Macrophages in Mesothelioma

Improving immunotherapy in pulmonary oncology

Lysanne Annelie Lievense

Colofon

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Macrofagen in mesothelioom

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Kunst

Wat we willen:
Momenten
Van helderheid
Of beter nog: van grote
Klaarheid

Schaars zijn die momenten
En ook nog goed verborgen

Zoeken heeft dus
Nauwelijks zin, maar
Vinden wel

De kunst is zo te leven
Dat het je overkomt

Die klaarheid, af en toe

Martin Bril
Verzameld werk. Gedichten (2002).

CONTENTS

Chapter 1

Introduction

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Dendritic cell-based immunotherapy in mesothelioma
R Cornelissen, LA Lievense, ME Heuvers, AP Maat, RW Hendriks, HC Hoogsteden, JPJJ Hegmans, JGJV Aerts
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INTRODUCTION

Tumor microenvironment

In 1889 the assistant surgeon Stephen Paget posed his 'seed and soil' hypothesis of the spreading of cancer cells from the primary site to one or more sites elsewhere in the body (metastasis). With this visionary paper he distanced himself from the then prevailing theory that cancer cells can home in all tissues and persuade the surrounding cells to grow similarly [1]. Paget proposed that the distant sites where travelling cancer cells, 'seeds', grow out are not a matter of chance, but are influenced by the nourishment of the local 'soil'. His observation that breast cancer metastasizes predominantly to the liver led to the hypothesis that some organs provide a better 'soil' for certain cancer cells than others. Paget's statement that 'the ploughman's observation of the properties of the soil may also be useful' was proven right almost 100 years later by Hart and Fidler who showed, that the outcome of metastasis is dependent on both tumor cell properties and host factors [2]. That local properties of the host don't only play a role in the determination of the distant metastasis site but also in the outgrowth of the primary tumor has become clear during the past decade.

The path that a normal cell takes to become malignant is highly variable and depending on several host factors, including environmental factors, polymorphisms and mutations in susceptibility genes, age and immunity. In addition, tumor development depends on factors in the microenvironment; interactions between malignant cells, stromal cells, extracellular-matrix components, various inflammatory cells, and a range of soluble mediators contribute to tumor development and progression. Before tumors become clinically relevant, tumor cells and their products have interacted with, and affected, host cells for a period of time. Tumors of patients, especially in advanced-stage disease, consist of an intricate network of cell types, like stromal cells and endothelial cells that comprise blood vessels. Also many immunological cell types are attracted towards and penetrate into cancer cell areas. These recruited host immune cells influence tumor responses in opposing ways and have shown to be a critical regulator of tumor biology: this dual role of the immune system in cancer can either suppress or promote tumor growth [3].

Tumors can evade potential immune destruction by the induction of an immunosuppressive microenvironment via secreting factors or creating specific conditions (e.g. hypoxia). In addition, it has become clear that the immune system can facilitate tumor progression by sculpting the immunogenic phenotype of tumors as they develop [4]. The concept that the immunogenicity of tumors changes due to the anti-tumor immune response is named 'cancer immunoediting' [5,6]. The immunosurveillance concept is now accepted by the scientific community and "avoiding immune destruction" is included as the latest hallmark of cancer [7]. Outgrowth of a tumor is divided in three phases often referred to as the three E's of immunoediting (Elimination, Equilibrium, Escape). In the first phase, tumor cells are recognized by the immune system and eliminated or controlled in their growth. In the equilibrium phase

the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. In the escape phase the immunologically sculpted tumor expands in an excessive manner leading to physical symptoms of cancer by the host [8].

There is accumulating evidence that cancer cells can recruit and subvert normal immune cells to serve as active collaborators in their neoplastic program [9]. This immunosuppressive effect has also been described in thoracic malignancies [10-14]. Intriguingly, immunotherapeutic approaches have recently shown that modulation of the patients' immune system is possible and can be of benefit for patients with lung cancer and mesothelioma [15-18]. Identification and targeting of the factors and cell types that play a role in the tumor microenvironment is essential to further improve and refine novel (immuno)therapies. In the microenvironment of a progressing tumor, regulatory T cells, myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) have an important role in facilitating tumor growth and immune escape by suppressing antitumor effector cells [13, 19-22]. Regulatory T cells and MDSCs are regarded as immune suppressive, as they are capable of inducing T cell apoptosis and T cell tolerance [20, 23]. TAMs are the major component of immune cell infiltration in the tumor microenvironment [21, 24-26]. Recently, there is a lot of evidence emerging regarding the clinical significance of these tumor-associated macrophages in various cancer types and TAM infiltration is correlated with patient outcome in several malignant tumors [27-31].

Macrophages and their role within tumors

Macrophages in general

Elie Metchnikoff was the first to describe in 1882 that there are leukocytes which are able to engulf and destroy harmful bodies such as bacteria. His discovery of phagocytosis was groundbreaking in a time where leukocytes were thought to take up bacteria in order to spread disease and made him the co-winner (together with Paul Ehrlich) of the 1908 Nobel Prize in Physiology or Medicine. Metchnikoff promoted the concept of natural, innate host defense by cellular mechanisms such as phagocytosis and recruitment of phagocytes during acute and chronic inflammation [32, 33]. Since their initial description, macrophages are now known to exert many essential functions in tissue development and homeostasis beside phagocytosis.

Macrophages are present in all tissues in mammals and these tissue-resident macrophages are derived from three sources; yolk sac, fetal liver and hematopoetic stem cells in the bone marrow [34-36]. The relative contributions of embryonic sources (yolk sac and fetal liver) versus hematopoetic sources vary per tissue [37]. Tissue-resident macrophages are specialized to perform many tissue-specific functions; e.g. osteoclasts (macrophages of the bone) are specialized in bone resorption [38], lung alveolar macrophages recycle surfactant molecules [39] and splenic red pulp macrophages process heme and iron from senescent red blood cells [40]. In concordance, abnormality of tissue-resident macrophages can be linked to various

pathologies such as osteopetrosis, alveolar proteinosis and a disturbed iron homeostasis. Furthermore, macrophages are known to have a critical role in e.g. atherosclerosis [41], type 2 diabetes [42], fibrosis [43] and cancer [44]. The maintenance of tissue-resident macrophages is organ specific. However multiple studies have revealed that tissue macrophage compartments are established prenatally and persist throughout adulthood through local proliferation without substantial input from adult hematopoiesis [45-47]. Substantial replacement of tissue-resident macrophages by monocyte-derived cells has only been reported for specific organs undergoing profound challenges such as the skin and gut which are constantly exposed to microorganisms [48]. However, these data are currently only based on experiments in mice, which have a limited lifespan.

When tissues are damaged during injury or infection, patrolling inflammatory monocytes are recruited from the circulation and differentiate into macrophages once they infiltrate the affected tissue [49, 50]. Depending on the local signals which the macrophage encounters, it can adopt a variety of phenotypes ranging from a proinflammatory cytotoxic phenotype to a phenotype capable of tissue remodeling and repair. The diversity and plasticity of phenotype and function are characteristic and unique features of macrophages [51-53]. The mechanisms that regulate the phenotypic switch in macrophages have a major impact on the progression or resolution of many chronic diseases, like tumor formation.

Tumor-associated macrophages (TAMs)

In cancer, monocytes and myeloid progenitors infiltrate tumoral tissues and polarize in a broad range of phenotypes depending on the local environment [54]. Macrophages in tumors are usually referred to as tumor-associated macrophages and their presence can be substantial (up to 60% of the tumor stroma) [55]. A hallmark of macrophages is their plasticity, an ability to either aid or fight tumors depending on the tumor environment, which has given them the reputation of a double-edged sword in tumor biology [56]. At the extremes of this spectrum are the M1 macrophages (classical activation) and M2 macrophages (alternative activation). Although the terms M1 and M2 macrophages are an oversimplification, they can be used to explain the opposing effects of different macrophage subsets. In general, M1 macrophages act as soldiers of the host and M2 macrophages act as workers of the host, as was illustratively described by Solinas et al [57]. M1 macrophages defend the host from viral and microbial infections, fight against tumors, produce high amounts of inflammatory cytokines, and activate the immune response [57, 58]. M1 macrophages originate upon encounter with interferon-γ (IFN–γ) and microbial stimuli such as lipopolysaccharide (LPS) and are characterized by IL-12 and IL-23 production and consequent activation of polarized type 1T-cell response, cytotoxic activity against phagocytozed microorganisms and neoplastic cells, expression of high levels of reactive oxygen species, and good capability as antigen-presenting cells. On the other hand, M2 macrophages promote scavenging of debris, angiogenesis, remodeling and repair of wounded/damaged tissues. Distinct types of M2 macrophages differentiate when mono-

cytes are stimulated with e.g. IL-4, IL-10 or glucocorticoids. Hallmarks of M2 macrophages are IL-10 production, CCL17, CCL22, and CCL2 secretion, matrix metallo-proteases (MMP) production, high expression of mannose receptor (CD206), scavenger receptor (CD163) and galactose-type receptor and poor antigen-presenting capability [57,59].

In addition, M2 cells control the inflammatory response by downregulating M1-mediated functions. The loss of equilibrium between M1 and M2 cell numbers may lead to pathological events: an M1 excess could induce chronic inflammation, whereas numbers of M2 could promote severe immune suppression [57-59]. The equilibrium between M1 and M2 polarization can in part be regulated by the bidirectional interaction with lymphocytes, as was reviewed by Biswas and Mantovani (Figure 1) [52].

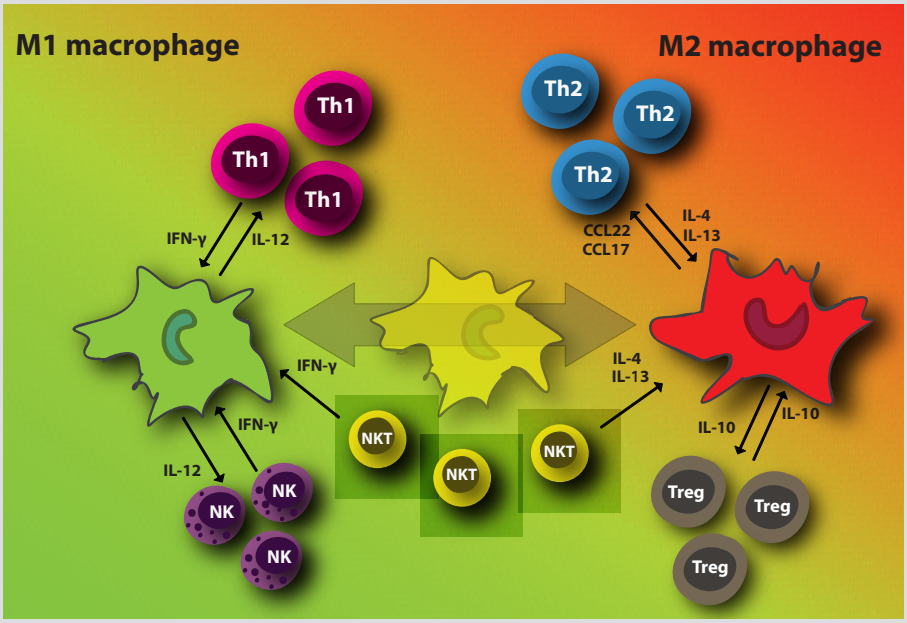

Figure 1. The macrophage phenotype can be regulated by interactions with lymphoid cells. Th1: type 1 T helper cell, Th2: type 2 T helper cell, NK: Natural Killer cell, NKT: Natural Killer T cell, Treg: regulatory T cell. An important hallmark of macrophages is their plasticity. Different lymphoid cell subsets are capable of skewing macrophages towards the opposing ends of their phenotypic continuum [35]. M1 polarization can be driven by interferon-γ production by Th1 cells and NK cells. M1 macrophages in their turn produce IL-12 which stimulates the activation of these cells. M2 polarization can be driven by IL-4 and IL-13 production by Th2 cells and IL-10 production by Tregs. The reciprocal production of IL-10 and the chemokines CCL17 and CCL22 by M2 macrophages stimulates Tregs and Th2 cells respectively. NKT cells can induce both M1 and M2 polarization, depending on their activation status [60].

When myeloid cells are attracted to the tumor, they are influenced by several local signals able to shape the cells as needed by the tumor. In an early phase of tumor development, the TAMs mainly consist of an M1-like phenotype and later in the tumorigenic process, when the tumor changes its local environment there is a skewing towards the M2 phenotype [61-63]. This takes place especially at those regions in the tumor that are hypoxic (Figure 2) [64, 65]. A subpopulation of TAMs gather in hypoxic sites in the tumor as a result of chemoattractants produced by tumor cells [66]. Exposure to hypoxia *in vitro* stimulates TAMs to acquire a pro-angiogenic M2 phenotype with high production of pro-angiogenic factors like VEGF and MMP-9 [67]. This preferential polarization is also a result of the absence of M1-orienting signals, such as IFN-γ or bacterial components in the tumor environment as well as the presence of M2 polarization factors. In addition to the formation of new blood vessels TAMs also play a role in the formation of new lymphatic vessels [68].

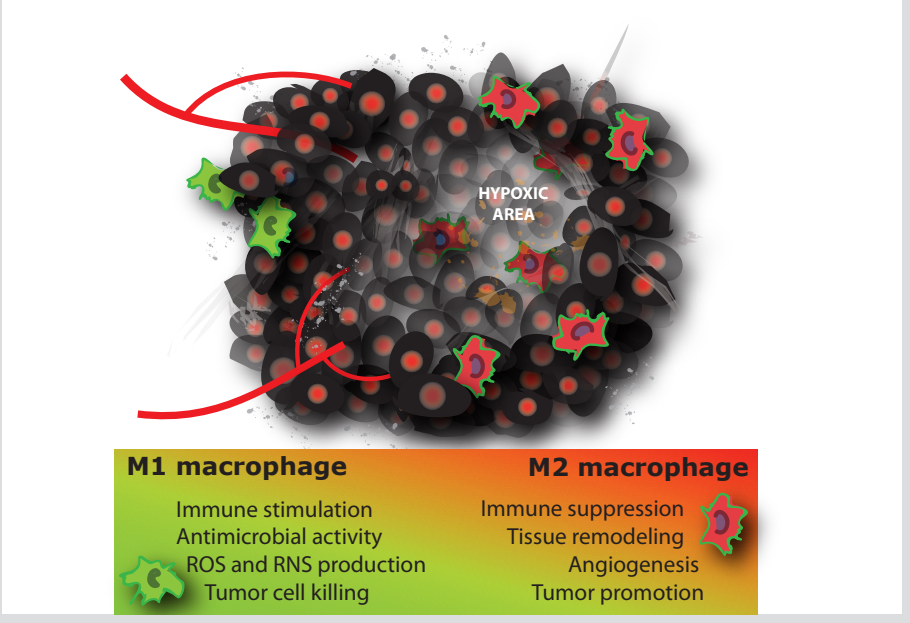

Figure 2 Opposing effects of M1 and M2 tumor-associated macrophages (TAMs) in the tumor environment. ROS: Reactive Oxygen Species, RNS: Reactive Nitrogen Species. The classically activated M1 macrophages and alternatively activated M2 macrophages are at the opposing ends of the polarization continuum and have opposing effects in tumor biology. M2-TAMs are abundantly present within the tumor, especially in those areas that are oxygen deprived.

Malignancies induce lymph angiogenesis, which leads to lymphatic and subsequently distant metastasis. Many of the pro-angiogenic factors produced by TAMs of the M2 phenotype can also contribute to lymh angiogenesis. The association between TAMs and lymph node

metastasis has also been confirmed in multiple clinical studies [69-71]. In addition to the secretion of paracrine mediators, evidence is emerging on the potential of macrophages to transdifferentiate into lymphatic endothelial cell progenitors (M-LECP) that structurally contribute to sprouting lymphatic vessels [68].

TAMs can influence fundamental aspects of tumor biology such as subversion of adaptive immunity, promoting tumor angiogenesis and supporting cancer cell survival, proliferation, invasion and tumor dissemination [57, 72]. In concordance with the pro- and anti-tumor effects that TAMs can elicit, clinical studies regarding the prognostic value of TAMs in multiple tumor types have also shown contradictory results depending on the prevalent TAM phenotype.

Pulmonary oncology with emphasis on malignant mesothelioma

Malignant mesothelioma

Asbestos was named by the Ancient Greeks, its name meaning "inextinguishable". It has been said that the Greeks also noted its harmful effects: "sickness of the lungs" in asbestos quarry slaves or slaves that wove asbestos into cloth, leading to a recommendation not to buy these slaves as they often "died young". The use of asbestos declined during the Middle Ages, but it regained popularity during the Industrial Revolution in the late 1800s. At the turn of the twentieth century, researchers began to notice a large number of deaths and lung problems in people living in asbestos mining towns and during these first decades of that century, a growing number of articles appeared in medical journals [73-75]. Some authors already suggested a link between inhalation of asbestos fibers and carcinogenesis [76, 77]. The term mesothelioma entered the medical literature in 1931 when it was identified by Klemperer and Rabin [78]. However, it was not until 1960 that the link between asbestos fibers and mesothelioma became incontrovertible with an article published in Lancet entitled "Primary Malignant Mesothelioma of the Pleura" by Eisenstadt and Wilson [79]. Over the last decades, the association between asbestos exposure and subsequent development of mesothelioma has been extensively studied in multiple animal species via inhalation of, or subcutaneous, intrapleural, and intraperitoneal inoculation with asbestos fibers [80-83]. Inhaled asbestos fibers present within the lung cause infiltration of macrophages into the pleural space, which try to phagocytose these inhaled foreign bodies [84]. In the effort to clear asbestos fibers, reactive oxygen species are generated, causing DNA damage to nearby cells. Subsequently, inflammatory cytokines and increased recruitment of immune cells to sites of inflammation within the pleura are induced [85-88]. Given the large size of the asbestos fibers, macrophages fail to clear the asbestos fibers, resulting in continued generation of reactive oxygen species and secretion of pro-inflammatory cytokines, a process often called "frustrated phagocytosis" [89]. In addition to this pro-carcinogenic and pro-inflammatory substance release, asbestos fibers can sometimes directly penetrate the cells and injure chromosomes [90]. Also, the retained asbestos fibers may adsorb other carcinogens on their surface [91]. As a result DNA alterations occur, such as inactivation of *p16INK4a/p14ARF*, *NF2/Merlin,* and *LATS2*, and the activation

of *YAP* [92,93].

