

Five µm-thick sections were prepared from FFPE tissue blocks. Prior to staining, the sections were baked in an oven for one hour at 60°C to facilitate attachment and to soften the paraffin. IHC was carried out on a Benchmark Ultra XT® autostainer (Ventana Medical Systems Inc.) with standard antigen retrieval methods (CC1, pH 8.0; Ventana, #950-124). The UltraView or OptiView DAB detection kit (Ventana, #760-500 or #760-700) and, in case of double stainings for CD4/CD8 and CD4/FoxP3, the UltraView universal alkaline phosphatase red detection kit (Ventana, #760-501) were used according to the manufacturer's instructions. Sections were counterstained with hematoxylin as part of the automated staining protocol. After staining, slides were washed in reaction buffer (Ventana, #950-300), dehydrated in graded alcohol, cleared in xylene, mounted with Quick-D Mounting Medium (Klinipath, #7280) and coverslipped. IHC staining protocols from the manufacturer's datasheets were used for the following antibodies from Ventana: anti PD-1 (clone NAT105, #760-4895), anti CD4 (clone SP35, #790-4423), anti CD8 (clone SP57, #790-4460), anti CD45RO (clone UCHL-1, #790-2930). The protocols for PD-L1 (clone SP142, 1:50, Spring Bioscience, #7309457001), CD68 (clone KP-1, 52' CC1, 12'

antibody incubation, Ventana, #790-2931) and granzyme B (polyclonal, 32' antibody incubation, Ventana, #760-4283) were slightly adapted from the datasheet. This was also the case for the antibodies used from Abcam® (Abcam®): anti LAG-3 (clone 11E3, 1:100, 36' CC1, 60' antibody incubation, #ab4065), anti TIM-3 (polyclonal, 1:500, 52' CC1, 28' antibody incubation, #185703) and anti FoxP3 (clone 236A/E7, 1:50, 36' CC1, #ab20034). Positive controls were included in each staining run. Human placenta was included as positive control for endogenous PD-L1, while human tonsil was used as positive control for the other markers. We looked at the presence of lymphocytes, lymphoid aggregates (score 0= 0 aggregates; 1= 1-5 aggregates; 2= 5-10 aggregates; 3= >10 aggregates) and the density of the stroma (score 0= scarce; 1= low density; 2= intermediate density; 3= high density [57]). A lymphoid aggregate was defined as 50 or more lymphocytes clustered together. Expression of each marker in the tissue was divided into five categories (0= <1%; 1= 1-5%; 2= 5-10%; 3= 10-50%: 4= >50%). All sections were scored by two observers, of whom one pathologist. Samples were considered to be positive in case of ≥1% positive cells [7,8] with specific staining of any intensity (0= no expression, 1= weak, 2= moderate, 3= strong) and any distribution (membrane and/or cytoplasm) [7]. These criteria were used for IHC scoring of all the different markers. An Olympus BX41 microscope was used for scoring of the tissue sections. Pictures were made using the Leica acquisition software v4.

Statistics

Spearman correlation coefficients were calculated to investigate the association between the expression of immune cell markers and immune checkpoints on MPM tissue samples. Overall survival was assessed from the date of diagnosis to the date of last contact or decease. Survival rates were visualized using Kaplan-Meier curves. The influence of immunological and clinicopathological parameters on survival was assessed using Cox proportional hazards models. Variables that showed significance (10%) in univariate analyses were considered for the multivariate model. We used tests based on the scaled Schoenfeld residuals to assess the proportional hazards assumption. The association of

immunological and clinicopathological variables with response to chemotherapy was analysed using logistic regression on 32 of 54 samples (treatment or response data not available for the other samples). Variable selection was performed by assessing significance on the 10% level in univariate analyses. Depending on the number of selected variables, forward or backward model building was used in the multivariate models. P-values ≤0.05 were considered statistically significant. All statistical analyses were performed using R statistical software version 3.2.2.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1. Lymphocyte infiltration of MPM tissue samples. Pictures were taken from slides of the 2 different MPM patients **(A)** and **(B)**. The lymphocyte infiltration of both tissue samples is representative for those in our overall MPM cohort. The images show the presence of lymphoid aggregates, CD4+ (red) and CD8+ (brown) lymphocytes in the tumoral stroma and in between the tumor cells. Tumor cells are round, epitheloïd and pleiomorfic with patient **(A)**, while they are smaller with less pleiomorphisme with patient **(B)**. Original magnification: left column pictures 100x; right column pictures 200x.

