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Mesothelioma environment comprises cytokines and Treg cells that suppress immune responses

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[3] Nonstandard abbreviations used: CTL, Cytotoxic T lymphocyte(s); DC, Dendritic cell; HGF, hepatocyte growth factor; MM, Malignant mesothelioma; T_{reg}, regulatory T. See table 1 for abbreviations of the cytokines.

Abstract

Malignant mesothelioma is a cancer with dismal prognosis. Our objective was to address the role of the immune system, tumor microenvironment and potential immunosuppression in mesothelioma.

Expression profiles of 80 cytokines were determined in the supernatant of mesothelioma cell lines and the original patient's pleural effusion. Influx of immune effector cells was detected by immunohistochemistry.

Angiogenin, VEGF, TGFβ, ENA-78 and several other proteins involved in immune suppression, angiogenesis and plasma extravasation could be detected in both supernatant and pleural effusion. Surrounding stroma and/or infiltrating cells were the most likely source of HGF, MIP-1δ, MIP-3α, NAP-2, and PARC that can cause leukocyte infiltration and activation. There was a massive influx of $CD4^+$ and $CD8^+$ T lymphocytes and macrophages, but not of dendritic cells, in human mesothelioma biopsies. We further demonstrated that human mesothelioma tissue contained significant amounts of $Foxp3^+CD4^+CD25^+$ regulatory T cells. When these $CD25^+$ regulatory T cells were depleted in an *in vivo* mouse model, survival was increased.

Mesothelioma is infiltrated by immune effector cells, but also contains cytokines and regulatory T cells that suppress an efficient immune response. Immunotherapy of mesothelioma might be more effective when combined with drugs that eliminate or control regulatory T cells.

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Introduction

Malignant mesothelioma (MM) is a highly aggressive neoplasm most often seen in patients with a history of asbestos exposure. There is a latency period of 20 to 40 years between the expose to asbestos fibers and the first symptoms of disease. With median survival durations of 9-12 months from onset of symptoms, the prognosis is poor. To date, there is no standard curative therapy for MM. Combined modality approaches such as extrapleural pneumonectomy followed by radiochemotherapy result in high local recurrence rates and questionable survival benefit [1]. As MM is a weakly immunogenic tumor, various groups have attempted to perform immunotherapy using cytokines or adjuvants to boost tumor immunity, with variable success (review[1]). In a previous study, we evaluated the therapeutic efficacy of tumor lysate-loaded antigen presenting dendritic cells (DCs) given before and/or after an intraperitoneal tumor challenge with the mouse mesothelioma cell line AB1. Dendritic cells pulsed with tumor lysate or exosomes were effective in inducing protective cytotoxic CD8 T cell responses and increasing survival, even when given after tumor implantation [2]. In these studies, DC treatment had a better outcome when DCs were injected early in tumor development indicating that tumor load played an important role in survival. Although the exact sequence of events in mesothelioma induction and progression is still unknown, a range of defects that develops both inside and outside the mesothelial cell could be involved in escape of the tumor from immune destruction. According to the immune surveillance theory, large tumors escape immune recognition by down regulating MHC class I or by altering expression of tumor antigens, thus leading to an escape from cytotoxic killing by CD8 cells [3]. This theory has recently been challenged as spontaneously arising tumors in mice remain immunogenic and rather escape immune recognition by inducing anergy in tumor infiltrating lymphocytes [4] or by attracting regulatory T (T_{reg}) cells that suppress antitumoral responses. It is now well established that certain tumors and the surrounding

stroma generate an immunosuppressive microenvironment to suppress the effector arm of the anti-tumoral immune response (CTL response inside the tumor) and the inductive arm of the immune response, i.e. the potential of antigen presenting DCs to induce CTL responses.

We have taken an unbiased approach using a proteomics platform and determined the presence of an array of 80 cytokines and chemokines in mesothelioma cell lines and pleural fluids of the original patients from which the cell lines were generated, allowing us to study which factors were tumor derived and which were derived from infiltrating immune cells or surrounding stroma. Tumors were heavily infiltrated with $CD4^+$ and $CD8^+$ T cells and macrophages, but DCs were strikingly absent. To our surprise we found Fox $p3^+$ $CD25^+$ regulatory T cells that were previously shown to promote tumor progression in other cancer models. Removal of these cells led to increased survival in a transplantable mouse model of mesothelioma. These findings suggest multiple levels by which MM escapes immune recognition.

Methods

Patient material and preparation of cell lines for analysis

Pleural fluid was collected after informed consent from histologically proven mesothelioma patients (n=6) who presented with large pleural effusions. Indication for pleural fluid evacuation was in most cases exertional dyspnea relief. Patients were all males between 67 and 88 years of age and were treated with best supportive care. Thoracocentesis was performed using fine needle aspiration inserted in the pleural cavity and collected in sterile tubes without anticoagulant. Pleural cells were removed from pleural effusions by centrifugation at 3000xg for 20 min at 4°C and the supernatant was stored in aliquots at –80°C. Long term mesothelioma cell lines can be generated from these pleural effusions as described previously [5]. Four mesothelioma cell lines were extensively characterized as described earlier [5]. This included determining the cellular DNA content, immunohistochemistry, tumorigenicity *in vivo*, virus and/or bacterial contamination, karyotyping, and HLA typing. Cell lines were kept in long-term cell culture (> 50 passages) while using for supernatant testing. From these patients, pleural fluid was still available for analysis. In separate patients (n=4), tumor biopsies were obtained through medical thoracoscopy and processed for immunohistology.

Proteomics platform to analyze cytokines and chemokines

An antibody-based cytokine array system (RayBiotech, Inc., Norcross, GA, USA) was used to determine cytokine expression profiles in supernatant of mesothelioma cell lines (n=4) and the corresponding patient's pleural effusions (n=6). Cell lines were grown in T175 culture flasks to 80 % confluence, then medium was replaced by 12 ml RPMI containing 1% FCS

and incubated at 37°C. Next day, supernatant was collected and centifuged 20 minutes at 3000xg to remove the cells. Samples were concentrated using Centricon YM3 columns (Millipore, Billerica, MA, USA). As a negative control, 12 ml of the above mentioned medium was prepared in the same way as the cell supernatant. 1 ml of these concentrated samples were applied on the Ray Bio membranes (human cytokine array V [table 1]). The detection was done according the manufacturer's protocol. Quantification of cytokine expression was performed in duplicate by two independent observers (JH, AH) using a standard scale of 6 spots with increasing density (score 0 to 5, landing lights (positive controls) were scored as 4).