Treatment options in mesothelioma are scarce and prognosis is poor, with a median survival of only 9-12 months [94]. The classic triad in cancer therapy; surgery, radiotherapy and chemotherapy, is also applied in mesothelioma [95]. Surgery for mesothelioma is a controversial subject. Only a minority of patients is eligible for surgical treatment and the performed clinical studies are too limited and diverse to draw definite conclusions regarding the survival benefit of surgery in mesothelioma [96-98]. Debates regarding the optimal surgical technique and multimodality setting are ongoing [99]. Radiotherapy is commonly used following surgery, in the palliative setting or as a means to prevent local tumor outgrowth at intervention sites. However, there is a distinct lack in randomized trials which support the clinical benefit of radiotherapy in mesothelioma. Novel forms and multimodality settings of radiotherapy in mesothelioma are currently being investigated and will hopefully lead to evidence-based indications of this treatment option in mesothelioma. Currently, chemotherapy is the only treatment for mesothelioma that has been proven to improve survival in randomized controlled trials. The landmark study is the publication by Vogelzang and colleagues in 2003 in which they compared cisplatin chemotherapy alone with a combination of cisplatin and pemetrexed resulting in a survival of 10 months in the control group and 13.3 months in the intervention arm [100]. This led to the approval of the combination of cisplatin and pemetrexed as 'standard of care' for the treatment of patients with unresectable mesothelioma. It should be noted that similar outcomes were reached with cisplatinum and raltitrexed compared to cisplatinum alone, confirming that a combination of cisplatin and an antifolate is superior to cisplatin alone in patients with mesothelioma [101]. In addition, no head-to-head chemotherapeutic comparison has been performed in mesothelioma, for example the comparison between the current standard regimen of cisplatinum/pemetrexed to cisplatin/raltitrexed, gemcitabine/cisplatinum, mitomycin, vindesine/cisplatin or vinorelbine. However, for every separate agent previously studied, the survival improvement was modest.

Several targeted agents have been extensively studied in mesothelioma. Epidermal Growth Factor Receptor (EGFR) inhibitors were thought to be a promising target for mesothelioma therapy since studies showed that EGFR was highly expressed in malignant mesothelioma [102, 103]. However, most likely due to absence of sensitizing mutations in the EGFR tyrosine kinase domain, the results of clinical trials were disappointing [104, 105]. Among anti-angiogenic agents, thalidomide is the most extensively studied drug. After many trials the phase 3 NVALT 5/MATES (Maintenance Thalidomide in Mesothelioma Patients) with thalidomide as switch-maintenance in non-disease progressive patients after first line pemetrexed chemotherapy could unfortunately not prove a survival advantage [106]. Phase 2 clinical trials of Vascular Endothelial Growth Factor (VEGF) tyrosine kinase inhibitors have shown at best modest activity in mesothelioma [107, 108]. The addition of bevacizumab, a VEGF monoclonal antibody, to standard chemotherapy has been recently shown to induce a modest but significant survival benefit in a randomized controlled phase 3 trial in newly diagnosed

mesothelioma patients [109]. This result warrants routine use of bevacizumab in addition to cisplatin and pemetrexed in unresectable mesothelioma [110]. An increasing amount of pre-clinical data highlighting the effectiveness of histone deacetylase inhibition in mesothelioma cell lines and mouse xenograft models has led to a number of early phase clinical trials in patients with mesothelioma [111]. The results of these efforts initiated a multicenter, randomized, placebo-controlled phase III study of the histone deacetylase inhibitor vorinostat in patients with advanced mesothelioma, which, unfortunately, did not improve survival compared with placebo as second-line therapy [112]. Furthermore, a recent randomized, placebo-controlled phase II trial investigating the Focal Adhesion Kinase inhibitor defactinib in mesothelioma patients was discontinued due to lack of efficacy.
In conclusion, there is no promising chemotherapeutic or targeted agent at the horizon which will profoundly improve survival in patients with mesothelioma. Clearly, there is a need for a new approach in the treatment of mesothelioma.

Lung cancer
Lung cancer is the most common cause of cancer-related death among males and females worldwide and non-small cell lung cancer (NSCLC) accounts for the majority of these cases [113]. On average, the 5 year survival rate for NSCLC is approximately 17%, with a rate around 70% for stage I [114]. The treatment of NSCLC is based on the patient's clinical signs and symptoms, tumor stage and subtype, medical and family history, and data from imaging and laboratory evaluation. The majority of patients (70%) is diagnosed with advanced (stage IV) disease, and until recently, palliative chemotherapy with platinum doublet therapy was the optimal treatment for these patients [115-117]. However, chemotherapy has a limited impact on long-term survival of NSCLC patients and the five-year survival rate is poor [118].
Recently the concept of driving mutations in NSCLC has dramatically changed the field of lung cancer treatment. Identification of these genotypic anomalies including activating mutations and fusion genes has set the stage for personalized medicine for distinct subsets of genetically defined NSCLC [119]. It involves tailoring treatment according to the genetic profile and molecular makeup of each patient, depending on the availability of targeted drugs. To date, several prognostic and predictive mutations have been identified in NSCLC; including oncogenic activation of epidermal growth factor receptor EGFR (HER1 / ErbB-1); translocation of EML4-ALK or CD74-ROS; point mutations in BRAF, PIK3CA, and MEK1; amplification of MET [120-123]. Patients with mutations have benefited from the development of target-specific therapy; e.g. gefitinib or erlotinib are effective EGFR tyrosine kinase inhibitors; crizotinib is used for ALK activation and sunitinib can be used when PDGFR is amplified [124, 125]. Objective response rates of 55 to 90 percent are observed when patients were selected based upon molecular criteria [121]. One of the most disappointing findings is the fact that tumors develop resistance to these agents [126]. The development of this resistance can either be mutation dependent, for instance genetic alteration of the drug target, or mutation independent, for

instance via transformation of histology.
Small cell lung cancer (SCLC) accounts for approximately 15% of the lung cancer cases [127, 128]. In general, SCLC is initially sensitive to chemotherapy and radiotherapy [129]. However, responses are often short-term and recurrence rates are high [129, 130]. Unfortunately, approximately 70% of patients diagnosed with SCLC have extended disease at presentation [131]. These patients are treated with platinum-doublet chemotherapy and have a median survival of 10-12 months [132, 133]. The development of targeted therapies for SCLC has proven to be challenging, mainly due to the complex and not fully uncovered biology of SCLC [133, 134].
The limited treatment options and poor prognoses of lung cancer and mesothelioma emphasize the need for novel treatments. Therefore, immunotherapeutic approaches are being investigated in these pulmonary malignancies.

Immunotherapy in pulmonary oncology

Setting the stage for immunotherapy

Paul Ehrlich stated in 1909 that cancer would be quite common if it wasn't for the protective effects of the immune system [135]. However, it were Richard Prehn and Joan Main who showed in 1957 that tumors induced by chemical carcinogens in mice were recognized by the immune system and rejected upon a secondary encounter with the same tumor [136]. With this observation, the potential of the immune system to eradicate tumors was demonstrated. Cancer immunotherapy attempts to activate or enhance the anti-tumor response of the immune system of the patient. Developments of therapeutic antibodies, cancer vaccines, and cell-based immunotherapeutic approaches reveal both the promise and relative infancy of these agents to extend the life of patients with cancer. In 2010, sipuleucel-T (Provenge; Dendreon Corporation) received the first FDA approval of a cancer vaccine for the treatment of metastatic castration-resistant prostate cancer [137]. It employs an adjuvant component to enhance the function of antigen presenting cells and immune effectors such as T cells. This was followed with the FDA approval in 2011 of the drug ipilimumab (Yelvoy, Bristol-Meyers Squibb) for the treatment of metastatic melanoma through potentiating T cell activity [138]. Both agents, whose activity is discussed in more detail below, demonstrate improved survival in randomized phase III trails and reignited enthusiasm for the field of active immunotherapy. With the many clinical programs currently underway, new approvals for therapeutic cancer vaccines by FDA and other ruling authorities as EMA are expected in the coming years. Immunotherapy is now considered as the fourth wave in cancer therapy after conventional treatments and targeted agents.

Types of immunotherapeutic approaches

Immunotherapy attempts to stimulate or restore the body's natural ability of the immune system to fight cancer. There are various strategies to activate the immune system and these are classified here into the following categories: biological response modifiers, monoclonal antibodies, peptide or tumor cell vaccines, and cellular immunotherapy (Figure 3). There is

no consensus regarding which of the four categories is the optimal approach for pulmonary malignancies, this will probably be highly dependent on the tumor characteristics of each individual patient.

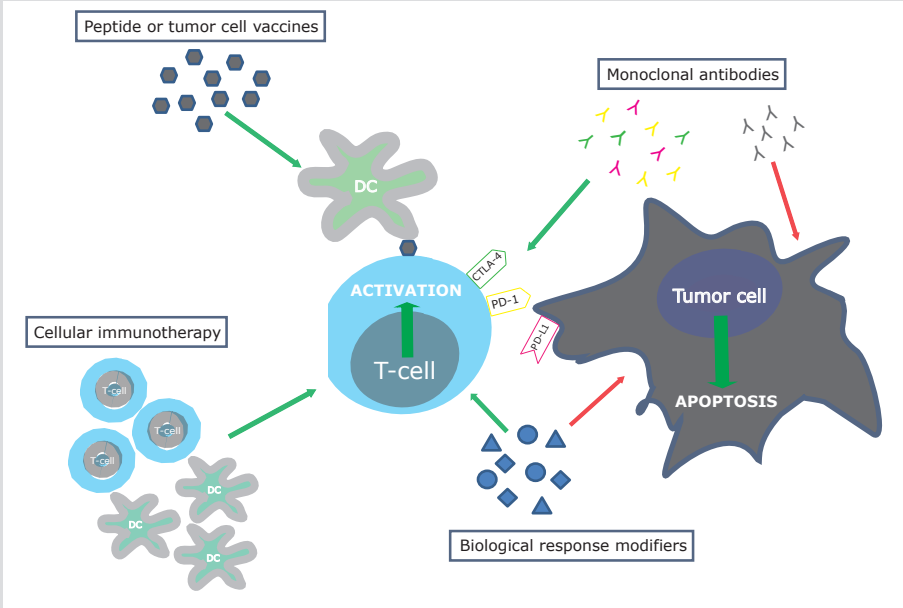

Figure 3 Immunotherapeutic approaches. Different immunotherapeutic approaches are currently being developed for the treatment of lung cancer and mesothelioma. All approaches aim to elicit an anti-tumor immune reponse and they can often work complementary and/or synergistically. Biological response modifiers can nonspecifically enhance the immune response, either by directly stimulating the immune system and/or by the direct induction of tumor cell apoptosis. Monoclonal antibodies bind specifically to one epitope and can be directed against numerous tumor- or immune cell related targets. Tumor vaccines are designed to deliver tumor antigens to antigen-presenting cells, which can subsequently induce a tumor specific immune response. Cellular immunotherapy includes the adoptive transfer of autologous or allogeneic activated immune cells.

Biological response modifiers

Biological response modifiers are compounds, which can aspecifically, enhance the immune response, either by directly stimulating the immune system and/or by the direct induction of tumor cell apoptosis. These compounds can activate the anti-tumor immune response via the direct stimulation of pro-inflammatory immune cells or via the inhibition of detrimental suppressive immune cells like regulatory T cells or myeloid-derived suppressor cells. The observation that lung cancer patients who developed an empyema after pneumonectomy seemed to have a longer survival gave rise to studies involving different biological response modifiers in the 1970's [139]. The idea that bacterial infection in the area of the draining

lymph nodes of the resected tumor could lead to immune destruction of residual tumor cells provoked studies involving the intrapleural injection of bacterial antigens to induce immune activation. Bacillus Calmette-Guèrin (BCG) is a vaccine against tuberculosis that is prepared from a strain of attenuated live bovine tuberculosis bacillus and its potential for cancer immunotherapy has been thoroughly investigated. McKneally *et al.* were the first to study the effect of postoperative injection of BCG into the pleural space of early stage lung cancer patients [140].Their observation that intrapleural BCG injection resulted in an improved survival lead to numerous studies regarding aspecific immune stimulation with this vaccine. Currently, BCG is most often investigated as an adjuvants instead as a single therapeutic agent in lung cancer patients [141]. In contrast, in patients with superficial bladder cancer, the use of intravesical BCG is now well-established [142]. In addition to BCG, heat-killed mycobacterium vaccae (SRL 172) has been investigated as a nonspecific immunostimulant in lung cancer and mesothelioma patients in combination with chemotherapy [143, 144], unfortunately no survival benefits were reported. Mycobacterial adjuvant-based agents have been shown to activate antigen-presenting cells and induce a Th1-type immune response, partly due to the binding of components of the cell wall of Mycobacteria to Toll-like receptors (TLRs) [145]. TLRs are membrane glycoproteins and belong to a family of pattern recognition receptors (PRRs) that recognize specific microbial molecular structures, pathogen associated molecular patterns (PAMPs). Recognition of a PAMP belonging to a micro-organism by a TLR leads to activation, maturation and induction of proinflammatory cytokines. Immature dendritic cells express numerous TLRs and aspecific immune activation via the stimulation of these TLRs has been extensively researched. In lung cancer specifically, TLR9 plays an important role and has been described to be overexpressed in lung cancer tissue [146]. Synthetic TLR9-activating compounds (e.g. PF-3512676, CpG-ODN) have been clinically tested in combination with chemotherapy in lung cancer patients, unfortunately no clinical benefit was found [147, 148]. However, since preclinical studies have shown that the use of the TLR9 agonist CpG-ODN as an adjuvants in tumor vaccines reduces the number of regulatory T cells and increases the number of effector T cells, TLRs remain a potential target in the field of cancer immunotherapy [149].

In addition to compounds that aspecifically enhance inflammation, the administration of cytokines has been amongst the earliest approaches in cancer immunotherapy. Interferons have been one of the major cytokine families of interest given their direct antiproliferative and immunopotentiating effects. In the 1980s, the first clinical trials were conducted in which lung cancer patients were treated with different types of interferons (recombinant alpha and beta) [150, 151]. Since then, the potential of interferon therapy in lung cancer patients has been researched extensively in a number of clinical trials, however no clinical benefits were found [152-155]. Other proinflammatory cytokines of which their potential as a therapeutic target in lung cancer patients has been investigated are interleukin-2 (IL-2) and tumor-necrosis factor alpha (TNF-α). In general, treatment with the combination of IL-2 and TNF-α induced relatively grave toxicities and no survival benefits[156]. In mesothelioma patients, the local infusion

of granulocyte/macrophage- colony-stimulating-factor (GM-CSF) has been studied with very limited success [157]. Currently, the direct administration of proinflammatory cytokines in order to enhance the anti-tumor immune response has been mostly abandoned in lung cancer and mesothelioma patients with the exception of the use of colony-stimulating factors with the purpose of the treatment of chemotherapy-induced neutropenia [158, 159].

Monoclonal antibodies

Monoclonal antibodies bind specifically to one epitope and their application as potential immunotherapeutic agents has received a lot of attention recently. The use of monoclonal antibodies directed against tumor growth related antigens on the tumor cell like epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) has been well established in lung cancer patients [160, 161]. In addition to the direct effect of the inhibition of growth factors and/or their receptors, antibodies bound to the tumor cell surface can induce antibody-dependent cell-mediated cytotoxicity (ADCC) [162]. Mesothelin is another tumor-specific antigen which is an attractive target for treatment with monoclonal antibodies because of its expression on several epithelial tumors including mesothelioma and lung cancer. Clinical studies with monoclonal antibodies against mesothelin are currently ongoing in lung cancer and mesothelioma patients [163, 164].

In addition to monoclonal antibodies directed against antigens specifically expressed by tumor cells, antibodies that are directed against tumor products have been clinically implemented. In lung cancer and mesothelioma patients, the monoclonal anti-VEGF antibody bevacizumab has been extensively investigated in clinical trials. In a meta-analysis, Cui *et al* showed that bevacizumab accompanied by chemotherapy improves clinical outcomes compared to other targeted therapies in chemotherapy-naïve lung cancer patients [165]. Bevacizumab has been developed in order to target blood vessel growth of tumors, however evidence shows that bevacizumab also has an immunomodulating effect and enhances circulating CD8 T cells in treated cancer patients [166]. This two-fold effect makes bevacizumab an interesting compound to study in combination with other immunotherapies.