Figure 2. Immunohistochemical staining patterns of different immunomarkers in MPM tissue. (A) CD4+ (red) and CD8+ (brown) lymphocytes in stroma; **(B)** CD68+ histiocytes stained with strong intensity in the cytoplasm surrounding negative tumor cells; **(C)** CD45RO+ lymphocytes in stroma with moderate to strong intensity;**(D)** CD4+FoxP3+ lymphocytes in stroma with brown nuclear staining for FoxP3 and red cytoplasmic staining for CD4; **(E)** stromal cells expressing granzyme B in their cytoplasm surrounding negative tumor cells; **(F)** PD-L1+ tumor cells with membrane accentuation; **(G)** PD-1+ tumor cells stained with strong intensity in the cytoplasm;**(H)** tumor cells and lymphocytes in stroma with strong TIM-3 staining in the cytoplasm; **(I)** Absence of LAG-3 staining in MPM tissue; **(J)** LAG-3+ lymphocytes in human tonsil showing strong cytoplasmic staining. All tissue sections were counterstained with hematoxylin (blue color). Original magnification 1000x.

LA, lymphoid aggregate; TC, tumor cells; TIL, tumor infiltrating lymphocytes; ST, stroma.

Figure 3. Predicted probability plots for the significant predictive factors CD45RO and PD-1. Plot of response on chemotherapy (y-axis) versus: **(A)** CD45RO expression on lymphocytes in the stroma (x-axis), and **(B)** PD-1 expression on lymphocytes in the stroma (x-axis). Observations within our cohort are represented by the empty dots (right y-axis). The full dots show the observed chance on partial/complete response within each expression category. The curve depicts the estimated probability of partial/complete response based on a univariate logistic regression model (p=0.017, p=0.0514; left y-axis).

After multivariate adjustments CD45RO expression on stromal lymphocytes remained an independent good predictive factor (p=0.0076).

Figure 4. Kaplan-Meier overall survival according to CD4, PD-1 and TIM-3 expression in the lymphoid aggregates. Univariate analysis showed prognostic significance for: **(A)** CD4 (p=0.008), **(B)** PD-1 (p=0.029), and **(C)** TIM-3 (p=0.001) expression in the lymphoid aggregates. After multivariate adjustments CD4 and TIM-3 expression in the aggregates remained independent good prognostic factors (p=0.015 and p=0.002).

Tables

Table 1. Clinicopathological parameters of unpretreated and chemotherapy pretreated mesothelioma patients.