Immunohistology on tumor biopsies

Tumor biopsies were taken using medical thoracoscopy and embedded in Tissue-Tek II OCT medium (Miles, Naperville, IL, USA), snap-frozen, and stored at -80°C. Tissue sections (6 µm) were cut on a HM-560 cryostat (Microm, Heidelberg, Germany) and immunostaining was carried out using antibodies (table 2). Binding of the antibodies was detected by immuno-alkaline phosphatase (AP) anti-alkaline phosphatase (APAAP) method (DAKO, Glostrup, Denmark). Naphtol-AS-MX-phosphate (0.30 mg/ml, Sigma-Aldrich) and new fuchsine (160 mg/ml in 2 M HCl, Chroma-Gesellschaft, Köngen, Germany) were used as substrate. The specificity of the antibodies was checked by using a protein concentration-matched non-relevant monoclonal antibody and phosphate buffered saline (PBS). Double staining of Foxp3 (rat IgG2a) and CD3 or CD25 (both mouse IgG1) was performed using AP-conjugated goat anti-rat (Sigma) and rat APAAP (DAKO) followed by horse radish peroxidase (HRP) conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL, USA). Naphtol-AS-MX-phosphate and 1 mM Fast Blue substrate were used as substrate for AP and NovaRed substrate for HRP according to manufacturer's instructions (Vector,

Burlingame, CA, USA). Alexa Fluor 647 labeled anti-human CD4 and FITC conjugated CD25 (both BD Biosciences, San Jose, CA, USA) were used in a dilution of 1:100 and 1:20, respectively. Signals were captured on a Zeiss confocal laser-scanning microscope (LSM510NLO).

Tumor growth of murine mesothelioma after in vivo depletion of $CD4^+CD25^+$ T cells in BALB/c mice

Female 6-10 weeks old BALB/c (H-2d) mice (Harlan, Zeist, The Netherlands) were housed under pathogen-free conditions at the animal care facility of the Erasmus MC, Rotterdam. Experiments were approved by the local Ethical Committee for Animal Welfare and complied to the Guidelines for the Welfare of Animals in Experimental Neoplasia by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR). The AB1 cell line, a mouse mesothelioma cell line was kindly provided by Prof. Bruce W.S. Robinson. For *in vivo* depletion of $CD4^+CD25^+$ T cells, 0.5 ml of anti-CD25 antibody (PC61) ascites fluid (kindly provided by G. Oldenhove, Université Libre de Bruxelles, Belgium) was given intraperitoneally on day –25 to each mouse (n = 12). The ascitis fluid was purified by affinity chromatography on protein A-Sepharose 4B as described by Lowenthal *et al*. [6]. As control 0.5 ml of PBS was used (n = 12). On day 0, mice were subjected to a lethal dose of 0.5×10^6 AB1 tumor cells. The occurrence of tumor growth, body weight, physical well-being, and survival were measured for two months as described earlier [2].

Statistical analysis

Data are expressed as mean ± standard deviation. Comparisons between groups were made by Mann Whitney U-test for independent samples. A two-tailed p-value of less than 0.05 was

considered significant. Data presented as percentage of tumor-free animals were analysed with Kaplan-Meier survival curves, using the log-rank test to determine statistical significance. Statistical analysis was performed using SPSS (Chicago, USA) for Windows™ version 11.0.

Results

Expression profile of pleural effusions and cell line supernatants on cytokine and chemokine array

An antibody-based cytokine array system (RayBiotech, Inc.) was used to determine cytokine expression profiles in pleural effusion (n=6) and in the supernatant of mesothelioma cell lines derived from the same patients (n=4). No cytokine expression was found in the negative (medium) control. Cytokine expression levels in pleural effusions (PF) and corresponding supernatant of mesothelioma cell lines (SN) are summarized in table 3. Forty-nine cytokines were not detected in effusion or in supernatant of cultured mesothelioma cell lines. HGF, MIP-1δ, MIP-3α, NAP-2, and PARC were exclusively present in pleural effusions and were not detected in mesothelioma cell supernatant. Therefore, these cytokines are possibly produced and secreted in pleural effusions by stromal cells and/or inflammatory cells. Some effusions also expressed cytokine levels of eotaxin-2, IL-12, leptin, MIF and OSM. In contrast, supernatants of mesothelioma cell lines contain high levels of GRO and RANTES. These proteins seem to be mainly secreted by tumor cells and the amount present in effusion may thus be correlated with the amount of tumor cells.

Cellular recruitment in human mesothelioma tumors

The local release of cytokines and chemotactic factors by tumor cells and surrounding stroma suggest an accumulation of leucocytic infiltrate in the vicinity of the tumor by recruitment from circulating blood cells. Therefore, immunohistochemical techniques were performed to determine the inflammatory component in solid tumor tissue. Mesothelioma tumor tissue can be regarded as complex tissues, composed of localized HBME-1 positive tumor fields, separated by stroma and many $CD31^+$ blood vessels (figure 2). Although some patient-to-patient heterogeneity was noted, leukocyte infiltration was always detected. Macrophages

(CD68) and NK cells (CD16) constitute the major part of the inflammatory cell infiltration. Interactions between cancer cells and host immune T cells ("pan-T" (CD3), T helper/inducer (CD4), and T suppressor/cytotoxic lymphocytes (CD8)) were visualized inside, at the rim and in the stroma of mesothelioma specimens. However, dendritic cells (using antibodies for Langerin, fascin, S100, CD1a, BDCA2, and CD209), eosinophils (BMK13), mast cells (anti-chymase), B cells (CD24), and neutrophils (CD15) were rarely detected (figure 3).