The blockade of immune checkpoints using monoclonal antibodies can be considered one of the major breakthroughs in cancer research of the past years [167]. In order to control the immune response and to mitigate collateral tissue damage the immune system is harnessed with a negative feedback system. T cells have the capacity to upregulate co-inbitory receptors in order to inhibit the immune response and mediate immune tolerance. Multiple immune-inhibitory pathways (checkpoints) and their accompanying inhibitory co-receptors have been identified. In chronic infection and in cancer, expression of these inhibitory co-receptors is enhanced and associated with an anergic state in T cells [168]. Antibodies that bind to these co-receptors can block inhibitory signals and therefore augment T cell activation and proliferation.

The development of antibodies which bind to co-inhibitory molecules activated during T

cell activation has led to the possibility to prevent T cell inhibitory mechanisms and therefore enhance the anti-tumor immune response [169]. The first monoclonal antibody against a co-inhibitory molecule that showed clinical efficacy in cancer patients was anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4, ipilimumab) [138]. Currently, ipilimumab is approved for the treatment of metastatic melanoma [170]. Following the success in melanoma, numerous clinical trials are being conducted investigating checkpoint inhibitors in lung cancer and mesothelioma. The programmed death protein 1-protein death ligand 1/2 (PD-1 – PD-L1/2) pathway and CTLA4 are currently the most studied immunotherapeutic targets in these malignancies. In non-small cell lung cancer (NSCLC), anti-PD-1 antibodies have become part of the approved treatment arsenal [171, 172]. In mesothelioma the efficacy of checkpoint inhibition has not yet been proven. The implementation of these immune checkpoint inhibitors is hampered by serious immune-related toxicities (e.g. colitis, pneumonitis) and relatively low response rates. Therefore, the development of robust, predictive biomarkers is pivotal for the clinical implementation of monoclonal antibodies against co-inhibitory receptors. Interestingly, in multiple cancers including lung cancer, the mutational landscape (e.g. neoantigen load) has been demonstrated to determine the sensitivity to checkpoint blockade [173, 174].

Tumor vaccines

The research regarding cancer vaccines has made great progress since the discovery of human tumor antigens which can be recognized by T cell receptors [175]. Tumor vaccines are designed to deliver tumor antigens to antigen-presenting cells, which can subsequently induce a tumor specific immune response by the adaptive immune system. These vaccines can consist of various types of antigen sources (e.g. proteins, liposomal complexes and cell-based vaccines). An antigen candidate needs to meet certain criteria in order to potentially be able to elicit a specific anti-tumor immune response. Tumor specificity, frequency and homogeneous expression in tumor cells, role as an oncogene and intrinsic immunogenicity are essential features of antigens which determine the success [176]. In lung cancer and mesothelioma, a broad spectrum of approaches using various antigen sources have been undertaken to develop cancer vaccines. Melanoma-associated antigen A3 (MAGE A3) is an antigen which is specifically expressed by several human tumors, including NSCLC. Activation of the MAGE genes is known to take place in early carcinogenesis of the lung, however the physiological function of MAGE gene products is unknown [16, 177]. MAGE A3 is detected in approximately 35-50% of NSCLCs and its expression has been shown to be inversely correlated with survival [178, 179]. Vaccines composed of recombinant MAGE A3 (and adjuvants) have shown promising results in lung cancer patients in phase 2 studies but unfortunately demonstrated no survival benefit in a large phase 3 trial [180, 181]. In addition to large proteins like MAGE, smaller peptides can also be used in tumor vaccines. The WT1 (Wilms' tumor suppressor gene 1) peptide vaccine is composed of four WT1 analogue peptides. WT1 can be expressed in both lung cancer and mesothelioma and vaccination with this peptide has been shown

to induce T cell mediated immune responses in lung cancer and mesothelioma patients [182]. Treatment of mesothelioma patients with a WT1 vaccine demonstrated promising effects in a phase 2 study[183] and a phase 3 trial is currently being conducted. Liposomes are known to be potent vaccine delivery systems [184]. The best known cancer vaccine which makes use of this delivery technique is L-BLP25 or Stimuvax. This liposomal vaccine aims to generate an immune response against mucin 1 (MUC1), a cell surface glycosylated phosphoprotein that is frequently overexpressed by epithelial tumors including NSCLC [185 186]. The L-BLP25 contains the BLP25 lipopeptide and a liposomal delivery system, which facilitates uptake by antigen-presenting cells [186]. A phase 2 trial with advanced stage NSCLC patients showed survival benefits and paved the way for a large phase 3 trial (the START trial) [187]. Unfortunately, Stimuvax failed to increase overall survival in this trial [188]. Viruses can be genetically modified in order to express certain antigens and/or co-stimulatory cytokines and are therefore useful as 'viral vaccines' in cancer immunotherapy. The earlier described MUC1 protein can also be targeted by the TG4010 viral vaccine. This vaccine consists of attenuated vaccinia virus Ankara which is genetically modified to express MUC1 and IL-2 as adjuvants [189]. In a phase 2 study it was shown that TG4010 enhances the effect of chemotherapy in advanced NSCLC patients, a phase 3 trial is currently being conducted [190, 191]. There is a variety of cell-based vaccines under development for the treatment of lung cancer and mesothelioma. Cell-based vaccines can be autologous or allogeneic and transfected or not with immunostimulatory compounds. Autologous tumor cell vaccines are ideal antigen sources because they are capable of inducing an immune response to a large variety of antigens expressed by the patient's tumor. However their practical implementation is complex and challenging for large scale development [192]. An allogeneic tumor cell vaccine that reached phase 3 clinical trials in NSCLC is belagenpumatucel-L (Lucanix). Lucanix consists of four irradiated NSCLC cell lines modified with transforming growth factor β2 (TGF-β2) antisense plasmid. TGF-β is known to be associated with the immune escape of tumors and increased levels of TGF-β are associated with a worse prognosis in NSCLC patients [193 194]. The addition of the TGF-β2 antisense plasmid aims to stimulate the vaccine-induced immune response by inhibition of the production of TGF-β by the tumor. It is possible to use a combination of tumor cell lines as vaccine cocktail because NSCLC tumor cell lines are described to share immunogenic epitopes with primary tumors [16]. A phase 2 study showed clinical response rates of 15% amongst advanced stage NSCLC patients [195]. Unfortunately, in a phase 3 study belagenpumatucel-L did not meet its predefined endpoint in the entire patient population [196]. However, in specific subgroups of patients marked improvements in survival were achieved resulting in a current continued development of belagenpumatucel-L for specific indications.

Cellular immunotherapy

Cellular immunotherapy includes the adoptive transfer of autologous or allogeneic activated immune cells. Initially, adoptive immunotherapy was used for relapses after allogeneic

bone marrow transplantation in leukemia patients [197]. Recent advances have facilitated the application and clinical success of this method in various solid tumors [198]. The most prominent success story regarding cellular immunotherapy is sipuleucel-T, a vaccine for prostate cancer that consists of autologous peripheral blood mononuclear cells (PBMCs) including antigen-presenting cells that have been activated *ex vivo* with a recombinant fusion protein (PA2024, a prostate antigen that is fused to GM-CSF) [137]. After it was demonstrated in a phase 3 clinical trial that sipuleucel-T prolonges survival in metastatic castration-resistant prostate cancer patients, FDA approval followed in 2010. The general goal of adoptive cellular immunotherapy is to induce a tumor-specific immune response via the infusion of e.g. tumor-antigen loaded dendritic cells or specifically activated T cells. Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system and they have emerged as the most powerful initiators of immune responses. Because of their capacity to engulf tumor antigens and activate T cells in an antigen-specific manner, the use of DCs as immunotherapeutic agents is very promising. In DC-based immunotherapeutic approaches, DCs are generated *ex vivo* from monocytes and after arming with tumor-associated antigens, reinjected into the patient with the intention to restore proper presentation of tumor-associated antigens and T cell activation. This concept has been researched in NSCLC and has shown promising results regarding the elicited immune response, safety and tolerability, despite the small sample sizes of the trials [199-201]. In mesothelioma, treatment with autologous tumor-lysate pulsed DCs was shown to be safe and elicited an anti-tumor immune response in two phase 1 clinical trials [17, 202]. A phase 3 clinical trial with allogeneic tumor-lysate pulsed DCs is currently being conducted in mesothelioma patients.

Based on the principle that tumors can share common antigens, T cell therapy aims to develop a 'universal' T cell which recognizes tumors in different patients [203]. Different sources and activation procedures can be used in specifically harnessing the T cells response. Adoptive transfer of T cells gene-engineered with antigen-specific T cell receptors (TCRs) has proven its feasibility and therapeutic potential in the treatment of various malignancies [204]. Clinical TCR-engineered T cells currently tested in lung cancer and mesothelioma are directed against e.g. MAGE-A3 and WT1. The adoptive transfer of tumor-specific T cells expressing chimeric antigen receptors (CAR) is being extensively studied in multiple tumor types. Impressive results have been achieved with CAR T cells expressing CD19 in patients with advanced B cell malignancies; with up to 95% response rates [205, 206]. In mesothelioma and lung cancer, the potential of mesothelin-targeted CAR T cells is being studied [207].

Targeting the tumor microenvironment

Within this research field, there is much attention for activating effector and memory T lymphocytes because the release of their cytotoxic granules containing perforin and granzymes upon stimulation can lead to death of tumor cells by apoptosis. Indeed, the infiltration of NSCLC with effector T cells (CD3+CD8+) and memory T cells (CD45RO+) is associated

with longer disease-free survival and/or a better overall survival [62,208-212]. However, many other leukocyte types infiltrate the tumor environment: natural killer (T) cells, neutrophils, B- and T lymphocyte subsets, myeloid derived suppressor cells, macrophages and dendritic cells. The net effect of the interactions between these various cell types and their secreted products within the environment of an established tumor participates in determining anti-tumor immunity, angiogenesis, metastasis, overall cancer cell survival and proliferation [60]. Therefore, in addition to the activation of the anti-tumor T cell response, there is increasing interest to modify this immunological balance, e.g. by targeting immune suppressive cell types or factors. Different approaches are currently studied to overcome the earlier described immunosuppressive environment and to enhance the cytotoxic T cell response. We developed dendritic-cell based therapy with the intention to potentiate the anti-tumor immune response and ultimately improve outcome in mesothelioma patients. It was demonstrated that this approach was safe and effective in mesothelioma patients [17,202,213]. However, in order for patients to fully benefit from the potential of immunotherapy, optimal priming of the local tumor environment is pivotal.

TAMs in malignant mesothelioma

After the introduction of asbestos fibers in the lung, macrophages are recruited and activated in an attempt to clear the fibers. As the macrophages are unable to eliminate the asbestos fibers, a chronic state of inflammation occurs during which the secretion of free radicals causes genotoxic damage and the transformation of normal mesothelial cells to malignant mesothelioma is facilitated, a process often called 'frustrated phagocytosis' as stated earlier [89]. It is known that established mesothelioma tumors contain an abundance of intratumoral leucocytes and the phenotype of these cells is topic of recent studies [214]. Our group demonstrated that the inflammatory cell infiltration is rich in macrophages [13]. The massive abundance of TAMs in the microenvironment of malignant mesotheliomas suggests a pivotal role for these cells in the tumor biology of mesothelioma. It is known that normal human mesothelial cells and established human mesothelioma cell lines can produce large amounts of cytokines, including IL-6, IL-8, granulocyte-colony stimulating factor (G-CSF), and granulocyte-macrophage-colony stimulating factor (GM-CSF) [215,216]. These cytokines recruit monocytes and MDSC to the tumor mass, where they can differentiate into macrophages. Unlike in NSCLC, not much is known about the phenotype and function of these macrophages in malignant mesothelioma. *In vitro* studies showed that interaction with mesothelioma cells shifts mature macrophages toward a M2 phenotype. Izzi *et al* showed that upon cocultivation with mesothelioma cells, macrophages released a significant amount of prostaglandin E2, an arachidonic acid metabolite with anti-inflammatory properties [90,217]. The production of this prostaglandin stimulates the development of regulatory T cells, promoting an immunosuppressive tumor microenvironment [218,219]. Our group investigated the effects of macrophage depletion on tumor progression in a murine model of mesothelioma [220]. Liposome-encapsulated clodro-

nate is readily taken up by phagocytic cells, including macrophages, and induces cell-specific apoptosis after clodronate is set free into the cytoplasm of cells. Depletion of macrophages with clodronate inhibited tumor growth, indicating that macrophages have a significant role in the onset and progression of tumor in our murine mesothelioma model. This has been confirmed by other studies [221]. In addition, we investigated the role of zoledronic acid (ZA), a bisphosphonate with antitumor properties, on the myeloid differentiation to TAMs in our murine mesothelioma model. ZA inhibits farnesyl diphosphate (FFP) synthase thereby leading to a dysfunction of small GTPases which are necessary for normal function of macrophages [222]. Despite the fact that ZA lead to a reduction in TAMs and impairment of polarisation towards the M2 phenotype, no improvement of survival was observed [220]. This was most likely due to the fact that the reduction of TAMs was associated with an increase in the number of immature myeloid cells, another immunosuppressive cell type, illustrating the complex interactions of immune cells in the tumor microenvironment.

Burt *et al* published a study in which they investigated the prognostic significance of circulating blood monocytes and tumor-associated macrophages in 667 pleural mesothelioma patients who underwent cytoreduction between 1989 and 2009 [223]. They found that higher numbers of circulating monocytes are associated with poor survival in patients with both epithelial and nonepithelial mesothelioma. In addition, higher densities of TAMs were associated with poor survival in patients with nonepithelial mesothelioma. These TAMs demonstrated an M2-immunosuppressive phenotype with high expression of CD163 and CD206 [223]. Because of the high amounts of TAMs present in mesothelioma, manipulation of these cells is a promising therapeutic target in this lethal disease.

TAMs in non-small cell lung cancer

The link between lung carcinogenesis and chronic immune activation is well established. Compelling evidence has accumulated that histological assessment of infiltration patterns of different host immune response components in NSCLC specimens helps to identify different prognostic patient subgroups [224, 225]. TAMs have been implicated in promoting tumor growth, progression and metastasis in various solid tumors [27-31]. Several studies investigated the prognostic value of macrophage infiltration in NSCLC, however the results have been contradictory. These discrepancies could reflect differences in the number, grade and stage of tumors included in the various studies and the methods used to assess macrophage infiltration but most importantly the difficulties to differentiate between M1 and M2 subsets, which have varied considerably. Welsh *et al* were the first to recognize the importance of the microanatomical localization of macrophages in the tumor [226]. They showed that the infiltration of macrophages in tumor islets was related with a good prognosis in contrast to infiltration in the tumor stroma, which was related with a bad prognosis in NSCLC. These findings indicate that the localization of tumor-associated macrophages is critical in determining the relationship to prognosis and this has been confirmed by other studies [227]. In

more recent studies the phenotype of TAMs related to survival has been taken into account. Several studies have shown that macrophages in the tumor islets which are associated with extended survival are mainly of the cytotoxic M1 phenotype [228, 229]. This supports the view that immune responses in the tumor islets play a crucial role in preventing NSCLC progression [228]. On the other hand, TAMs of the M2 phenotype which are located in the tumor stroma have been related to treatment response to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) and were shown to be independent negative predictors of survival in advanced NSCLC treated with an EGFR-TKI [230]. In addition, macrophages of the M2 immunosuppressive phenotype were correlated with lymph node metastasis and poor prognosis in NSCLC [231, 232]. More specifically, several studies have shown a possible role for the expression of the immunosuppressive cytokine interleukin-10 (IL-10) by TAMs in the progression and prognosis of NSCLC [233, 234]. IL-10 is produced by a number of cells including neoplastic cells and macrophages and Il-10 production is a specific hallmark of TAMs with the M2 phenotype. High production of IL-10 has been described to enable tumors to evade immunosurveillance and the potential importance of IL-10 in cancer is supported by reports of an association between high IL-10 levels in serum or in tumors and worse survival in lung cancer patients [235]. Zeni *et al* showed that high IL-10 expression by TAMs and not by tumor cells was a predictor of advanced tumor stage and was associated with worse overall survival [233]. Wang *et al* showed similar results in their study reporting that high levels of IL-10 in TAMs significantly correlated with stage, tumor size, and lymph node metastasis [234]. In another study Wang *et al* showed that the expression of MMP-9 on TAMs isolated from NSCLC samples was higher in patients with late stage disease compared to early stage disease, which illustrates the importance of phenotypic analysis of TAMs and their potential to create a microenvironment that facilitates tumor progression [236].

The aforementioned studies show compelling evidence for the prognostic value of TAMs and their role in the tumor progression of NSCLC, both beneficial and detrimental. To the best of our knowledge there are no clinical studies available on the role of TAMs in small cell lung cancer (SCLC). The low incidence (15% of all lung cancer cases) and fast progression resulting in little opportunity to obtain histology are possible explanations for this lack in evidence [128].

TAM-targeted therapy

Given the emerging evidence regarding the prognostic value of TAMs in both mesothelioma and NSCLC, the therapeutic targeting of macrophages represents a valuable strategy to complement existing treatments. There is a rationale for re-educating the TAM compartment and the (combined) treatment of cancer may benefit from therapies that interfere with the attraction or the activation of M2 macrophages or polarize the M2 towards the M1 subtype. Influencing the number and functionality of M2 macrophages may improve survival when combined with standard or other immunotherapeutic regimens.