Characteristics	unpretreated (n,%)	pretreated (n,%)	p-value
Number of samples (N)			
	40	14	
Age (years)			0.043
Median	69	63	
Range	42-81	45-73	
Sex			0.890
Male	34 (85%)	11 (79%)	
Female	6 (15%)	3 (21%)	
Histological subtype			0.151
Epitheloid	26 (65%)	13 (93%)	
Sarcomatoid	5 (13%)	0	
Biphasic	9 (22%)	1 (7%)	
Smoker			0.502
No	7 (18%)	4 (29%)	
Yes	24 (60%)	6 (42%)	
No data	9 (22%)	4 (29%)	
Professional asbestos exposure			0.464
No	10 (25%)	1 (7%)	
Yes	23 (57%)	8 (57%)	
No data	7 (18%)	5 (36%)	
Survival			0.412
Alive	9 (22%)	5 (36%)	
Dead	31 (78%)	9 (64%)	
Laterality			0.812
Left	11 (28%)	5 (36%)	
Right	29 (72%)	9 (64%)	
Surgery			0.114
No surgery	33 (82%)	11 (79%)	
Diagnostic VATS	3 (8%)	1 (7%)	
P-D	1 (2%)	2 (14%)	
EPP	3 (8%)	0	
Stage			0.814
I-II	13 (33%)	6 (43%)	
III-IV	22 (55%)	7 (50%)	
No data	5 (12%)	1 (7%)	
Hemoglobin (g/dL)			0.272
< 14.6 (low)	5 (12%)	9 (64%)	
≥ 14.6 (high)	35 (88%)	4 (29%)	
No data	0	1 (7%)	
White blood cell count (x10^{3}cells/µL)			0.745
< 15.5 (low)	37 (93%)	13 (93%)	
≥ 15.5 (high)	3 (7%)	0	
No data	0	1 (7%)	
Platelet count (x10^3 cells/µL)			1.000
< 400 (low)	26 (65%)	9 (64%)	
≥ 400 (high)	14 (35%)	4 (29%)	
No data	0	1 (7%)	
Neutrophil / lymphocyte ratio			0.542
< mean★	16 (40%)	6 (43%)	
≥ mean★	24 (60%)	6 (43%)	
No data	0	2 (14%)	

★, mean of unpretreated samples = 3.3; mean of pretreated samples = 3.2. EPP, Extrapleural Pneumonectomy; P-D, Pleurectomy-Decortication; VATS, Video Assisted Thoracoscopic Surgery.

Table 2. Expression of immune checkpoints and immune cell markers in FFPE tissue from unpretreated and pretreated MPM patients.

% samples	CD8+	CD68+	CD45RO+	CD4+	CD4+ FoxP3+	Granzyme B+	PD-1+	PD-L1+	TIM-3+	LAG-3+
Unpretreated										
TOTAL (N=40)	100	100	100	85	70	33	75	68	63	0
Tumor cells (n=40)	0	0	0	0	0	0	10	40	40	0
Immune cells in stroma (n=40)	100	100	100	75	60	33	65	53	40	0
Lymphocytes in lymphoid aggregates (n=26)	100	0	96	88	50	11	69	31	54	0
Germinal centers within lymphoid aggregates (n=7)	0	0	0	0	0	0	86	29	0	0
Prereated										
TOTAL (N=14)	100	100	100	100	79	43	71	57	79	0
Tumor cells (n=14)	0	0	0	0	0	0	0	0	36	0
Immune cells in stroma (n=14)	100	100	100	71	50	43	71	29	29	0
Lymphocytes in lymphoid aggregates (n=10)	100	0	100	100	80	0	70	60	60	0
Germinal centers within lymphoid aggregates (n=3)	0	0	0	0	0	0	33	100	0	0

Percentages of all positive samples are shown per marker. Expression on tumor cells, immune cells in the stroma (lymphocytes, macrophages, histiocytes, plasma cells) and lymphocytes in lymphoid aggregates and in germinal centers within lymphoid aggregates is depicted separately.

Table 3. Within patient combined expression of PD-1, PD-L1 and TIM-3 on tumor cells and TIL subtypes.

% unpretreated samples		Tumor cells			% treated samples		Tumor cells
		PD-1+	PD-L1+	TIM-3+			TIM-3+
TILs	PD-1+	10	28	33	TILs	PD-1+	21
	PD-L1+	10	28	23		PD-L1+	7
	TIM-3+	5	15	23		TIM-3+	7
	CD8+	10	40	40		CD8+	36
	CD4+	10	30	33		CD4+	29
	CD4+FoxP3+	10	28	30		CD4+FoxP3+	21

Percentages of unpretreated and chemotherapy pretreated samples showing tumoral expression of PD-1, PD-L1 or TIM-3 together with the T cell markers, PD-1, PD-L1 or TIM-3 on TILs.