Regulatory T cells can be discriminated based on the expression of CD4 and CD25, combined with the transcription factor Foxp3. Mesothelioma tissue sections were analyzed by fluorescence microscopy for the phenotypic evidence of $CD4^+CD25^+$ double positive cells. Especially at the rim of tumor areas $CD4^+CD25^+$ T cells were detected (figure 4G, and 4H). The transcription factor Foxp3, identified by Sakaguchi as a hallmark of naturally arising $CD4^+CD25^+$ T_{reg} cells [7], confirmed the presence of these cells in the vicinity of the tumor (figure 4A). Double stainings showed that the expression of Foxp3 strongly correlated with CD3 (figure 4B) and CD25 expression (figure 4C, and 4D).

Effects of in vivo $CD4^+CD25^+$ T cell depletion on tumor growth

We used the mesothelioma mouse model as described in a previous study [2] to examine the impact of $CD4^+CD25^+$ T cell depletion on tumor progression. Preliminary studies indicated that in BALB/c mouse, CD25 expression was restricted to $CD4^+$ T cells and represented a small fraction of total cells in the blood, lymph nodes and spleen (2%–10%). Kinetic studies have shown that injection of the depleting antibody led to the selective loss of $CD4^+CD25^+$ T cells for at least 30 days and that replenishment of the population was observed 50 days after treatment [8]. We first investigated the effect of the *in vivo* administration of anti-CD25 ascites fluid on the $CD25^+$ population in blood, as revealed by flow cytometry. After a single

i.p. injection of 0.5 ml PC61 ascites fluid, this population of cells decreased in blood from 3.5% to 1% after 25 days (figure 5).

In this protocol, BALB/c mice were injected i.p. with PBS or PC61 ascites fluid 25 days before tumor cell inoculation. On day 0, all mice were injected i.p. with a lethal dose of $0.5x10^6$ AB1 tumor cells. First signs of terminal illness (typically formation of ascites, ruffled hair, or marked loss of condition) appeared after 6 days in both groups (figure 6). Mice were subjected to extensive autopsy that always showed solid tumor formation within the peritoneal cavity, which was accompanied in a few cases by thick, yellow-stained ascites. The nature of the solid tumors varied from numerous small nodules spreading throughout the mesentery and peritoneal lining to a single large mass. Within 40 days, all mice from the PBS group showed evidence of ill health or overt tumor growth. The administration of anti-CD25 antibodies prolonged the median survival from 19 to 33 days. Strikingly, 5 of the 12 mice (41%) treated with depleting antibody PC61 remained tumor free for 2 months. Mice were then sacrificed and checked for tumor growth. No tissue abnormalities or formation of tumors could be detected.

Discussion

Cancer, and in particular mesothelioma, is a chronic disease. When mesothelioma becomes clinically visible 20 to 40 years after asbestos exposure, tumor cells and their products have already been interacting with and affecting host cells for a considerable time to ensure the survival of the tumor. The paths that mesothelial cells take on their way to become malignant is unknown and probably highly variable dependent of several host factors, including environmental factors, polymorphisms and mutations in susceptibility genes, age and immunity. Tumor development also depends on factors in the microenvironment. Interactions

between malignant cells, stromal cells, extra cellular-matrix components, various inflammatory cells, and a range of soluble mediators contribute to tumor development and progression. Mesothelioma tumor of patients with advanced-stage disease is composed of more than just cancer cells – it consists of an intricate network of cell types, including endothelial cells that comprise blood vessels and stromal cells. Many immunological cell types surround and penetrate the cancer cell areas, yet tumors escape immune destruction. There is accumulating evidence that cancer cells can even recruit and subvert normal cell types to serve as active collaborators in their neoplastic program [9]. Understanding these multiple factors that come into play at the tumor microenvironment level may help to better understand and design immunotherapy protocols. Therefore, we undertook an unbiased view at what inflammatory cell types were present inside mesothelioma, and which cytokines and chemokines were produced by mesothelioma cell lines and present in corresponding pleural fluid. One remarkable observation of our immunohistological studies was that mesothelioma is heavily infiltrated with many immune effector cells. Macrophages, NK-cells, and T-lymphocytes, both T helper/inducer (CD4), and T suppressor/cytotoxic (CD8) cells, constituted the major part of the inflammatory cells. Not surprisingly, our protein array demonstrated the production of many chemokines in both MM cell line supernatant and pleural fluid (IP10, MIF, MCP1, ENA78, MIP1β, IL8, GRO, RANTES) or exclusively in pleural fluid (MIP-1δ, MIP-3α, NAP-2, and PARC) with a potential to attract these cell types. The role of macrophages, T cells, and other immune cells in mesothelioma biopsies is still unknown. The influx, probably caused by changes in the microenvironment of the tumor, can favour inflammation, angiogenesis, and tumor growth thus leading to tumor progression. In contrast it can also negatively impact on tumor growth. For example, the presence of macrophages in tumor islets of non-small-cell lung cancer and the presence of infiltrating effector memory T cells in colorectal cancer is associated with an increased survival [10, 11].

Understanding the outcome of the anti-tumoral immune response, leading to tumor progression or regression, and understanding the role of individual cell types on outcome is of major importance for the design of future clinical trials.

On the contrary, eosinophils, mast cells, B cells, and neutrophils were rarely detected. Mast cell infiltration and their density in tumor islets have been described as predictor of survival in non-small-cell lung cancer (NSCLC) [10]. The difference in mast cell density in NSCLC and mesothelioma may be caused by discrepancies in type, stage, and size of the tumors and the methods used to assess these cells. Welsh *et al.* used anti-tryptase to detect all mast-cells while we stained for the chymase+ mast cell. This disprecancy between tryptase and chymase has also been illustrated in malignant breast tumours [12]. Clinical follow-up data comparing chymase+ and tryptase+ in human mesothelioma biopsies in combination with other immune cell markers may reveal more information for their prognostic significance.