Several strategies are currently investigated that influence TAMs at multiple levels. For exam-

ple, blockade of factors and cytokines secreted by tumor or immune cells to limit the induction of M2 macrophages have been studied.
These strategies include inhibition of prostaglandin E2 synthesis (cox-2 inhibitor [237]), anti-CCL2 [238], anti-TGF-b [239] and anti-IL-6 (Siltuximab [240]), however these approaches are at a preclinical stage or have met with only limited success so far. Targeting TAM recruitment with CSF1R-signaling antagonists improves efficacy of cytotoxic therapies in murine models of solid tumors [241, 242]. Drastic reduction in TAM density has been achieved using an oral DNA vaccine encoding the entire murine Legumain gene (overexpressed by TAMs in murine tumor stroma), this vaccine provided effective protection against tumor cell challenge in a murine breast tumor model [243, 244]. It has been shown that inhibiting IkB kinase (IKK) reprogrammes the M2 phenotype to the M1 subset [245, 246]. Also CD40 therapy seems to skew tumor-infiltrating (not the resident) macrophages through CD40-ligation towards the M1 phenotype [247]. In addition, the TLR7 agonist Imiquimod has been associated with a switch from M2 tot M1 macrophages in a murine model for gynaecological tumors [248]. Furthermore, recently depletion of B cells has been demonstrated to be associated with a phenotypic switch in macrophages leading to a CD8 recruitment [249]. As stated earlier, the effects of bisphosphonates (Zoledronic acid) on TAMs were investigated but the impaired polarization was associated with increased MDSC levels which diminished the effects on survival [220, 250]. U'Ren *et al* have recently shown in a murine tumor model that endogenously produced type I interferons suppress the generation of TAMs [251]. Therapeutic administration of high doses of recombinant IFN-γ has been used to inhibit tumor angiogenesis and growth [252]. The study of U'Ren *et al* suggests that in addition to the direct inhibitory effect of IFN-γ on endothelial cell migration [252, 253], the inhibition of tumor angiogenesis by recombinant IFN-γ may be due in part to inhibition of M2 macrophage generation. An important feature of TAMs with the M2 phenotype is their ability to stimulate angiogenesis and thereby aid to the evolution, invasion and metastasis of tumors. The abnormal vasculature of tumors impedes the delivery of chemo- and immunotherapeutic agents. Moreover, the resulting abnormal microenvironment with hyper- and hypoxic regions reduces the efficacy of radiation, chemo-, and immunotherapies, selects for more malignant clones, and facilitates disease progression [254]. Thus, restoration of the normal structure and function in blood vessels, referred to as vascular normalization, is emerging as a new concept in cancer treatment [255]. As stated earlier, exposure to hypoxia stimulates TAMs to acquire a pro-angiogenic phenotype and induces the production of pro-angiogenic and tissue remodelling factors such as VEGF, placental growth factor (PlGF), and MMP-9 [256]. Rolny *et al* recently showed compelling evidence that histidine-rich glycoprotein (HRG) is capable of polarizing M2 macrophages towards a M1 phenotype partly by downregulating macrophage-derived PLGF in multiple murine tumor models [257]. HRG is a multidomain plasma protein synthesized by hepatocytes and has important function in regulation of tumor angiogenesis and immunity [258]. After overexpression of HRG in cancer cells, tumor vessels became normalized, resulting in decreased

hypoxia and improved delivery of chemotherapeutic agents and decreased metastasis [257]. Critically underlying this effect was the ability of HRG to skew TAM polarization away from the pro-angiogenic and immunosuppressive M2-phenotype. Future studies will have to reveal whether HRG can live up to its potential as an anticancer drug in humans.

Hypoxia-inducible factor (HIF) is one of the most important links between low oxygen pressure and macrophage polarization and targeting of its signal transduction pathway could be a potential novel therapeutic target [65]. The hypoxic cytotoxin Tirapazamine which is activated only at very low oxygen levels has been tested in lung cancer but showed no clinical benefits, however the treatment success may depend on the selection of patients with high levels of tumor hypoxia as we found in a study on hypoxia in NSCLC [259, 260]. Instead of the blockade or manipulation of hypoxia-induced pathways as an attempt to influence macrophage polarization another possible strategy is to directly decrease the hypoxic conditions in the tumor microenvironment. Nitroglycerin, due to its vasoactive effects, tends to redistribute the blood supply to the tumor, increasing tumor blood flow, hereby theoretically decreasing hypoxia. In a randomized phase 2 trial by Yasuda *et al* nitroglycerin has been successfully combined with chemotherapy in NSCLC, enhancing chemotherapy response, possibly due to better delivery of the anti-cancer drugs in the tumor [261, 262]. However, a recent randomized phase 2 study investigating addition of nitroglycerin patches to first-line carboplatin-paclitaxel-bevacizumab showed no survival benefit in stage IV NSCLC patients [263]. Whether treatment with nitroglycerin patches can also influence macrophage polarization will have to be subject of future studies.

Furthermore, an extensive review was published on the influence of aerobic exercise on the polarization of macrophages in breast cancer [264]. Several animal studies have indirectly shown the ability of exercise training to induce an anti-tumor effect of macrophages, however the exact mechanism and *in vivo* attribution of potential exercise-induced polarization remains to be elucidated. A possible explanation could be the exercise-induced enhanced tissue blood flow and inherent decrease of hypoxia, which skews the TAMs towards a more M1-like phenotype.

The emerging understanding of TAM biology and in particular their plasticity has lead to the development of numerous potential TAM-targeted therapies. Despite the current lack of a strategy that has proven its clinical value, the amount of literature emerging regarding TAM-targeted therapy illustrates the great potential of this cell type. Therapeutic targeting of macrophages could represent a valuable strategy to complement existing treatments of mesothelioma and lung cancer.

Aims and outline of the thesis

In the previous chapter, the establishment and importance of the tumor microenvironment was described. Tumor-associated macrophages are a pivotal part of this environment and have the capacity to greatly influence the natural course of disease and the outcome of cancer (immuno)therapy. Especially in mesothelioma, a disease with a profound influx of macrophages, their phenotype, function and potential as a therapeutic target could be of great value to improve future prognostic and therapeutic strategies. In **chapter 2** and **chapter 3** of this thesis, the clinical value of the phenotype of tumor-associated macrophages in mesothelioma was investigated. We studied the phenotype of macrophages in tumor biopsies of mesothelioma patients and linked this phenotype to the survival of mesothelioma patients and their likelihood to develop local tumor outgrowth at an intervention site. The immunosuppressive function of macrophages in a pivotal part of the mesothelioma environment, pleural effusion, was investigated in multiple *in vitro* studies in **chapter 4.** Furthermore, **chapter 5** illustrates the immunological dynamics in pleural effusion and the relation between the immunological composition of pleural effusions and the pleural tumors. After the demonstration of the clinical relevance and *in vitro* suppressive mechanisms of tumor-associated macrophages in mesothelioma, their potential as a therapeutic target (monotherapy and in combination with dendritic cell-based immunotherapy) was investigated in mesothelioma mouse models in **chapter 6 and 7**. The potential of the novel immunotherapeutic strategy using checkpoint blockade in mesothelioma and lung cancer was reviewed and put into perspective in **chapter 8 and 9**. Furthermore, a meta-analysis evaluating the efficacy of different immunotherapeutic approaches in NSCLC was included as **chapter 10**. All presented findings are evaluated and put into perspective in the general discussion in **chapter 11**.

Together, this work provides a profound translational insight regarding the clinical value, function and potential as a therapeutic target of tumor-associated macrophages in mesothelioma. Furthermore, this thesis thoroughly evaluates the general potential of immunotherapy in pulmonary oncology.

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Chapter 2

Ratio of intratumoral macrophage phenotypes is a prognostic factor in epithelioid malignant pleural mesothelioma

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ABSTRACT

Background: The tumor micro-environment and especially the different macrophage phenotypes appear to be of great influence on the behavior of multiple tumor types. M1 skewed macrophages possess anti-tumoral capacities, while the M2 polarized macrophages have pro-tumoral capacities. We analyzed if the macrophage count and the M2 to total macrophage ratio is a discriminative marker for outcome after surgery in malignant pleural mesothelioma (MPM) and studied the prognostic value of these immunological cells.

Methods: 8 MPM patients who received induction chemotherapy and surgical treatment were matched on age, sex, tumor histology, TNM stage and EORTC score with 8 patients who received chemotherapy only. CD8 positive T-cells and the total macrophage count, using the CD68 pan-macrophage marker, and CD163 positive M2 macrophage count were determined in tumor specimens prior to treatment.

Results: The number of CD68 amd CD163 cells was comparable between the surgery and the non-surgery group, and was not related to overall survival (OS) in both the surgery and non-surgery group. However, the CD163/CD68 ratio did correlate with OS in the total patient group (Pearson r -0.72, $p<0.05$). No correlation between the number of CD8 cells and prognosis was found.

Conclusions: The total number of macrophages in tumor tissue did not correlate with OS in both groups, however, the CD163/CD68 ratio correlates with OS in the total patient group. Our data revealed that the CD163/CD68 ratio is a potential prognostic marker in epithelioid mesothelioma patients independent of treatment but cannot be used as a predictive marker for outcome after surgery.

INTRODUCTION

Malignant pleural mesothelioma is invariably a lethal tumor with a median survival of 9-12 months after the first signs of illness. It is one of the diseases caused by exposure to asbestos fibers. The incidence varies from two to 30 cases per 1 000 000 population worldwide. Most patients are older than 60 years, a reflection of the latency period of 30–50 years after asbestos fiber inhalation.

Chemotherapy is offered to patients as standard of care treatment, as it currently is the only treatment that improved survival in randomized controlled trials in mesothelioma patients [1,2]. The survival benefit of chemotherapeutic treatment is in general modest with 2-3 months but long-term survivors do exist.

For decades, clinicians have tried to improve survival by removal of the pleural-based lesions. In order to try to completely remove the disease, a pneumonectomy with the complete removal of the visceral and parietal pleura is considered necessary, a so-called extra-pleural pneumonectomy (EPP). EPP is mostly performed in a multi-modality setting with induction chemotherapy and adjuvant radiotherapy. Selection of patients appeared crucial in the case-series that were published [3]. A less invasive procedure, that does not include the removal of the affected lung but of the visceral and parietal pleura, if necessary pericardium and diaphragm, an extended pleurectomy/decortication (PD), is also performed in patients. Whether surgery does lead to increased survival remains a matter of continuous debate, but it is evident that long-term survival after surgery occurs [4,5]. On the other hand, there are also patients in whom survival after surgery is extremely short. This points out the need for a biomarker to provide insight in which patients may benefit from surgery and which patients do not.

Gordon *et al.* described a four-gene expression ratio test that can predict good prognosis after surgery [6], however this test still has to be validated in a clinical setting. Suzuki *et al.* found in a patient group with predominantly surgical therapy that chronic inflammation in stroma is an independent predictor of survival [7], while other groups found a subset of immunological cell types to predict for better outcome in patients receiving surgical treatment with a special focus on CD8 tumor infiltrating lymphocytes [8,9]. The question remains whether these factors are prognostic or predictive for the effect of surgery.

The role of immune cells, like CD8 cells, within the tumor microenvironment has become a major area of interest in the last decade. It is now established in certain tumor types, that these infiltrating immune cells are capable of influencing tumor progression. One of the other involved immunological cell types are macrophages, which are known to have a dual role in cancer depending on their phenotype. Tumor associated macrophages (TAMs) can be divided in classically activated (M1) macrophages and alternatively activated macrophages (M2). M1 macrophages, following exposure to interferon-γ (IFN-γ), can secrete chemokines and promote T cell proliferation, thus activate type 1 T cell responses and have antitumor activity

and tissue-destructive activity. However, M2 TAMs promote the development and metastatic capacity of tumors due to the production of multiple cytokines such as interleukin (IL)-1, IL-6 and IL-10, vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) [10]. In mesothelioma, Burt *et al* showed that higher densities of tumor-infiltrating macrophages are associated with poor survival in patients after surgery, however, this was only in patients with non-epithelioid MPM [11].

A large proportion of M1 macrophages in the total macrophage count that can aid in tumoricidal activities could provide a better tumor control, since the overall balance in the tumor microenvironment shifts to an anti-tumor response. If the TAMs largely consist of M2 macrophages, this balance can shift to an overall pro-tumor micro-environment. The importance of the percentage of M2 macrophages of the total macrophage count (i.e. the CD163/CD68 ratio) and M1/M2 ratio has been found in other tumor types recently, such as melanoma, non-small cell lung carcinoma and angioimmunoblastic T-cell lymphoma [12–17]. In most of these studies, the ratio of M1/M2 macrophages predicts survival and metastatic ability of these cancers. Overall, a larger M2 component of the total macrophage count is inversely correlated with survival.

With CD8 T-cells and TAMS being the key immune cells in the tumor microenvironment [18,19], we analyzed if T cells and macrophage subtypes could be useful as a predictive marker to select mesothelioma patients for surgical treatment. Furthermore, the prognostic value of the different macrophage subtypes and CD8 positive tumor infiltrating lymphocytes (TILs) were tested.

MATERIALS AND METHODS

Patients and specimens

The Erasmus Medical Center ethical commission gave approval for this study. Diagnostic paraffin-embedded tumor specimens were used from 8 MPM patients who underwent an extended PD during the course of a phase I clinical trial following induction chemotherapy in our institute between 2008 and 2010 (a local study which is identified as Erasmus MC Cancer Institute MEC number 2008-405). The clinical trial randomized patients to P/D or best supportive care. Consent was obtained to use patient material for future research. Unfortunately, from the patients randomized to the best supportive care arm, adequate histology was not available in all cases. Therefore, we selected 8 MPM out of the total 89 patients that only were treated with chemotherapy during the course of the trial. The selection was matched to the surgical cases upon survival, EORTC prognostic score [20] and histology. Patient information was anonymized end de-identified prior to analysis. Histopathological diagnoses were established by pathologists from our institute and confirmed by the National Mesothelioma Pathology Board. Clinicopathological information was collected from patient charts. The TNM stage was based on the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) classification. Overall survival (OS) analysis of patients who underwent either chemotherapy or chemotherapy and PD was conducted. OS was defined as the time from the completion of chemotherapy to death. Three patients are still alive at the time of submitting this manuscript, since these are the 3 patients with the longest survival, last contact date was used instead of date of death.

Immunohistochemistry

The following primary antibodies were used: anti-human CD8 (clone C8/144B, Dako, Glostrup, Denmark), anti-human CD68 (clone KP-1, Dako), and anti-human CD163 (clone 10D6,Leica Biosystems Novocastra, Newcastle, UK). Paraffin-embedded tumor specimens were cut into sequential 5 µm thick sections and deparaffinized and stained using a fully automated Ventana BenchMark ULTRA Stainer (Ventana, Tucson Arizona, USA) according to manufacturers' instructions at the pathology department. Binding of peroxidase-coupled antibodies was detected using 3,3' - diaminobenzidine (DAB) as a substrate and the slides were counterstained with haematoxylin. The specificity of antibodies was checked using isotype-matched controls.

Evaluation of CD8, CD68 and CD163 stainings

The number of CD8-positive T-cells, CD68-positive total macrophages and CD163-positive M2-type macrophages were independently assessed by two investigators (R.C. and L.L.) who were not informed of the patients' clinicopathological data. To examine TILs and TAMs, the number of cells per microscopic field of 0,025cm^2 with immunoreactivity to CD8, CD68

and CD163 were counted in three independent tumor areas with the most abundant immunoreactive cells. For each antibody, the same area was used. Only cells with a visible nucleus were counted. We defined the average value of the three times the number of TILs and TAMs were counted for each case.

In vitro measurement of CD80, HLA-DR, IL-10, IL-12, VEGF, PD-L1, CD163, iNOS (NOS2) and Arginase-1 in macrophages by quantitative real time PCR

We investigated the influence of mesothelioma-derived factors on the phenotype and function of macrophages. Monocytes obtained from peripheral blood of an healthy control were cultured in the presence of 20 ng/ml recombinant M-CSF (R&D systems, Abingdon U.K.) in RPMI medium (Life Technologies, Bleiswijk, the Netherlands) containing 5% normal healthy AB serum (NHS) during 6 days at 37 °C /5% CO_2. After six days of differentiation, macrophages were cultured in the presence of 30 % mesothelioma cell line conditioned media (CM) during two days (n=6). CM were obtained from mesothelioma cell lines at 80% confluency, centrifuged for 10 min at 400 x g to remove cells and debris. These long-term tumor cell lines were established from the cellular fraction of 6 mesothelioma patient's pleural effusions as described earlier [21]. As a control we used standardized M1 (medium supplemented with 100 ng/ml LPS [Sigma-Aldrich, Zwijndrecht, the Netherlands] and 20 ng/ml IFN-gamma [R&D systems) and M2 cultures (medium supplemented with 40 ng/ml IL-10 [R&D systems]). Cells were harvested and mRNA was isolated by RNeasy micro kit according to manufacturer's instruction (Qiagen, Hilden, Germany). cDNA was prepared from 1 ug RNA sample using First Strand cDNA synthesis kit (Thermo Fisher, Pittsburgh, PA, USA). cDNA (5 µL) was amplified by RT-PCR reactions with 1× Maxima SYBR green / ROX qPCR mastermix (Thermo Fisher) in 96-well plates on an 7300 real time PCR system (Applied Biosystems), using the program: 10 min at 95°C, and then 40 cycles of 20 s at 95°C, 1 min at 58°C and 30 sec at 72°C. The primer sets used for different sets of genes are listed in Table 1. Specificity of the produced amplification product was confirmed by examination of dissociation curves. Expression levels were normalized to the internal control β-actin.

Table 1 Primer sequences of genes associated with macrophage phenotype used in RT-PCR

Gene	Forward primer	Reverse primer
β-actin	CTGTGGCATCCACGAAACTA	AGTACTTGCGCTCAGGAGGA
CD80	AAACTCGCATCTACTGGCAAA	GGTTCTTGTACTCGGGCCATA
HLA-DR	AGTCCCTGTGCTAGGATTTTTCA	ACATAAACTCGCCTGATTGGTC
IL-10	TCAAACTCACTCATGGCTTTGT	GCTGTCATCGATTTCTTCCC
IL-12	GCGGAGCTGCTACACTCTC	CCATGACCTCAATGGGCAGAC
VEGF	CACACAGGATGGCTTGAAGA	AGGGCAGAATCATCACGAAG
PD-L1	TATGGTGGTGCCGACTACAA	TGCTTGTCCAGATGACTTCG
CD163	GCGGGAGAGTGGAAGTGAAAG	GTTACAAATCACAGAGACCGCT
iNOS	ATTCTGCTGCTTGCTGAGGT	TTCAAGACCAAATTCCACCAG
Arg1	GTTTCTCAAGCAGACCAGCC	GCTCAAGTGCAGCAAAGAGA

Statistical analysis

The numbers of CD8 TILs and CD163 and/or CD68 TAMs were expressed as mean ± SD. Statistical differences between the means were analyzed by the Mann–Whitney U test. Correlations were made calculating the Pearson r correlation. Statistical calculations were performed using IBM SPSS Statistics version 21.0.0.1. Statistical significance was established at the $p < 0.05$ level, and all analyses were two-sided. Overall survival (OS) was calculated from the start date of treatment until patient death.

RESULTS

Patient characteristics

The median age of all participating patients was 62 years (range 36-75 years). There were 12 men and 4 women. All histologies were of the epithelioid subtype. The patient characteristics of the surgery and the non-surgery group are listed in Table 2. Chemotherapeutic treatment was given in both groups and consisted of 4 cycles of pemetrexed combined with either cisplatin or carboplatin. In case of surgery, P/D was performed 8 to 10 weeks after induction chemotherapy in all cases.

Table 2 Patient characteristics

	Surgery	Non-surgery
Patients (n)	8	8
Mean age (SD)	60 (11,9)	55 (7)
Male (n)	6	6
EORTC (SD)	1,025 (0,6)	0,88 (0,5)
EORTC high (n)	2	1
EORTC low (n)	6	7
PR after chemotherapy (n)	1	2
TNM		
T1-2 (n)	6	5
T3-4 (n)	2	3
N0 (n)	5	5
N1-2 (n)	3	3
M0 (n)	8	7

CD8 Tumor infiltrating lymphocytes in MPM

A representative image of immunohistochemical staining of CD8 TILs are shown in Figure 1. The mean CD8 numbers were comparable between the surgery and the non-surgery group (p=0.51) and no correlation was found between CD8 cell count and OS in the surgery group (p=0.88) and non-surgery group (p=0.96) nor for the whole group (p=0.73).

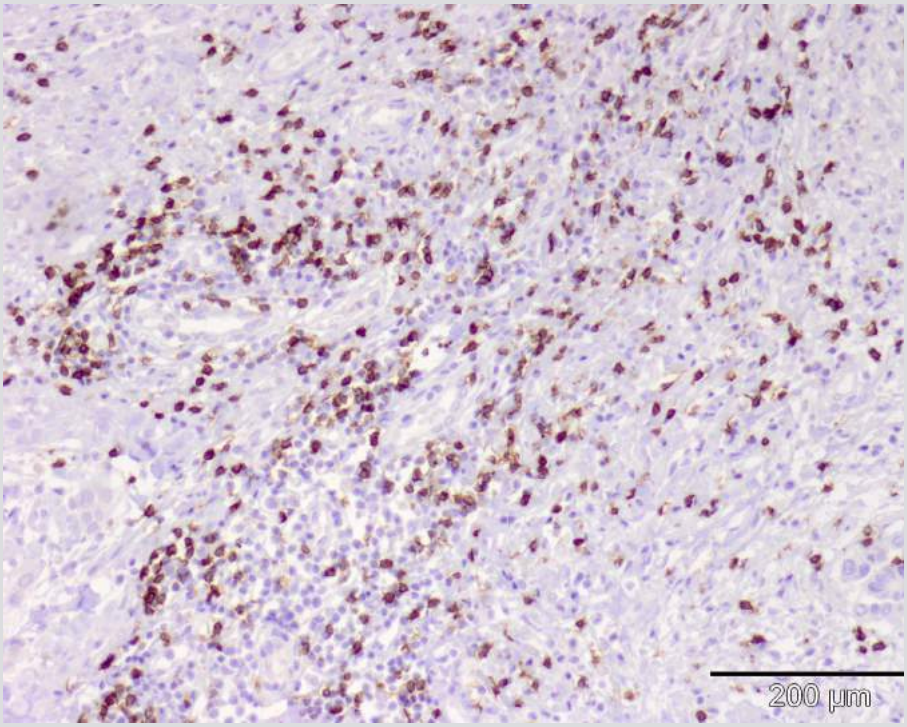

Figure 1 Representative image of CD8 staining in the tumor biopsy of one MPM patient.

CD68 and CD163 TAMs in MPM

Representative images of immunohistochemical staining of TAMs are shown in Figure 2a and 2b. The total count of CD68 was comparable between surgery and the non-surgery group (mean 211.3, SD 80.2 vs. mean 213.9, SD 100.4, p=1.0). Also, the total count of CD163 was comparable between surgery and the non-surgery group (mean 168.3, SD 80.2 vs. mean 164.1, SD 82.5, p=0.8).

The CD68 count did not correlate with OS (Figure 3a, Pearson r -0.07, p=0.81), the CD163 count showed an inverse trend with OS (Figure 3b, Pearson r -0.33, p=0.22).

CD163/CD68 ratio correlating with overall survival

We calculated the CD163/CD68 ratio, i.e. the number of M2 macrophages within the total macrophage count. This ratio was significantly negatively correlated with OS in the total patient group (Figure 4, Pearson r -0.72, $p<0.05$). A correlation analysis for the individual groups in regards to the CD163/CD68 and OS showed a significant correlation in the non-surgery group (Pearson r -0.91 [p = 0.001]) and a trend for the surgery group (Pearson r -0.65 [p = 0.08]).

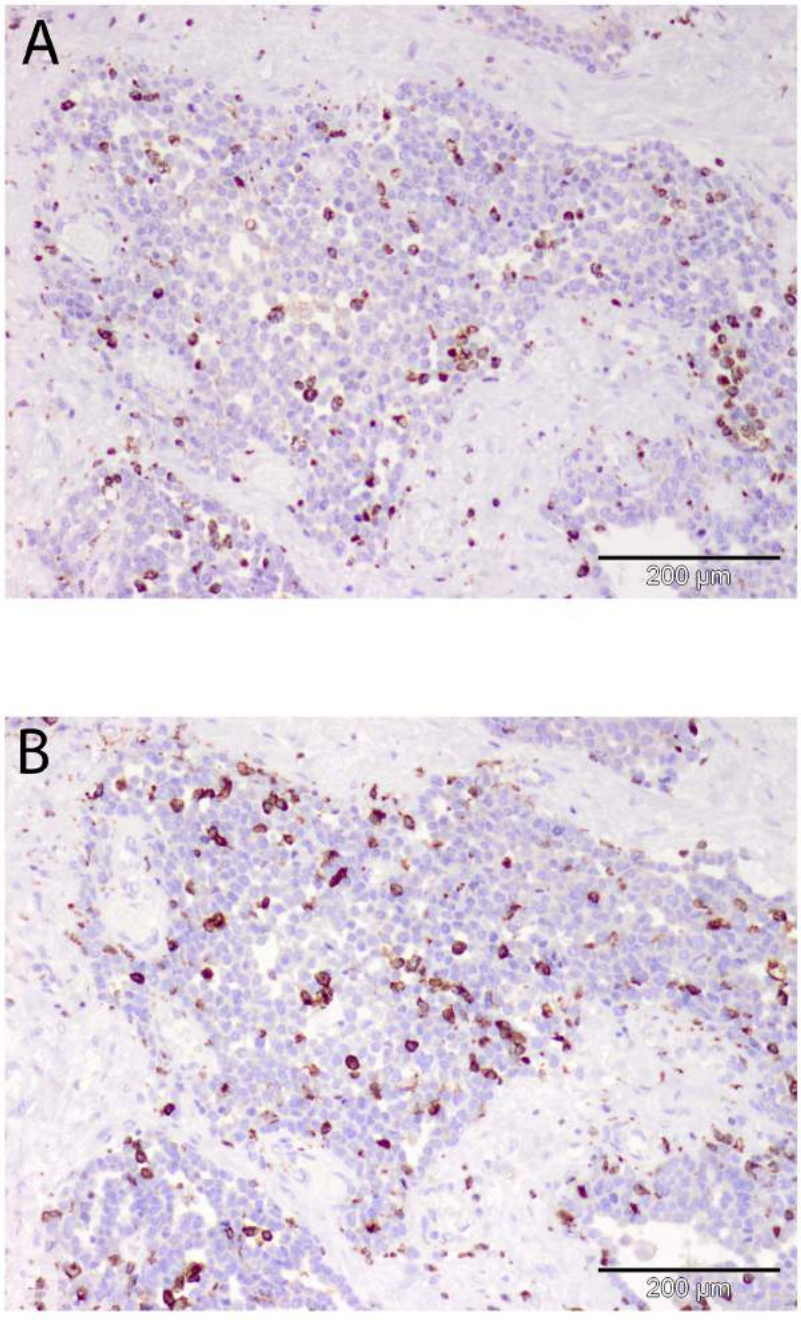

Figure 2 Representative images of CD68 (a) and CD163 (b) staining in the tumor biopsy of one MPM patient.

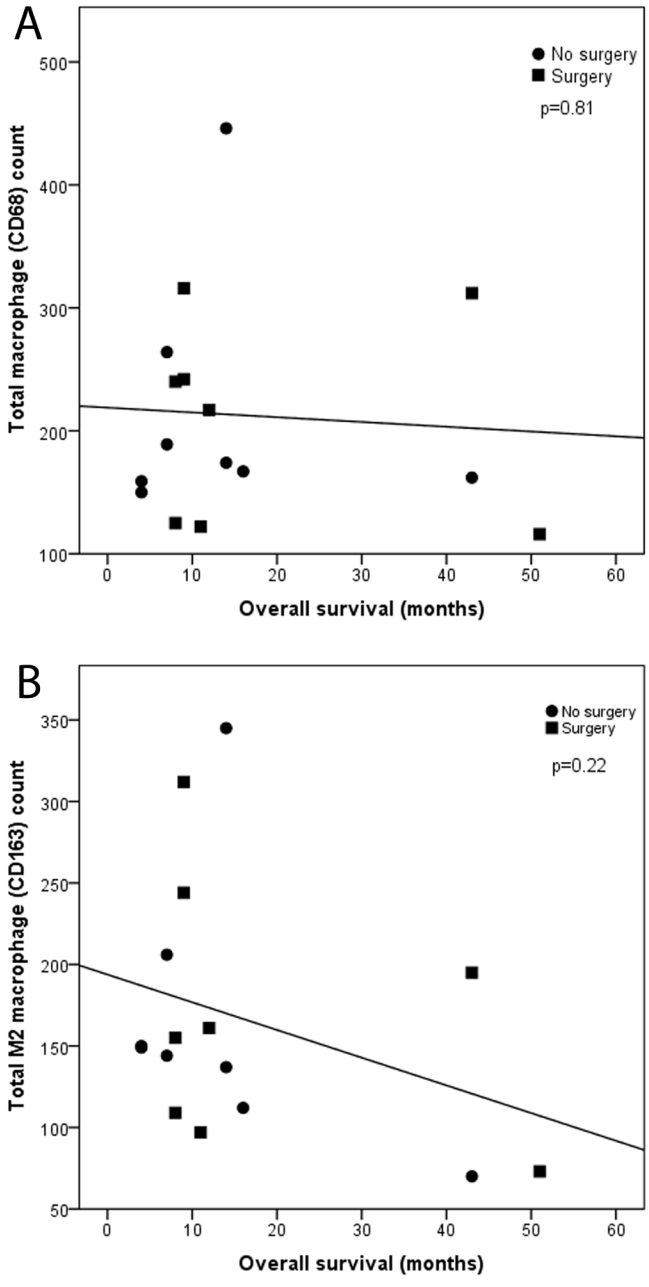

Figure 3 Correlation between CD68 (a) count or CD163 (b) count and OS in both surgery and non-surgery groups. The CD68 count does not correlate with OS (Pearson r -0.07, p=0.81), the CD163 count shows an inverse trend with OS (Pearson r -0.33, p=0.22).

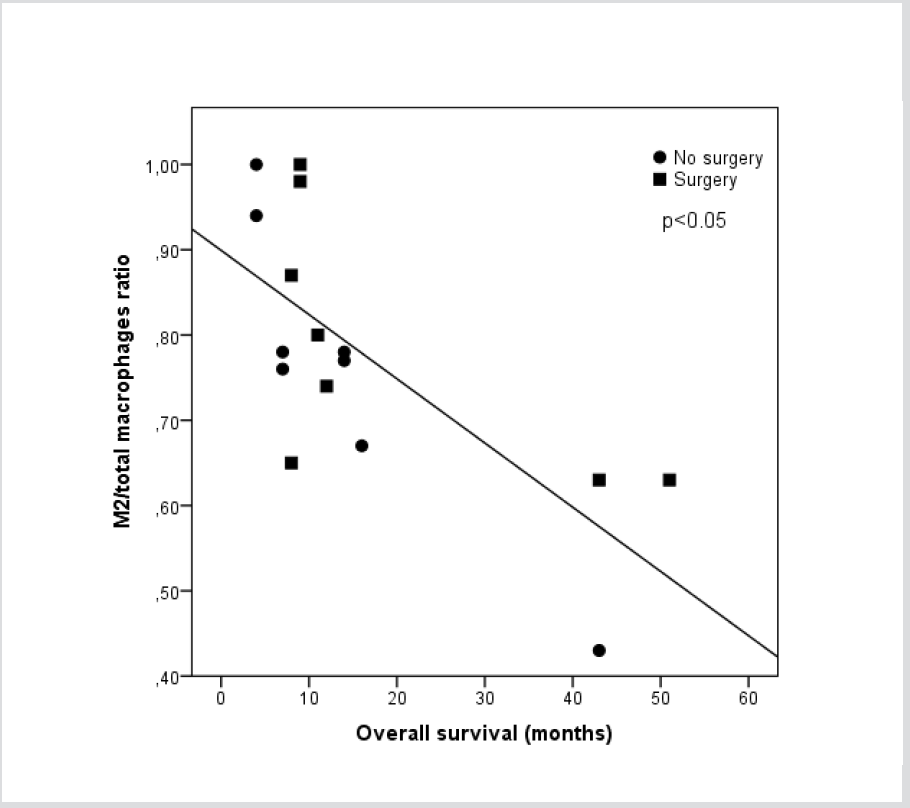

Figure 4 Correlation between CD163/CD68 ratio in tumor in both surgery and non-surgery patients and OS. This ratio is significantly negatively correlated with OS in the total patient group (Pearson r -0.72, p<0.05)

RT-PCR measurements for macrophage phenotype conditioned in mesothelioma environments

To investigate the influence of tumor-derived factors on macrophage phenotype, we cultured monocyte-derived macrophages in the presence of supernatant derived from six mesothelioma cell lines. Tumor cell supernatants (CM) induced macrophages towards a M2 prone phenotype with relatively high expression levels of the M2 cytokine IL-10 and low mRNA levels of the M1 markers IL-12, CD80 and HLA-DR. The standard M2 marker CD163 and the arginase1/iNOS ratio showed differential expressions dependent on the different CM. Furthermore, expression levels of the activation marker PD-L1 on macrophages cultured in CM were comparable to the M2 condition, in general these levels were lower than the M1 condition. Furthermore, results showed that CM have different abilities to influence macrophage phenotypes (Figure 5). Gene expression of IL-12 was only found when macrophages were cultured under M1 conditions and VEGF expression was low/absent in all conditions (data not shown). In conclusion, mesothelioma-derived factors influence macrophages towards a M2 phenotype to varying degrees.