As in many other cancer types, it was striking to see so many immune effector cells within the tumor while tumors were still not rejected. One of the aims of the current investigation was specifically to determine the presence of factors within both the mesothelioma and surrounding stromal cells that could suppress the immune response to the tumor. In addition to factors involved in angiogenesis (angiogenin, VEGF, GRO), and leukocyte attraction (chemokines) we discovered several factors that might suppress the anti-tumoral immune response, either by suppressing the antigen presenting capacity of dendritic cells or the effector arm of the immune system. One of the factors that was found in pleural fluids of MM patients was hepatocyte growth factor (HGF), also known as scatter factor. HGF is a multifunctional factor involved both in development and tissue repair, as well as pathological processes such as cancer and metastasis [13]. It was previously shown that HGF has a predominant role in mesothelioma cell invasion, stimulating simultaneously adhesion, motility, invasion and regulation of MMP and TIMP levels [14]. Strikingly, it was shown that

HGF has the potential to induce GRO and VEGF (also found in our analysis) in a number of tumor models including non-small cell lung cancer [15]. HGF might be a critical mediator of immune suppression in MM as it was recently shown that HGF suppresses the maturation status and antigen presenting capacity of lung dendritic cells [16]. Along the same lines, we discovered that VEGF was secreted by MM cell lines and was present in pleural effusions. VEGF has its main role in inducing angiogenesis to the benefit of the tumor, but has long been known to critically suppresses the function of DCs in inducing an anti-tumoral response by keeping these cells in an immature state or inhibiting their differentiation from monocytes [17]. Another striking observation in our studies was that $CD1a^+$ DCs could not be found inside the MM biopsies that we took. Additional DC markers (Langerin, fascin, S100, BDCA2, and CD209) were applied because of the debate on the use of CD1a[18, 19], and confirmed this rare detection of dendritic cells in mesothelioma biopsies. This is in contrast to many other tumors such as breast cancer or non-small cell lung cancer where $CD1a^+$ DCs are found within tumor lesions [20]. One possible explanation for the lack of DCs inside MM would be the presence of high levels of interleukin 6 (IL-6) produced by the MM cells, as shown in our study. IL-6 has been shown also by others to be universally expressed in MM cell lines [21]. IL-6 suppresses the development of DCs from $CD34^+$ progenitors and from monocytes in vitro [22]. Studies in multiple myeloma patients have demonstrated that high level IL-6 is responsible for a lack of circulating DCs in these patients [23]. In mice and humans, IL-6 also keeps DCs in a persistently immature state, and promotes the differentiation of macrophages from monocytes [22]. The absence of DCs inside tumors has been shown to affect tumor progression in a number of cancers, including lung cancer [24]. Dendritic cells inside tumors might be crucial for activating effector CD4 and CD8 cells to exert their effector function locally inside the tumor [25]. The absence of DCs might partially explain why tumors are not killed despite the presence of effector cells inside the tumor.

Consequently, increasing the levels of the DC differentiation and growth factor GM-CSF has been utilized in a number of cancers as a strategy to enhance the anti-tumoral response [26].

Another explanation for the presence of immune effector cells inside tumors despite a lack of antitumoral response has been the presence of naturally occurring regulatory T cells (T_{reg}) that suppress the antitumoral T cells. In a mouse model of spontaneous tumor development it was indeed shown that this is one of the predominant ways by which tumors evade immune recognition [4]. Naturally occurring T_{reg} cells play an important role in maintaining immunological balance by suppressing a wide variety of immune responses to self antigens, infectious agents and tumors [27]. This subset of $CD4^+$ T cells express high levels of CD25 (interleukin-2 receptor α-chain) is naturally anergic and requires stimulation through the T-cell receptor for induction of their cell-mediated suppressive function. The forkhead transcription factor Foxp3 is particularly important in development of these cells [28]. An increased frequency of T_{reg} cells has been observed in peripheral blood and tissues of patients with cancer [29]. In ovarian carcinoma, high numbers of infiltrating $CD4^+CD25^+Foxp3^+$ T_{reg} cells were associated with worse prognosis and these cells directly suppressed infiltrating CD4 and CD8 cell function [30]. Here, we similarly demonstrated that human mesothelioma biopsies harbor significant numbers of $CD4^+CD25^+$ T cells, and that these $CD25^+$ cells also expressed the T_{reg} transcription factor Foxp3. Others also described an increase in $CD4^+CD25^+$ cells in MM pleural effusion [31].

To address the function of these T_{reg} cells, we turned to the murine transplantable mesothelioma AB1 mouse model [2]. In this model, tumor growth was significantly reduced and survival increased when T_{reg} cells were depleted using a CD25-depleting antibody prior to tumor implantation. The median survival was prolonged from 19 to 33 days. Strikingly, 5 of the 12 treated mice (41%) remained tumor-free for 2 months. This is reminiscent of other experimental tumor models in which T_{reg} depletion using the same antibodies led to increased

tumor rejection [32, 33]. One way in which T_{reg} cells mediate their suppressive function is through signaling of the TGFβ receptor of CD8 T cells [34]. We could detect TGFβ2 in the pleural fluid and MM supernatant but currently we do not know whether TGFβ is produced by $CD4^+CD25^+$ T_{reg} cells. Another suppressive cytokine produced by T_{reg} cells is IL-10. Although we were unable to detect IL-10 in our protein array, we did observe weak staining for IL-10 on immunohistology (data not shown). Besides T_{reg} cells and immunosuppressive cytokines in the tumor microenvironment, there are other possibilities why the immune response against mesothelioma is generally so ineffective. Low or absent production of specific antigens, crypticity of epitopes or down-regulation of MHC expression keeps the immune system ignorant of the tumor. Presentation of antigen without adequate costimulation may induce clonal anergy or effector cells develop a non-destructive response or in a manner which leads to cell death. Future experiments in mice will explore the functional significance of these cytokines in T_{reg} mediated immunosuppression. Also we currently can only speculate why high levels of T_{reg} cells with suppressive function are found inside mesothelioma. We have previously described high levels of heat shock proteins 70 in MM derived tumor fractions [5]. It was shown that self-HSP derived peptides have the potential to expand $Foxp3^+CD4^+CD25^+$ T_{reg} cells [35]. Moreover, high levels of cyclooxygenase 2 (COX2) and prostaglandin-E2 (PGE2) have been found in mesothelioma, and are correlated with worse prognosis [36]. Tumor derived PGE2 specifically induces the Foxp3 gene expression and regulatory T cell function in human $CD4^+$ $CD25^-$ T cells [37].