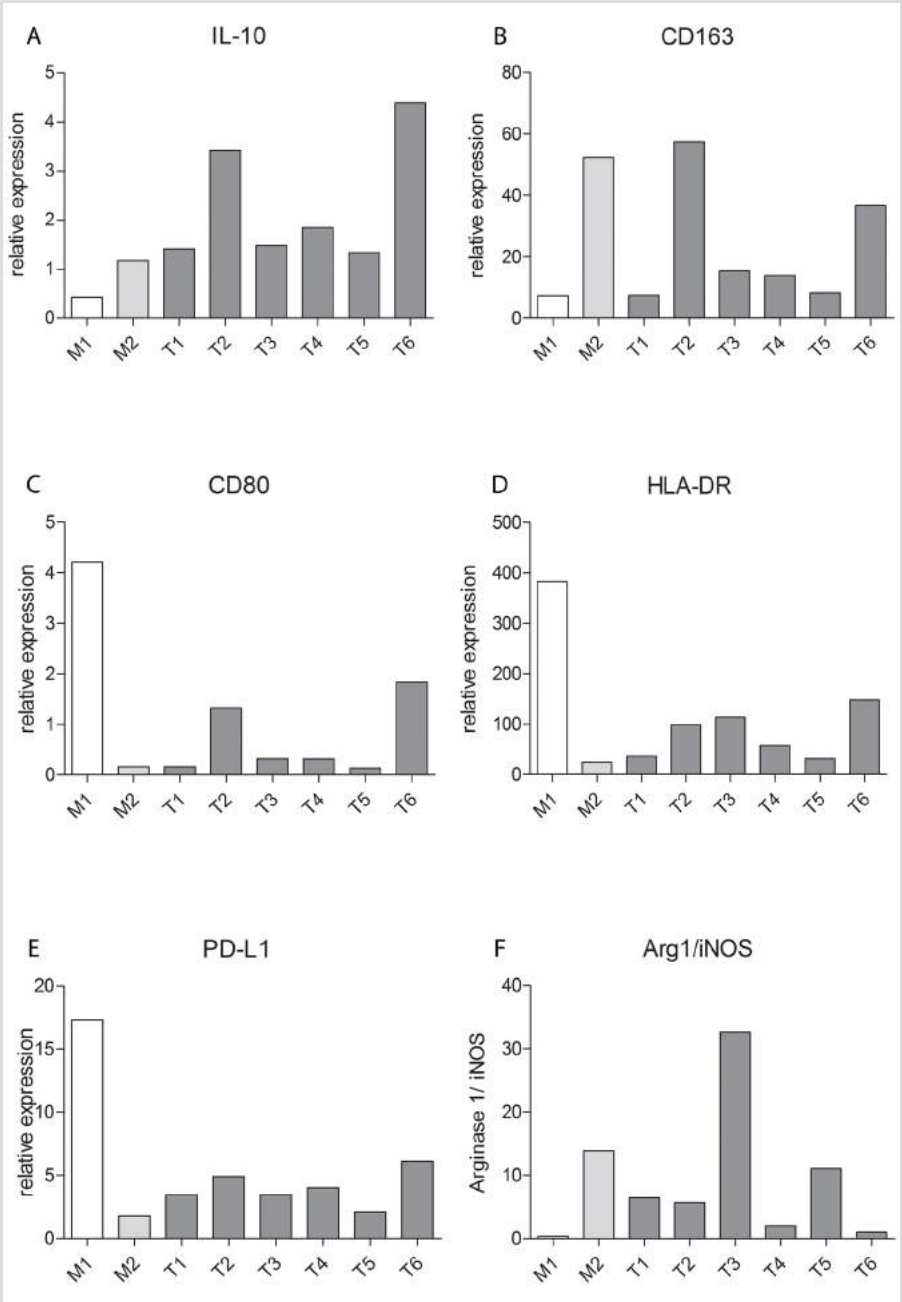

Figure 5 Tumor derived factors influence macrophages towards a M2 phenotype to varying degrees. Relative mRNA expression levels of IL-10 (a), CD163 (b), CD80 (C), HLA-DR (d), PD-L1 (e), and Arginase-1/iNOS (NOS2) ratio (f) in macrophages cultured in six mesothelioma cell line conditioned media (T1 -T6) compared to standard M1 and M2 conditions.

DISCUSSION

Macrophages in tumors are usually referred to as tumor-associated macrophages and their presence can be substantial (up to 60% of the tumor mass) [22]. A hallmark of macrophages is their plasticity, an ability to either aid or fight tumors depending on the tumor environment, which has given them the reputation of a double-edged sword in tumor biology [23]. At the extremes of this spectrum are the M1 and M2 macrophages. In an early phase of tumor development, the TAMs mainly consist of an M1-like phenotype and later in the tumorigenic process, when the tumor changes its local environment, there is a skewing toward the M2 phenotype [24–26]. Analysis of CD163/CD68 ratio in biopsy material before treatment showed a correlation with OS (combined groups: Pearson r -0.72 [$p<0.05$]; non-surgery group: Pearson r -0.91 [$p = 0.001$]; surgery group: Pearson r -0.65 [$p = 0.08$]). The total number of macrophages did not correlate with OS, indicating that the absolute number of macrophages does not influence tumor progression. The percentage of M2 macrophages of the total macrophage count was comparable between the surgery and non-surgery group and therefore, the CD163/CD68 ratio does not discriminate in favor of surgery in mesothelioma patients.

Although the terms M1 and M2 macrophages are an oversimplification of reality, it can be used to explain the opposing effects of different macrophage subsets. Our findings indeed correspond with the negative prognostic capacities of the M2 macrophages; a large proportion of these CD163 positive macrophages in the total macrophage count correlates with a decreased survival. This emphasis that the balance between M1 and M2 macrophages seems to play a crucial role in the prognosis of MPM patient.

As mentioned before, the importance of the CD163/CD68 and M1/M2 ratio is found in several other tumor types [12–17]. In our study, a similar outcome is found regarding M1/M2 ratio based on CD163/68 ratio and the prediction of survival in patients with mesothelioma. This gives a clinical correlation to the hypothesis of the anti-tumor effect of M1 TAMs and the pro-tumor effect of the M2 TAMs. To our knowledge, this is the first publication showing the importance of the CD163/CD68 ratio in mesothelioma. Furthermore, this ratio proved to be significantly correlated with survival in epithelioid mesothelioma. Previously, it was only shown that the absolute number of macrophages was prognostic in non-epithelioid mesothelioma after EPP [11].
In previous studies looking at the number of CD8 TIL's a high number of CD8 TIL was associated with a better outcome in mesothelioma patients after surgery [8(p8),9]. We could not reproduce these findings in our study. This could be due to the smaller numbers of surgical patients that were available for our study. Furthermore, the correlation between TIL count and survival was only found in patients that received chemotherapy and EPP, while in our study, P/D was performed.

The six mesothelioma cell lines showed evident heterogeneous effects on the macrophages in terms of macrophage polarization. Tumor-derived factors from cell lines induced M1 and M2 macrophage phenotypes in varying degrees, in concordance with the broad phenotype spectrum found in tumors. However, overall the tumor cell supernatants induced a more M2 prone phenotype with relatively high expression levels of IL-10 and low expression levels of M1 markers: IL-12, CD80 and HLA-DR. The standard M2 marker CD163 and the arginase1/iNOS ratio showed very differential results between the tumor cell lines. Furthermore, PD-L1 expression levels appeared to be relatively low. However, PD-L1 is known to be upregulated in a response to high IFN-γ levels as a negative feedback mechanism and therefore although PD-L1 is a co-inhibitory receptor, its presence can be indicative of an active T-cell response [27–29]. This was confirmed by the high PD-L1 level in the M1 condition. The *in vitro* experiments using tumor derived factors to influence macrophage phenotype complement the *in vivo* immunohistochemical findings by demonstrating that tumor-derived factors can directly modulate macrophage phenotype multiformity.

In addition to the impact of this finding on prognostic value of the OS of patients, macrophages may also reveal as a potential target for therapeutic intervention. Targeting the total macrophage population would not be the most optimal approach, since M1 macrophages would be decreased as well as the M2 macrophages. In an earlier trial we showed that this kind of intervention does not lead to increased survival in a murine model of mesothelioma [30]. There are several proposed strategies to counteract the M2 macrophages, including inhibiting M2 macrophage recruitment [31], M2 macrophage depletion [32] and blocking M2 tumor-promoting activity of TAMs [33]. However, since M2 macrophages remain the plasticity for polarization [34], re-polarization from M2 to M1-type could be the ideal method to tip the balance between M1 and M2 to a tumor-hostile situation. Recently, it has become clear that there is probably not one single compound that can achieve this goal [22]. A proposed strategy therefore is a combination of infusion of antibodies against CD40 in order to stimulate the secondary lymph node resident macrophages to migrate into the tumor tissue with IFN-γ to effectively reprogram tumor-induced M2-like macrophages into activated IL-12 producing M1 cells [35]. In addition, targeting the nuclear factor κB (NF-κB) signaling pathway, a crucial pathway in the activation of M2 TAMs, was shown to switch M2 TAMs to a M1 phenotype [36]. Furthermore, the combined use of Toll-like receptor 9 ligand CpG-ODN and anti-IL-10 blocking antibodies has been shown to induce the switch from M2 to M1 phenotype [37]. Also, several other therapeutic strategies are under investigation [38–41]. In mesothelioma, Fridlender et al. tested monocyte chemoattractant protein-1 (MCP-1/CCL2) blockade in a mouse model for mesothelioma and demonstrated an altered macrophage phenotype and improved survival. Currently there are no clinical compounds tested in mesothelioma patients which specifically aim at macrophage repolarization [42].

Our study has several limitations. First, the number of patients included is rather small. This is due to the fact that mesothelioma surgery in Europe is advised to be only performed in the setting of a clinical trial by the guidelines of the European Respiratory Society and the European Society of Thoracic Surgeons for the management of malignant pleural mesothelioma [43]. The results of the present trial are based on a trial randomizing patients between P/D or observation. This trial was stopped based on slow accrual. Furthermore, only patients with the epithelioid subtype of mesothelioma were selected for surgery. The trend seen in the surgery group between the CD163/CD68 ratio and OS should be confirmed in a larger patient group and we hope that our findings will encourage other researchers who have access to patients undergoing surgery to confirm the data presented in this manuscript. Second, a definitive M1 macrophage marker would enhance the findings of our manuscript for this would give a true insight in the M1/M2 macrophage ratio. NOS2 expression has proven be a useful marker for M1 macrophages in several tumor types [44–46]. However, for mesothelioma, Soini et al. and others [47,48] have demonstrated that NOS2 is highly expressed in healthy pleura as well as in cancerous mesothelioma tissues and mesothelioma cell lines. These findings complicate the use of NOS2 in pleural diseases as mesothelioma. Whether the unique capacity of mesothelial / mesothelioma tumor cells of synthesizing NOS2 is important to control a variety of infections in the pleural space in particular is unknown.

In conclusion, CD163/CD68 ratio was found to be a prognostic marker in a limited number of epithelioid mesothelioma patients, but not a predictive marker for outcome after surgery. This study emphasizes the importance of the balance between M1 and M2 macrophages in tumor behavior. In spite of not being a predictive factor for surgery in mesothelioma, we consider that the prognostic value may be of great importance in patients with mesothelioma. Repolarization of macrophages may be a new therapeutic target in mesothelioma complementing immunotherapeutic strategies.

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Chapter 3

Intratumoral macrophage phenotype and CD8+ T lymphocytes as potential tools to predict local tumor outgrowth at the intervention site in malignant pleural mesothelioma

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ABSTRACT

Objectives: In patients with malignant pleural mesothelioma (MPM), local tumor outgrowth (LTO) after invasive procedures is a well-known complication. Currently, no biomarker is available to predict the occurrence of LTO. This study aims to investigate whether the tumor macrophage infiltration and phenotype of and/or the infiltration of CD8+ T-cells predicts LTO.

Materials and Methods: Ten mesothelioma patients who developed LTO were clinically and pathologically matched with 10 non-LTO mesothelioma patients. Immunohistochemistry was performed on diagnostic biopsies to determine the total TAM (CD68), the M2 TAM (CD163) and CD8+ T-cell count (CD8).

Results: The mean M2/total TAM ratio differed between the two groups: 0.90±0.09 in the LTO group versus 0.63±0.09 in patients without LTO ($p<0.001$). In addition, the mean CD8+ T-cell count was significantly different between the two groups: 30 per 0.025 cm^2 (range 2-60) in the LTO group and 140 per 0.025 cm^2 (range 23-314) in the patients without LTO ($p<0.01$).

Conclusion: This study shows that patients who develop LTO after a local intervention have a higher M2/total TAM ratio and lower CD8+ cell count at diagnosis compared to patients who didn't develop this outgrowth. We propose that the M2/total TAM ratio and the CD8+ T-cell amount are potential tools to predict which MPM patients are prone to develop LTO.

INTRODUCTION

In patients with malignant pleural mesothelioma (MPM), local tumor outgrowth (LTO) at the intervention site of cytology or biopsy needles, chest tubes, thoracoscopy trocars or surgical incisions is a well-known complication of diagnostic and therapeutic procedures, associated with substantial morbidity [1–10]. Although this phenomenon in general is called tract metastatic disease or malignant seeding, this terminology may be misleading. The growth pattern of the 'malignant seeding' appears to be outgrowth of the primary tumor and not related to metastatic spreading of the tumor along the tract during the procedure. The reported incidence of LTO after an intervention is highly variable, with extremes ranging from 0% to 48% [1,2]. The risk of LTO is ascribed to be related to the invasiveness of the procedure and highest following thoracotomy (24%); 9–16% for thoracoscopy; and 0–22% for needle biopsy [3]. In addition, a recent study describing the occurrence of LTO after indwelling pleural catheter placement in 107 patients (60% MPM patients) showed that the duration of interval after catheter insertion was a major risk factor for development of LTO [4].

LTO lesions can be very painful and are resistant to analgesics. Surgical resection of LTO is rarely feasible and questionable, taking into account the pathophysiology of the disease. In spite of the proven, although in mesothelioma limited, effect of chemotherapy on tumor load [11], it is mostly ineffective in the treatment of these LTO sites once they have occurred[5]. A recent systematic literature review showed that there is no strong evidence to support radiotherapy in treating pain in MPM in general [12].

Whether chemotherapy prevents LTO in some patients is not known. Prophylactic irradiation of intervention track (PIT) was introduced in an attempt to prevent LTO and thus improve quality of life for these patients [13]. Three randomized controlled trials have addressed this subject showed conflicting results, which may be caused by the low incidence of LTO in the non-treatment arm [3,6–8].

The key issue for both patient care and to investigate new agents preventing LTO would be to identify patients prone for the development of LTO. We hypothesize that the development of LTO is related to immune characteristics within the tumor microenvironment. Immune cells are found to be a prognostic factor in MPM. Especially tumor infiltrating CD8+ T lymphocytes (TILs) [14,15] were described to inhibit tumor growth whilst tumor associated macrophages (TAMs) [16,17] can influence tumor growth.

Macrophages can develop towards an M1 or M2 subtype [18]. Classically activated (M1) macrophages have pro-inflammatory, tissue destructive, and anti-tumor activity. On the other hand, alternatively activated (M2) macrophages are oriented to tissue repair, tissue remodeling, and immunoregulation and therefore can be seen as pro-tumorigenic [19]. We hypothesize that M2 macrophages could play a role in the development of LTO. In contrast, M1 macrophages, together with CD8+ T lymphocytes, could be an indicator of an effective anti-tumor microenvironment, preventing LTO.

In this study we used the most widely applied T-lymphocyte subset marker and pan-macrophage marker for immunohistochemistry, CD8 and CD68; and CD163, a specific M2 scavenger receptor that is reliable for demonstrating M2 macrophages by immunohistochemistry [20–23].

The aim of this study is to investigate whether the macrophage number and phenotype or the CD8+ TIL number in the tumor microenvironment can predict the development of LTO and therefore aid to the selection of patients who could benefit from prophylactic interventions. To this end, we quantified TAM and CD8+ TIL numbers in diagnostic tumor biopsies of MPM patients who developed LTO and compared them to patients who did not develop LTO who were matched for other parameters including clinical outcome.

MATERIALS AND METHODS

Patients and specimens

Retrospectively, paraffin-embedded tumor specimens taken from the diagnostic procedures were obtained from 10 patients diagnosed with MPM between 2008 and 2012 who developed LTO (LTO+ group). LTO was defined as a clear growth of tumor mass in the tract of a previous diagnostic or therapeutic procedure while there was no evidence of pleural or metastatic disease progression. These 10 cases were matched with 10 cases with comparable age, tumor histology, diagnostic procedures, tumor treatment, and survival that did not develop LTO (LTO- group) after diagnostic or therapeutic procedures (Table 1). None of the patients did receive PIT. Histopathological diagnoses of mesothelioma were established by pathologists from our institute and confirmed by the Dutch Mesothelioma Panel (the national mesothelioma pathology board). Clinicopathological information was collected from patient charts. The TNM stage was based on CT scan and thoracoscopy report (if available) using the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) classification. Survival and treatment was recorded. Overall survival was defined as the time from the date of diagnosis to death. Because of the retrospective nature of the study protocol, no ethical institutional review board approval was necessary.

Immunohistochemistry

The following primary antibodies were used: mouse anti-human CD8 (clone C8/144B, Dako, Glostrup, Denmark), mouse anti-human CD68 (clone KP-1, Dako), and mouse anti-human CD163 (clone 10D6, Leica Biosystems Novocastra, Newcastle, UK). Paraffin-embedded tumor specimens were cut into sequential 5µm thick sections, deparaffinized and stained using a fully automated Ventana BenchMark ULTRA Stainer (Ventana, Tucson Arizona, USA) according to manufacturers' instructions at the pathology department. Binding of peroxidase-coupled anti-mouse antibodies was detected using 3,3' - diaminobenzidine as a substrate and the slides were counterstained with haematoxylin. The specificity of antibodies was checked using isotype-matched, non-relevant antibody controls.

Evaluation of slides

Amounts of CD8-positive TIL, CD68-positive TAM, and CD163-positive TAM of the M2 phenotype were independently assessed by two experienced investigators (R.C. and L.L.) and a pathologist (J-L.R) who were blinded to the patients' clinicopathological data. Three representative high-power fields (400x magnification) per slide were manually selected using a Leica DM2000 microscope (Leica Microsystems, Wetzlar, Germany). In the thoracoscopically obtained pleural biopsies, the tissue infiltrating tumor front was selected for counting of the immune cells [24]. In the two patients with only CT-guided needle biopsies, the regions with the most tumor cells were chosen for analysis. The number of cells per microscopic field

of 0.025 cm^2 with immunoreactivity to CD8, CD68 and CD163 were counted manually in three independent tumor areas. Cellular staining with a nucleus was counted as a positive cell. The same areas were used for analysis for each antibody. For each case, we defined the average value of the 3 counts in the slide as the number of TILs and TAMs. To assess interobserver reproducibility, the average of the 3 counts of the 3 observers was evaluated for comparability. If >10% difference was encountered (2 cases), J-L.R assessed the slides for final evaluation.

Statistical analysis

Mean densities of TILs and TAMs were compared between the LTO+ and LTO- group and p values were calculated with the Mann–Whitney U test. Correlations were made calculating the Spearman's rank correlation coefficient. Statistical calculations were performed using IBM SPSS Statistics 21. Statistical significance was established at the $p < 0.05$ level, and all analyses were two-sided.

RESULTS

Patient characteristics

The median age, sex, disease stage (I–IV), histological diagnosis (epithelioid, biphasic, or sarcomatoid), treatment (surgery and chemotherapy), and survival for the LTO+ and LTO- group are listed in Table 1. A representative CT scan of a patient who developed LTO is shown in Figure 1.

Table 1 Characteristics of the mesothelioma patients that developed LTO and the patients that did not develop LTO.

	LTO+	LTO-
Patients	10	10
Men	9	8
Average age at diagnosis (range)	61 (38-75)	60 (36-73)
Pathology		
Epithelial	9	9
Biphasic	1	1
Stage		
Stage I/II	6	6
Stage III/IV	4	4
Diagnostic procedures		
Pleuracentesis	4	5
CT guided biopsy	2	1
Thoracoscopy	9	9
Treatment		
platinum/pemetrexed	9	10
PR	1	1
SD	7	8
PD	1	1
second line chemotherapy treatment	1	1
experimental drug	2	1
pleurectomy/decortication	1	2
Average survival (range), months	18,9 (11-41)	19,2 (9-38)

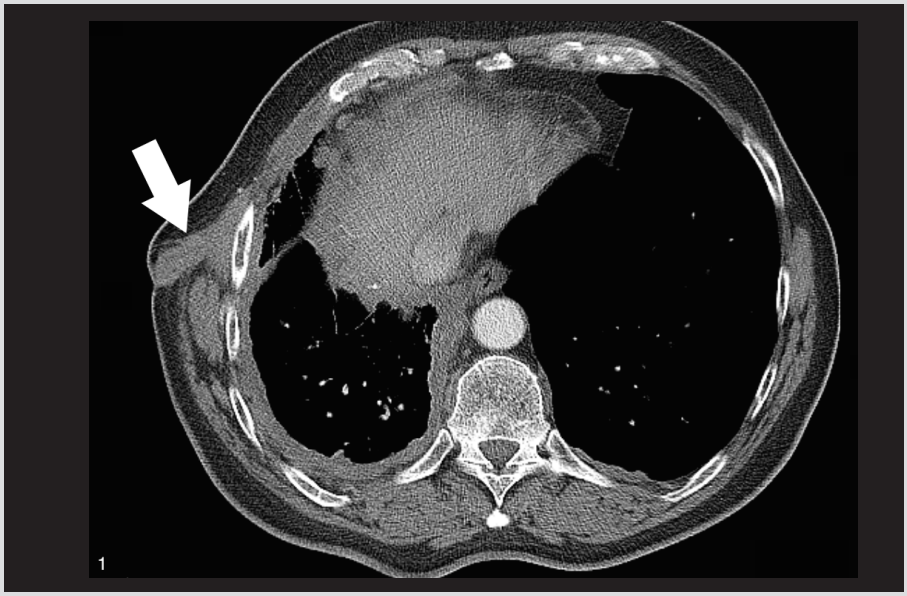

Figure 1 LTO on CT scan. CT scan of a patient with LTO after thoracoscopy on the right side. Outgrowth of tumor is seen in the thoracoscopy tract (white arrow).

Tumor-associated macrophages in MPM

Representative images of immunohistochemical staining of TAMs are shown in Figure 2a and 2b. The frequencies of CD68 (specific for all macrophages) and CD163 (specific for M2 macrophages) TAMs were comparable between the LTO+ and the LTO- group; CD68 mean 185.1/0.025 cm^2 (range 45-408) vs. 219.8/0.025 cm^2 (range 92-348) p=0.4, and CD163 mean 170.5/0.025 cm^2 (range 42-422) and 135/0.025 cm^2 (range 68-240) p=0.9. A larger proportion of CD163+ TAMs amongst the CD68+ TAMs may potentially reflect a more detrimental pro-tumor microenvironment. Therefore, we calculated the CD163/CD68 TAM (i.e. M2/total TAM) ratio for each patient in the groups with and without LTO development, as is shown in Figure 3a. The average M2/total TAM ratio in the LTO+ group was 0.9 (SD 0.09), compared with 0.63 (SD 0.09) in the LTO- group ($p<0.001$).

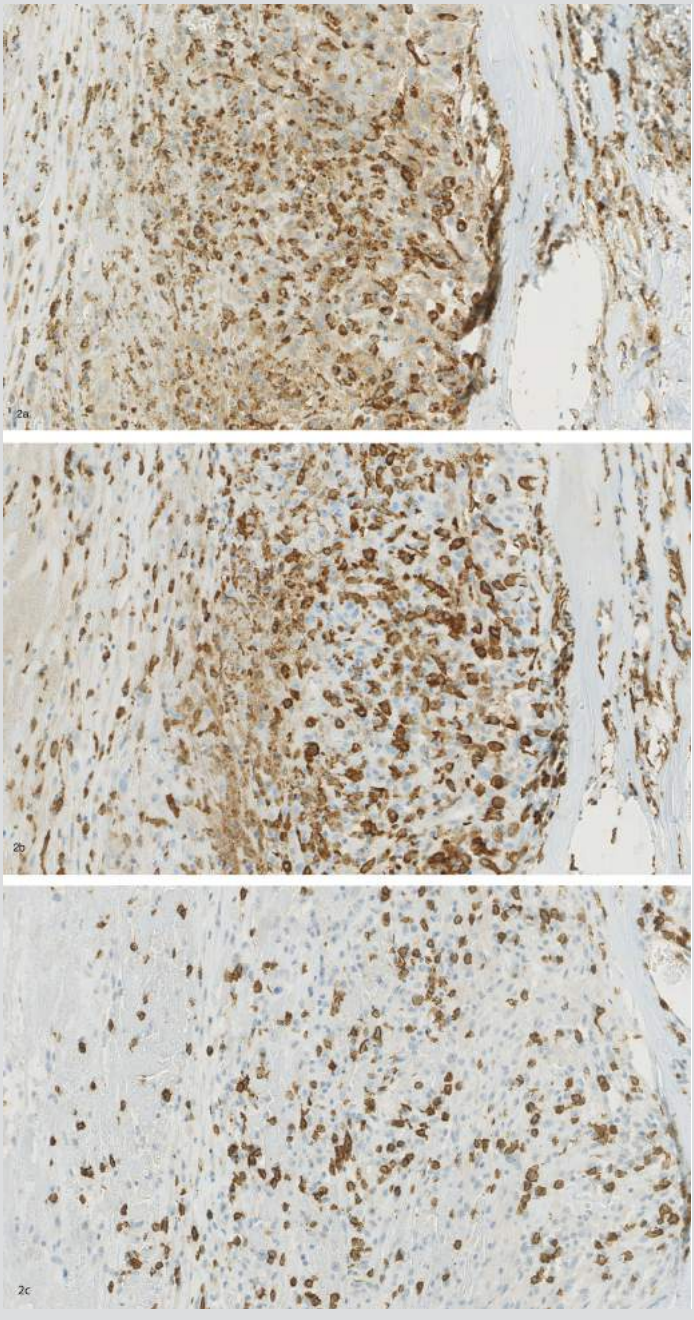

Figure 2
Immunohistochemical stainings of CD68 (2a), CD163 (2b), and CD8 (2c) in a mesothelioma tumor specimen. The brown color represents the CD68 staining (2a), the CD163 staining (2b), and the CD8 staining (2c) in mesothelioma tumor specimens (2a and 2b are in the same microscopic field). The blue colored cells are CD68, CD163, or CD8 negative cells.

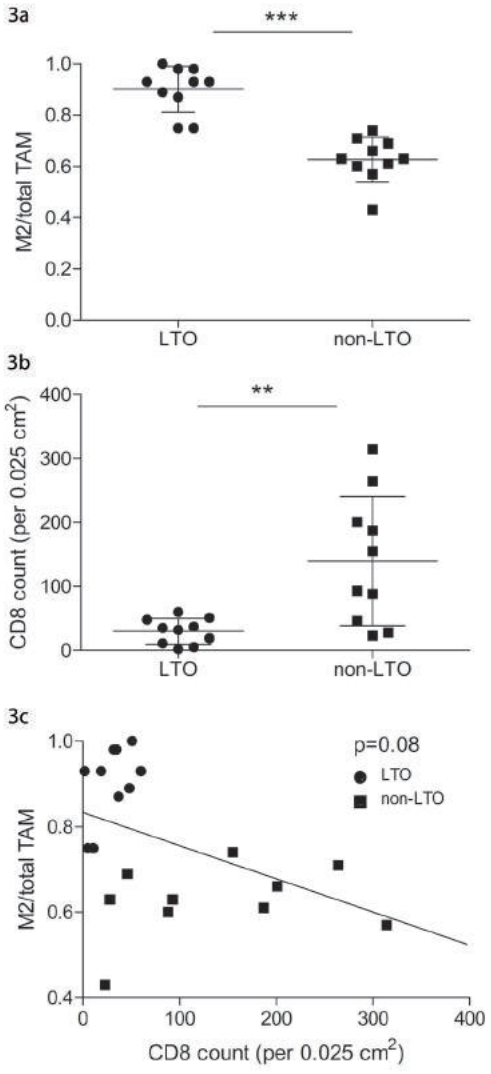

Figure 3 Increased proportions of M2 TAMs and educed numbers of CD8+ cells in diagnostic biopsies from in diagnostic biopsies of mesothelioma patients who develop LTO and correlation between M2/total macrophage ratio and CD8 lymphocyte count.

a) Ratio of CD163 positive cells (M2) and CD68 cells (all macrophages) of patients who developed local tumor outgrowth and those who did not, as determined by immunohistochemistry (N=10 for both groups) $p<0.001$, calculated by MWU test.

b) Quantification of immunohistochemical staining for CD8+ in diagnostic tumor biopsies from patients who did (LTO) or did not (non-LTO) develop local tumor outgrowth. N=10; ** $p< 0.01$.

c) Correlation between M2/total macrophage ratio and CD8 lymphocyte count. Squares are patients with local tumor outgrowth, circles without local tumor outgrowth. A near significant correlation was found between the M2/total macrophage ratio and the CD8 TIL count (Spearman's rho -0.40, p=0.08).

Tumor infiltrating lymphocytes in MPM

A representative image of an immunohistochemical staining of TILs is shown in Figure 2c. The CD8+ TIL counts are shown in Figure 3b. Patients who did not develop LTO had a higher number of CD8+ TILs (140/0.025 cm^2 (range 23-314)) compared with patients who did develop LTO (30/0.025 cm^2 (range 2-60))($p<0.01$).

CD8 and M2/total TAM ratio

The correlation between the CD8 TIL count and the M2/total TAM ratio is shown in Figure 3c. Although not statistically significant with a p-value of 0.08 (Spearman's rho -0.40), all patients who developed LTO were clustered in the area representing a high M2/total TAM ratio and a low CD8+ TIL count.

DISCUSSION

Within the tumor microenvironment, interactions among tumor cells, immune cells, stromal cells, endothelial cells, and the extracellular matrix are vital to tumor progression. MPM tumors contain a varying amount of intratumoral leukocytes [25]. An improved overall survival in patients with MPM tumors that contained a high number of CD8+ TILs was recently shown [26]. In addition to TILs present in the MPM tumor micro-environment, macrophage infiltration in MPM was shown by our group [27], and its prognostic role was also published [16]. The symbiotic relation between tumor cells and M2 TAMs has been extensively studied in the last decade [18,19,28,29].

In the current study we demonstrated the percentage of M2 TAMs of the total TAM count in diagnostic biopsies to be significantly higher in MPM patients who developed LTO after an invasive procedure and a significantly lower CD8+ TIL count was also found in patients who developed LTO. Although patient numbers were relatively low, this is the first time to our knowledge that the composition of the tumor microenvironment is investigated for its potential use to predict the occurrence of LTO in MPM patients after a diagnostic or therapeutic procedure and the first study showing possible markers for the prediction of the occurrence of LTO in mesothelioma. The total macrophage or M2 numbers did not differ between the two groups, indicating that the phenotype, rather than the total number of macrophages is important in LTO. This finding correlates with our earlier finding that the ratio of M2 macrophages of the total TAM count correlates with survival in epithelial mesothelioma [30].

When macrophages reach the tumor, they can be polarized to a continuum of phenotypes with the M1 or M2 phenotype at the ends of the spectrum [18,31]. In the presence of M2 polarizing cytokines and the absence of signals that give preferential polarization to a M1 TAM they polarize towards M2 [32]. With this increase in M2 of the total macrophage population, several M2-derived cytokines involved in the breakdown of extracellular matrix are increasingly released (for example VEGF and matrix metalloproteinase 9), which may aid to the process of the development of local outgrowth after an invasive procedure [33,34]. Vice versa, a more M1 TAM oriented microenvironment is more capable to suppress tumor growth by the production of e.g. tumor necrosis factor alpha, interleukin 12 and the interaction with other anti-tumor immune cells like cytotoxic T-cells. As stated earlier, CD8+ T-cells are capable of killing tumor cells directly via e.g. the production of perforin and granzymes. Therefore, in concordance with a more M1 TAM oriented microenvironment, in a tumor microenvironment where CD8+ TILs are abundantly present, LTO might be directly suppressed after an intervention.

When combining the M2/total TAM ratio and the CD8+ TIL count, our results suggest an interesting potential relationship between these cells. Although only a trend was seen and thus speculative; the diagnostic biopsies of patients who developed LTO showed the combination of a high M2/total TAM ratio and a low CD8+ TIL count compared to the non-LTO

group. Comparable results were found earlier in other tumor types [35–37]. These results point towards a complex interplay within the entire tumor microenvironment. Macrophages and T-lymphocytes are known to be able to cross-regulate each other's function and phenotype via multiple pathways [38], e.g. M2 macrophages are able to directly induce regulatory T-cells, resulting in suppression of tumor-specific cytotoxic T-cells function and number[39]. The interactions between macrophages and T-cells in the tumor microenvironment of mesothelioma patients will be subject of future studies.

Our study has several limitations. First, we could only test our hypothesis on a limited number of patients. Nevertheless, we show a statistically significant result in the M2/total TAM ratio and CD8+ TIL count between the LTO+ and the LTO- group and therefore this should be regarded as a preliminary method of predicting LTO.

Secondly, the immune cells that were determined in our study have been correlated to survival in previous studies [26,30]. While the patients in our study were matched for survival, future studies are needed to assess the magnitude of effect of these immune cells on both survival and occurrence of LTO.

Third, in this study we used single staining immunohistochemistry to identify the infiltration of TAMs and CD8+ T-cells in mesothelioma biopsies. Ideally, additional markers would be useful to identify M2 macrophages in more detail; however, other single immunohistochemical markers as CD206 are equivalent to CD163 or still subject of debate. Immunohistochemical staining using CD68 and CD163 to characterize TAMs and CD8 for T cell subsets has been demonstrated useful in numerous studies [20,21(p163),22–24,40]. Furthermore, immunohistochemistry is a relatively easy technique that allows characterization of the tumor microenvironment that would be feasible in a broad clinical setting. However, further studies will be necessary to validate this approach in a larger patient cohort and to establish proper cut-off values.

CONCLUSIONS

The macrophage phenotype ratio and CD8+ TIL count in diagnostic biopsies provides an opportunity to predict which MPM patients are prone to develop LTO after a local intervention. The M2/total TAM ratio and CD8+ TIL count showed a significant difference between the group that developed LTO at a later stage and the group that didn't. The presence of these intra-tumoral immune cells identifies patients who could benefit from prophylactic interventions (e.g. in a study of testing PIT). In addition, this study indicates that targeting M2 TAM function or enhancing CD8+ TILs activity are potential strategies to prevent LTO in malignant pleural mesothelioma.

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Chapter 4

Pleural effusion of patients with malignant mesothelioma induces macrophage-mediated T cell suppression

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ABSTRACT

Introduction: Clinical studies have demonstrated beneficial effects of immunotherapy in malignant pleural mesothelioma. The pleural cavity seems an attractive compartment to administer these types of therapies, however local immunosuppressive mechanisms could hamper their efficacy. Macrophages are abundantly present within the mesothelioma microenvironment. This study investigates the influence of the macrophage phenotype and their capacity to inhibit local immune responses and the decisive role of pleural effusion (PE) in this regard.