One way in which our data might be employed is in the design of better immunotherapeutic trials for mesothelioma. We have recently shown that immunotherapy using tumor-pulsed DCs is effective in preventing the outgrowth of murine mesothelioma, but that success is compromised in mice with larger tumor burden [2]. Inhibiting the immunosuppressive milieu of the tumor by tumor debulking or by blocking VEGF, IL-6 or HGF activation and/or

signaling might be used as an adjunct to DC immunotherapy. Even more challenging, the inhibition of T_{reg} function by low dose cyclophosphamide, specific Toll like receptor 8 agonists, COX2 inhibition or immunotoxins directed at CD25 might be used in combination with DC immunotherapy to increase the success rate of mesothelioma tumor eradication. Clearly, preclinical studies in mouse MM models will have to demonstrate the success of such a combined approach before designing a clinical trial.

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Table 1: 80 Cytokines probed for on the RayBio human cytokine array membranes

(abbreviation) [systemic name]

Angiogenin (Ang)
B-lymphocyte chemoattractant (BLC) [CXCL13]
Brain-derived neurotrophic factor (BDNF)
Chemokine-β-8-1 (Ck beta 8-1) [CCL23]
Eotaxin / Eotaxin-2 / Eotaxin-3 [CCL11 / CCL24 / CCL26]
Epidermal growth factor (EGF)
Epithelial neutrophil-activating protein (ENA)-78 [CXCL5]
Fibroblast growth factor (FGF)-4 / FGF-6 / FGF-7 / FGF-9
Fractalkine (FKN) [CX3CL1]
Fms-like tyrosine kinase-3 ligand (Flt-3 ligand)
Glial-derived neurotrophic factor (GDNF)
Granulocyte chemotactic protein 2 (GCP-2) [CXCL6]
Granulocyte colony-stimulating factor (GCSF)
Granulocyte–macrophage colony-stimulating factor (GM-CSF)
Growth-related oncogene (GRO) / GRO-α [CXCL1]
Hematopoietic growth factors, hepatocyte growth factor (HGF)
I-309 [CCL1]
IFN-γ-inducible protein (IP)-10 [CXCL10]
Insulin-like growth factor (IGF)-1
Insulin-like growth factor binding protein (IGFBP)-1 / IGFBP-2 / IGFBP-3 / IGFBP-4
Interferon(IFN)-γ
Interleukin (IL)-1α / IL-1β / IL-2 / IL-3 / IL-4 / IL-5 / IL-6 / IL-7 / IL-8 / IL-10 / IL-12 / IL-13 / IL-15 / IL-16
Leptin
Lymphotoxin-like, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed on T lymphocytes (LIGHT)
Leukemia inhibitory factor (LIF)
Macrophage Inflammatory protein (MIP)-1β / MIP-1δ / MIP-3α [CCL4, CCL15, CCL-20]
Macrophage colony-stimulating factor (MCSF)
Macrophage-derived chemokine (MDC) [CCL22]
Mesoderm-inducing factor (MIF)
Monokine induced by γ-interferon (MIG) [CXCL9]
Monocyte chemoattractant protein (MCP)-1 / MCP-2 / MCP-3 / MCP-4 [CCL2, CCL8, CCL7, CCL13]
Neutrophil activating peptide (NAP)-2 [CXCL7]
Neurotrophin (NT)-3 / NT-4
Oncostatin M (OSM)
Osteoprotegerin (OPG)
Placenta growth factor (PIGF)
Platelet-derived growth factor-BB (PDGF-BB)
Regulated upon activation, normal T-cell expressed and presumably secreted (RANTES) [CCL5]
Pulmonary and activation-regulated chemokine (PARC) [CCL18]
Stem cell factor (SCF)
Stromal cell-derived factor (SDF)-1 [CXCL12]
Thrombopoietin (TPO)
Thymus and activation-regulated chemokine (TARC) [CCL17]
Tissue inhibitor of metalloproteinase (TIMP)-1 / TIMP-2
Transforming growth factor (TGF)-β1 / TGF-β2 / TGF-β3
Tumor necrosis factor (TNF)-α / TNF-β
Vascular endothelial growth factor (VEGF)

Table 2: Source and specificity of antibodies used for the immunohistochemical staining of mesothelioma sections

Antibody	Present on	Source
BDCA2	Plasmacytoid dendritic cells	Miltenyi Biotech, Bergisch Gladbach, Germany
BMK-13	Resting and activated eosinophils	Monosan/Sanbio, Uden, The Netherlands
CD1a	Dendritic cells	DAKO, Glostrup, Denmark
CD3	"pan-T" lymphocytes	DAKO
CD4	T helper / inducer lymphocytes, monocytes	DAKO
CD8	T suppressor / cytotoxic lymphocytes	DAKO
CD11c	Monocytes, granulocytes, NK cells , macrophages, DC	BD Biosciences, San Jose, CA, USA
CD14	Monocytes (macrophages/granulocytes)	DAKO
CD15	Neutrophils (monocytes)	DAKO
CD16	Natural killer cells, neutrophils and basophils	DAKO
CD24	B cells, neutrophils, DCs	DAKO
CD25	Activated T cells and, at a lower density, on activated B cells	DAKO
CD31	Blood vessels and microvessels	DAKO
CD68	Macrophages (antigen presenting cells)	DAKO
CD209	Monocyte derived dendritic cells (DC-SIGN)	R & D Systems, Minneapolis, MN, USA
Chymase	Mast cells	Chemicon, Temecula, CA, USA
Foxp3	Regulatory T cells	eBioscience, San Diego, CA, USA
HBME1	Mesothelial cells	DAKO
HLA DR DQ DP	MHC class II expressing cells	DAKO
RCK108	Normal and malignant epithelial cells	DAKO
5B5	Fibroblasts	DAKO

Table 3: Cytokines investigated in pleural effusions and corresponding mesothelioma cell lines (for cytokine abbreviations see Table 1)

Not detected		**Detected**		Mean PF±sd : mean SN±sd
BLC	IL-7	**PF ↑↑**	HGF	1.50±1.50 : 0
Ck beta8-1	IL-10		MIP-1delta	1.67±0.52 : 0 *
EGF	IL-13		MIP-3alpha	0.83±1.33 : 0
Eotaxin	IL-15		NAP-2	2.33±0.52 : 0 *
Eotaxin-3	IL-16		PARC	3.50±0.55 : 0 *
FGF-4	LIF	**PF ↑**	Ang	3.17±0.41 : 2.00±0 *
FGF-6	LIGHT		Eotaxin-2	0.33±0.84 : 0
FGF-7	MCSF		IGFBP-1	1.50±1.05 : 0.50±0.57
FGF-9	MCP-2		IGFBP-2	1.83±0.98 : 1.25±1.5
Flt3L	MCP-3		IL-12	0.17±0.41 : 0
Fractalkine	MCP-4		IP-10	1.00±0.89 : 0.75±1.50
GCP-2	MDC		Leptin	0.50±0.84 : 0
GCSF	MIG		MIF	0.67±1.04 : 0.25±0.5
GDNF	NT-3		OSM	0.17±0.41 : 0
GM-CSF	NT-4		PIGF	0.83±0.75 : 0.50±1.00
GRO-alpha	PDGF-B	**PF ↔ SN**	Osteoprotegrin	0.83±0.75 : 0.75±0.50
I-309	SCF		VEGF	0.67±0.82 : 0.75±0.96
IFN-gamma	SDF-1		MCP-1	2.33±0.51 : 2.50±1.73
IGF-1	TARC		TGF-beta2	0.17±0.41 : 0.25±0.50
IL-1alpha	TGF-beta1		ENA-78	0.50±1.22 : 0.50±1.00
IL-1beta	TGF-beta3		TIMP-1	2.67±0.51 : 2.50±1.29
IL-2	TNF-alpha		IL-6	4.50±0.84 : 3.50±2.38
IL-3	TNF-beta		MIP-1beta	1.00±0.63 : 0.75±1.50
IL-4	TPO	**SN ↑**	BDNF	0 : 0.25±0.50
IL-5			IGFBP-3	0.33±0.51 : 0.75±0.50
			IGFBP-4	0.83±0.75 : 1.25±0.96
			TIMP-2	2.50±0.55 : 3.25±0.50
			IL-8	3.66±1.51 : 4.25±0.50
		SN ↑↑	GRO	1.50±0.84 : 2.75±0.5 *
			RANTES	0.17±0.41 : 1.25±0.96

PF = pleural fluid, SN = cell line supernatant

↔ equally expressed in PF and SN; ↑ moderate increase; ↑↑ strong increase

* : p-value < 0.05.

Figure legends:

Figure 1: Expression levels of cytokines present in effusion (circles) and in the supernatant of mesothelioma cell lines (squares) assayed by human cytokine antibody arrays. The relative expression levels were determined by density scoring as described in Methods. Horizontal bars represent mean values.

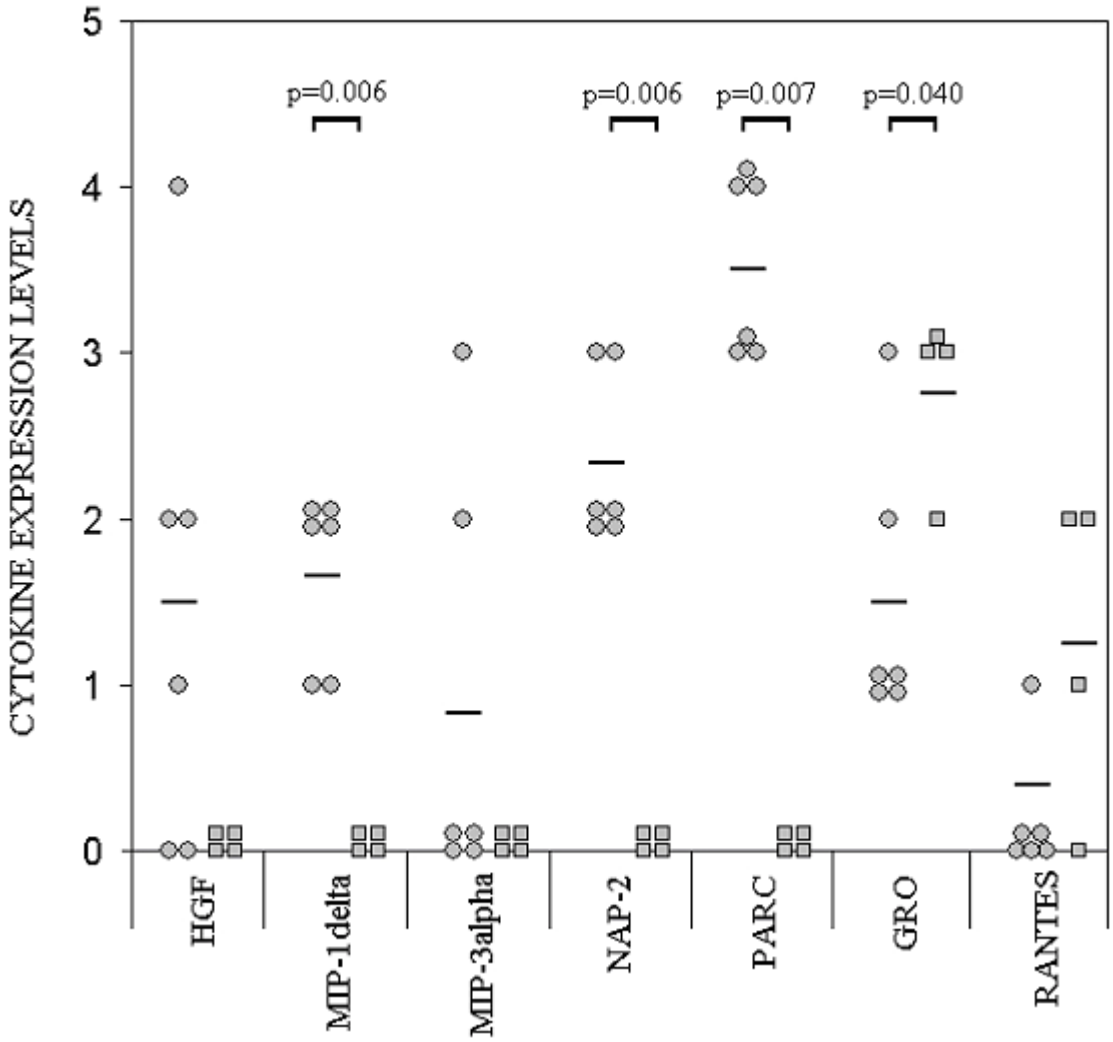


Figure 2: Tumor cells were localized in tumor fields as visualized by HBME1 and cytokeratin staining (RCK108, data not shown). Blood vessels (CD31) were present mainly at the periphery of the tumor and are considered as a key step in tumor growth. Macrophages

(CD68), NK cells (CD16), and T-lymphocytes, both T helper/inducer (CD4), and T suppressor/cytotoxic (CD8) cells, constitute the major part of the inflammatory cell infiltration.

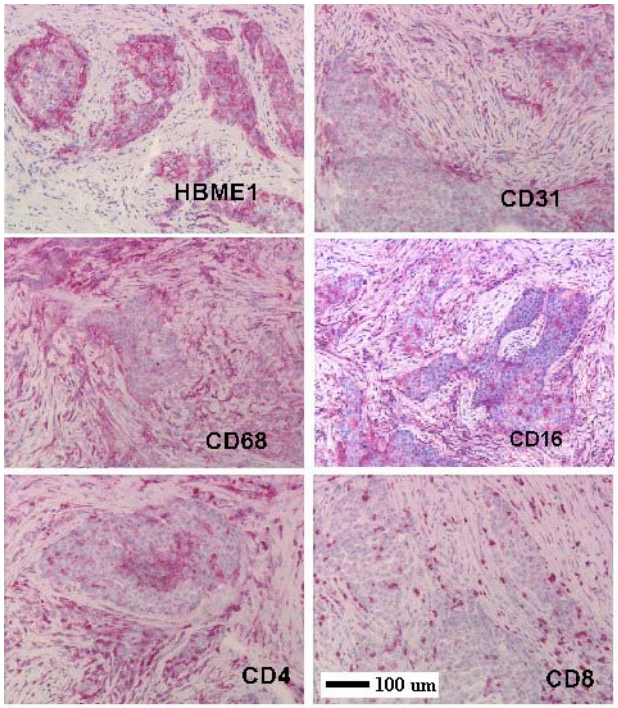


Figure 3: Dendritic cells (Langerin [A], CD1a [B]), eosinophils (BMK13 [C]), and B cells (CD24 [D]) were rarely detected. Counterstained with hematoxylin.

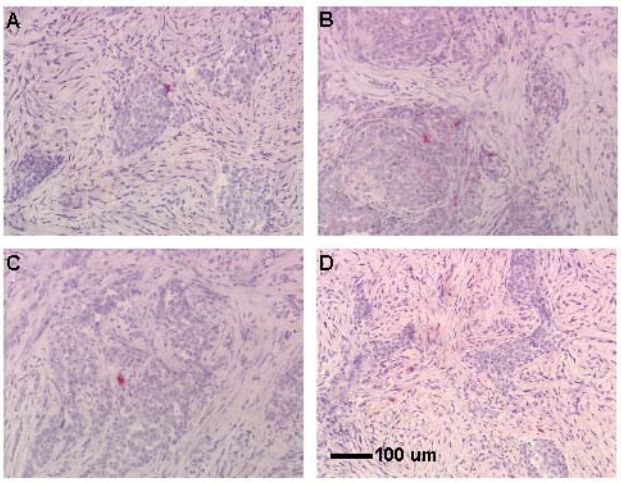


Figure 4: The transcription factor Foxp3 as hallmark of naturally arising $CD4^+CD25^+$ T_{reg} cells was expressed in the vicinity of the tumor (red staining). Counterstained with hematoxylin (A). Expression of Foxp3 (blue staining) strongly correlates with CD3 (red staining) (B) or CD25 expression (red staining) (C, D). No counterstaining. Magnification: x 100 (A, C) and x 200 (B, D). Fluorescent microscopy on mesothelioma tissue sections showing FITC labeled $CD25^+$ cells (E), Alexa Fluor 647 labeled anti-human $CD4^+$ cells (F) and $CD4^+CD25^+$ double positive cells (arrow) at 400x magnification (G) and at 100x magnification (H).

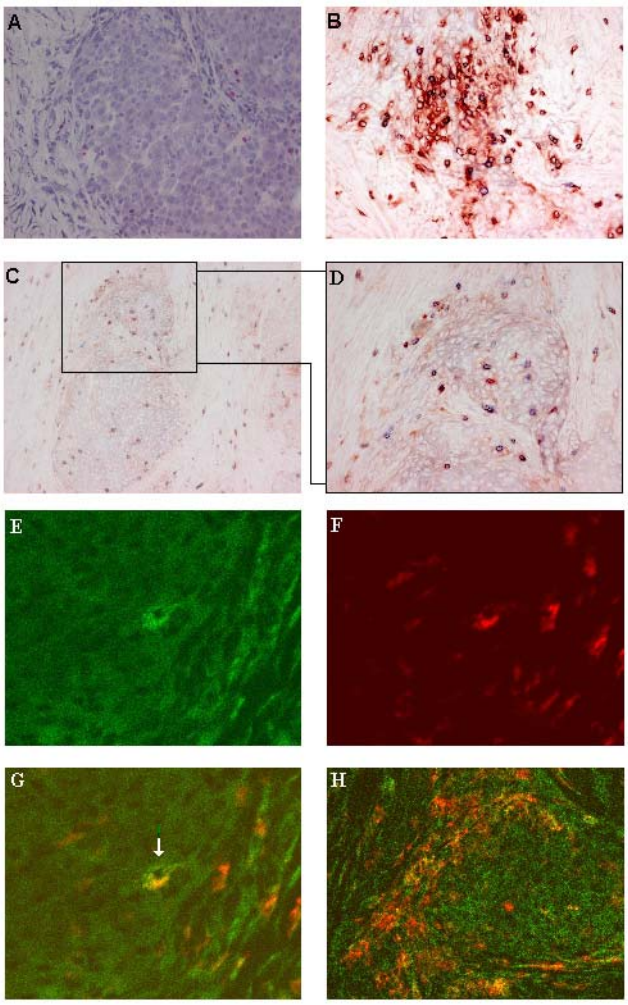


Figure 5: Flow cytometry analysis of blood from BALB/c mice that were untreated or treated with 0.5 ml PC61 ascites fluid on day 25 after antibody administration

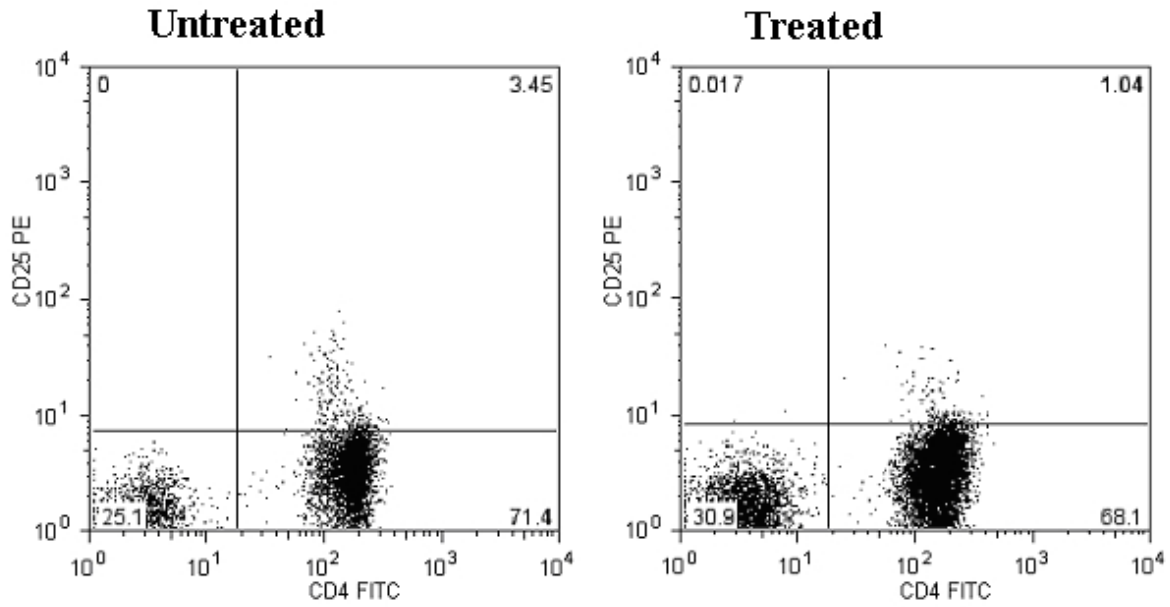
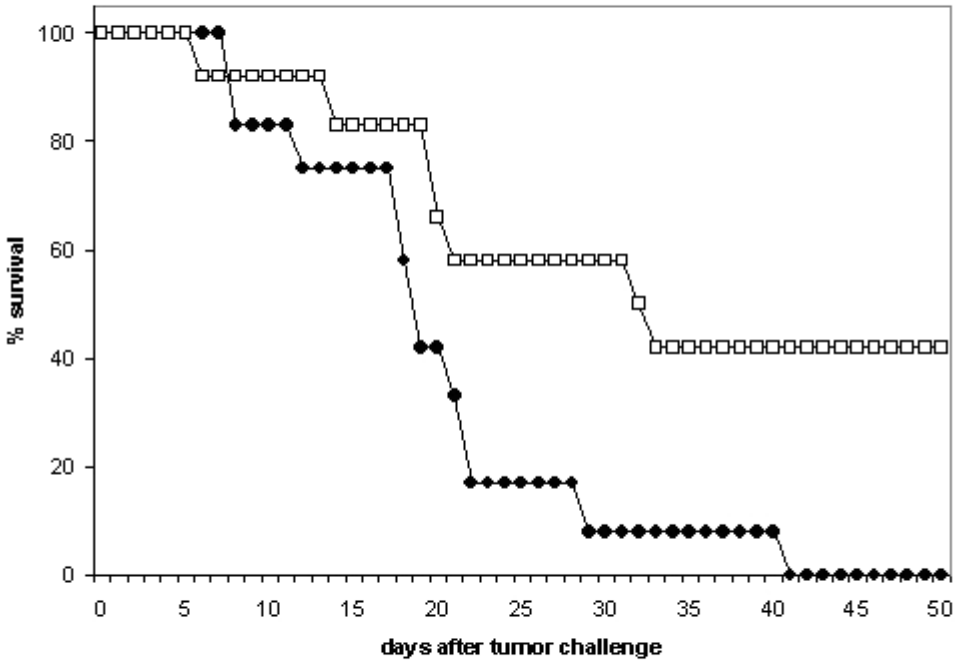


Figure 6: Kaplan-Meier survival plot shows the effect of depleting $CD4^+CD25^+$ T cells by the administration of anti-CD25 antibody (PC61) in the development of malignant mesothelioma in a mouse model. On day –25, mice were injected intraperitoneally with PBS (closed circles, n = 12) or depleting antibody PC61 (open squares, n = 12). On day 0, mice were subjected to a lethal dose of 0.5 x 10^6 AB1 tumor cells. Mice were scored when profoundly ill to U.K. coordinating Committee on Cancer Research regulations and by the Code of Practice of the Dutch Veterinarian Inspection. P-value < 0.028 by log-rank test.


100
80
60
40
20
0
% survival
0
5
10
15
20
25
30
35
40
45
50
days after tumor challenge

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