Methods: We cultured macrophages in the presence of PEs and investigated their phenotype. Macrophages and T cells were co-cultured in the presence of PEs and tumor cell line supernatants. The levels of 11 cytokines and the prostanoid prostaglandin E2 (PGE2) were measured in PEs and supernatants. The presence and phenotype of macrophages and T cell subsets was measured in the PE of mesothelioma patients.

Results: PE induced a tumor promoting M2 phenotype in macrophages, which was confirmed by the suppressive activity of macrophages on T cell proliferation during co-culture. PGE2 was identified as a potential inducer of the suppressive capacity of macrophages in PE. Macrophages isolated from PEs displayed an M2 phenotype and were negatively correlated with T cells in vivo.

Conclusions: The current study demonstrates that macrophages in PE can play a pivotal role in directly hampering the anti-tumor T cell immune response. This emphasizes the potential of macrophages as a therapeutic target in mesothelioma and indicates that the presence and phenotype of macrophages in PE should be taken into consideration in the application of (intrapleural) immunotherapies.

Key words: mesothelioma, pleural cavity, tumor-associated macrophages, immunosuppression, microenvironment

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a highly aggressive cancer with currently limited treatment options. The immune system is considered to play a major role in the pathogenesis, prognosis and, potentially, in the treatment of this devastating disease [1-6].

Despite encouraging results of immunotherapeutic approaches, responses are hampered by local and systemic immunosuppressive mechanisms [7,8]. Therefore, attention is focusing on the cellular and molecular mechanisms, which play a role in the immunosuppressive tumor environment. The pleural cavity is a convenient compartment to administer different treatment modalities in close approximation to the tumor and potentially minimizing systemic toxicities. Different intrapleural treatment options are now under investigation [9]. Pleural effusion (PE) accompanies mesothelioma in approximately 70% of the cases, predominantly in the epithelioid subtype [10]. PE consists of tumor cells and numerous types of immune cells and stromal cells[11, 12]. Immune cells invade both the tumor and PE of MPM patients [13-15]. These infiltrating immune cells can exert either beneficial or detrimental effects, depending on their phenotype [16]. Tumor-associated macrophages (TAMs) are a major component of the immune cell infiltration of the tumor microenvironment in mesothelioma patients [17]. Under the influence of various stimuli within the tumor microenvironment, TAMs can develop into a tumor-inhibitory (M1) or tumor-promoting (M2) phenotype [18, 19]. Others and we have found that the presence and M2 phenotype of TAMs in MPM tumor biopsies was related to a worse survival [20, 21].

Given the close proximity between PE and the pleural tumor, the pleural space is a pivotal part of the tumor environment in MPM and characterization of the local immunosuppressive mechanisms is essential to improve (local) immunotherapeutic approaches. The aim of the present study is to investigate the immunosuppressive properties of PE and its effect on the phenotype and function of macrophages.

MATERIALS AND METHODS

Collection and processing of pleural effusions

Thoracocentesis was performed using fine-needle aspiration inserted into the pleural cavity and the pleural effusion was collected in sterile containers without anticoagulant. Pleural cells were pelleted from PE and ficoll density gradient centrifugation was applied to separate the red blood cells from the leucocytes as previously described [22]. Six PE supernatants were selected for the *in vitro* experiments because accompanying long-term MPM cell lines were established from the cellular fractions of these PEs [13]. In order to obtain MPM cell lines, the original cellular fractions of the PEs were cultured in culture medium (RPMI) supplemented with 10% normal human serum. Cell line supernatants (SN) were collected around passage number 70 for all cell lines at 80% confluency. Supernatants were collected during passaging and centrifuged at 1200G during 10 minutes before use.

Isolation of healthy monocytes and T cells

Peripheral blood mononuclear cells (PBMC) were isolated from a buffy coat of a healthy donor (Sanquin, Amsterdam, The Netherlands) using ficoll density gradient centrifugation [22]. Monocytes and T cells were isolated with MACS® separation using a Monocyte Isolation Kit followed by a Pan T cell Isolation Kit (cat. no. 130-050-201 and 130-096-535, all Miltenyi Biotec). Purity of the isolated fractions was confirmed using flow cytometry (>97% pure, data not shown).

Macrophage cultures

For all conditions, normal monocytes from a healthy donor were differentiated to macrophages during a 6-day culture in the presence of 10% normal healthy AB serum and M-CSF (20ng/ml, R&D systems, cat.no. 216-MC) in RPMI-1640 medium containing GlutaMAX (GIBCO, ThermoFisher, cat.no. 61870-010). Subsequent polarization to the M1 or M2 phenotype occurred in the presence of lipopolysaccharide (LPS) (100 ng/ml, Sigma-Aldrich, cat. no. L3012) and IFN-γ (20 ng/ml, R&D systems, cat.no. 285-IF) for M1 or IL-10 (40ng/ml, R&D systems, cat.no. 217-IL) for M2 during 2 days. For the PE conditions, the differentiated macrophages were subsequently cultured during 2 days in the presence of 10% PE supernatant.

Gene expression analysis

Gene expression analysis of selected genes was performed on the macrophages after 8 days of culture as described earlier [21]. Specificity of the amplification product was confirmed by examination of dissociation curves. Expression levels were normalized to the internal control β-actin. The primer sequences are depicted in Table 1, Supplemental Data.

T cell co-culture and proliferation assay

Monocytes were seeded at 5.10^4 cells in wells of a 96-well plate and differentiated to macrophages during 6 days as described earlier. T cells isolated from the same healthy donor were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, ThermoFisher cat.no. C34554) as previously described [22]. The CFSE labeled T cells were co-cultured in a 1:1 ratio with the differentiated macrophages and stimulated using anti-CD3/anti-CD28 beads (Dynabeads® Invitrogen, ThermoFisher cat.no. 11131D) during 4 days. T cells and macrophages were co-cultured during 4 days in 10% normal healthy AB serum, 10% PE supernatants (PE, n=6) or 30% MPM cell line supernatants (SN, n=6). Cell division was quantified based on serial halving of CFSE intensity, algorithms provided by FlowJo software (Treestar) were used. Proliferation percentages were calculated as percentage of T cells recruited into cell division, as previously described [23].

Cytokine measurements

The levels of 12 cytokines were measured by a magnetic bead-based multiplex assay in the six PE supernatants and accompanying MPM cell line supernatants used for the *in vitro* experiments (11-plex and single plex (transforming growth factor beta (TGF-α)) Bio-Plex Pro™ Magnetic Cytokine Assay, Bio-Rad, specific 11-plex no longer available, TGF-α cat.no. 171V4001M). PGE2 levels were measured using a PGE2 Parameter Assay Kit (R&D systems, cat.no. KGE004B). MPM cell line supernatants were harvested at 80% confluency in all cell lines. The PE supernatants and MPM cell line supernatants were depicted undiluted in the cytokines measurements.

Subjects

MPM patients were selected from the patient databank which was set up for our immunotherapy trials [3]. The study was approved by the institutional Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands (NL24050.000.08). Thirty patients prior to treatment, whose diagnosis was confirmed by the Dutch National Mesothelioma Panel, were included in this study based on the availability of stored pleural effusions and accompanying viable cellular fractions. Clinical data were retrieved retrospectively. Patient survival was defined as the time between diagnosis and death.

Flow cytometry

Cryopreserved cellular fractions isolated from pleural effusions were defrosted and stained with two marker sets to identify different lymphoid-subsets and myeloid-subsets. The monoclonal antibodies used for flow cytometry are depicted in Table 2, Supplemental Data. All samples were stained with a Live/Dead® Fixable Aqua dead cell stain in Amcyan (Invitrogen, ThermoFisher cat.no. L34957). The analysis was performed using FlowJo software (Tree Star Inc.).

Statistical analysis

Data are expressed as mean ± standard deviation. Paired data were compared using the paired Wilcoxon rank test. Correlations were made calculating the Spearman's rank correlation coefficient. Statistical significance was established at the $p < 0.05$ level, and all analyses were two-sided. All statistical analyses were performed using IBM SPSS Statistics 21.

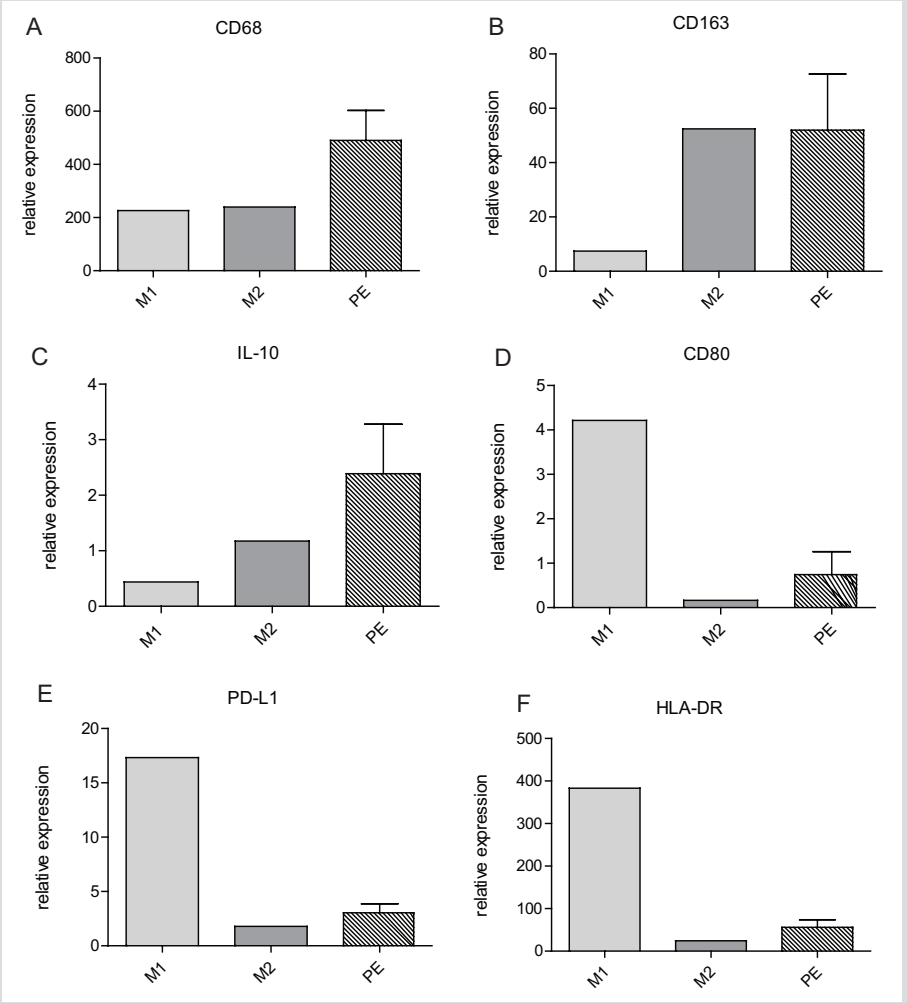

Figure 1 Expression of signature macrophage phenotype-related genes after culture under standard M1 or M2 condition or in the presence of PE supernatant (n=6). Panel A shows the gene expression of the general macrophage marker CD68, panel B and C show the expression of the specific M2 markers scavenger receptor CD163 and IL-10. Panel D shows the expression of the activation marker characteristic for the M1 phenotype CD80, panel E shows the expression of the activation marker PD-L1 and panel F shows the expression of the pro-inflammatory marker HLA-DR. Expression levels are calculated relative to the housekeeping gene β-actin.

RESULTS

Pleural effusions polarize monocytes towards a M2 macrophage phenotype

The influence of PE supernatants on the phenotype of monocyte-derived macrophages was investigated *in vitro*. Standard M1 or M2 polarizing culture conditions were used as controls. Gene expression of the standard macrophage marker CD68 was used to confirm proper macrophage maturation (Figure 1A). Overall, PE supernatants induced gene expression of M2 markers with a typical high gene expression of scavenger receptor CD163 (Figure 1B) and IL-10 (Figure 1C) and low gene expression of the activation markers CD80 (Figure 1D), PD-L1 (Figure 1E) and the typical pro-inflammatory marker HLA-DR (Figure 1F).

Macrophages suppress T cell proliferation only in the presence of pleural effusions

To investigate the immunosuppressive effect of PE on macrophages we performed macrophage – T cell co-cultures. In addition, we used corresponding MPM cell lines supernatants (SN) as a directly tumor cell derived reference. These MPM cell lines were originally established from the cellular fractions of the PEs used in this assay [13]. We co-cultured PE (10%, n=6) and SN (30%, n=6) polarized macrophages with autologous anti-CD3/anti-CD28 stimulated CFSE-labeled T cells. After 96 hours, T cells and macrophages were harvested and T cell proliferation was calculated. The co-culture performed in the presence of 10% normal human AB serum was used as a control. Figure 2A demonstrates that macrophages significantly reduce the proliferation of T cells in the presence of PE (n=6, p=0.03). This was in contrast with macrophages cultured in the presence of MPM cell line SN, in which co-culture with macrophages enhanced T cell proliferation. The results were comparable for both CD4 and CD8 T cells (data not shown). These results indicate that PE induces a functional immunosuppressive phenotype in macrophages which cannot be directly attributed to tumor cell derived factors. Figure 2B-F demonstrate representative flow cytometry histograms of each condition.

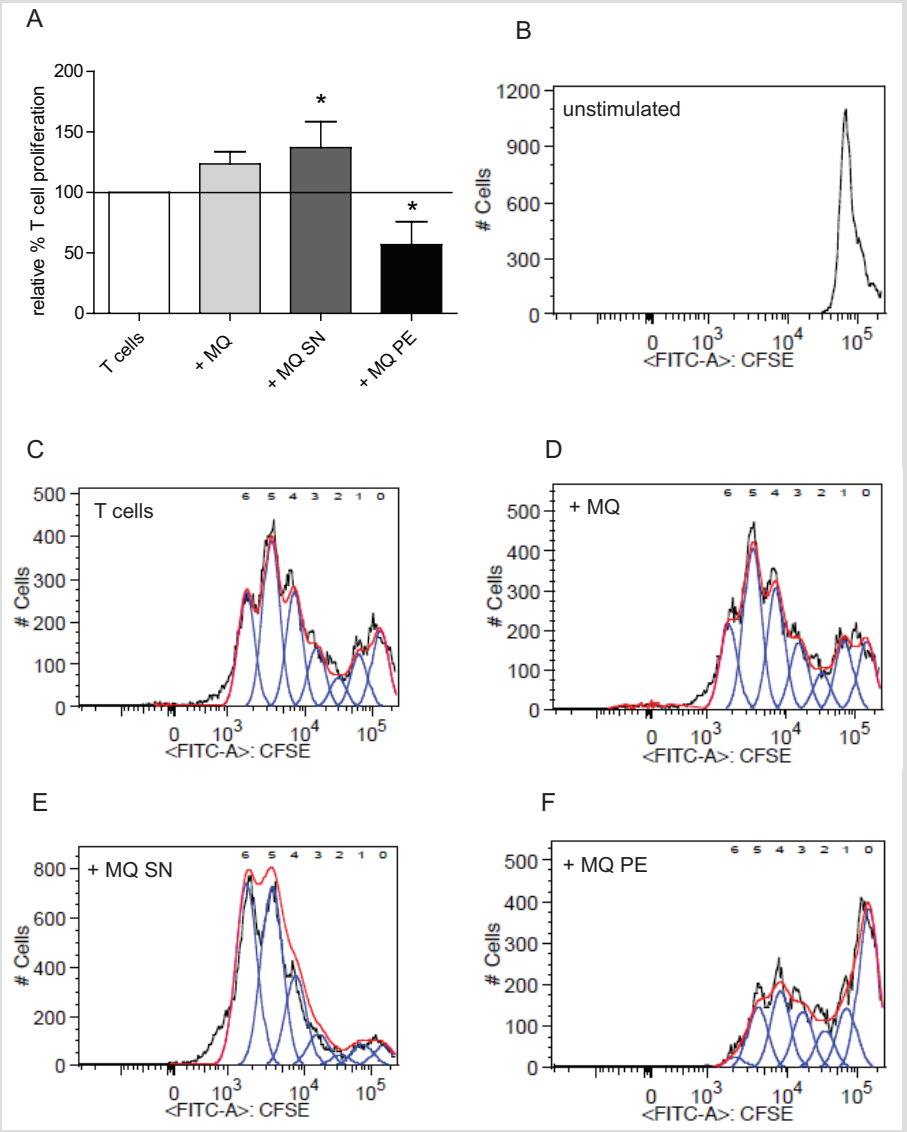

Figure 2 Macrophages suppress T cell proliferation in the presence of PE. The percentage proliferation of only T cells (control, in MPM cell line supernatant (SN) or in PE) was set at 100% and the T cell proliferation during co-culture with macrophages was calculated relative to the basic proliferation of only T cells (Panel A). Co-culture with macrophages under control conditions (n=3, 10% NHS) did not show a statistically significant increase or decrease in T cell proliferation (Panel A). Co-culture with macrophages in the presence of SN demonstrated an increase in T cell proliferation (n=6, $p<0.05$, Panel A). Co-culture with macrophages in the presence of PE induced a suppression of T cell proliferation (n=6, $p<0.05$, paired Wilcoxon test, Panel A). Panel B-F demonstrate representative flow cytometry histograms of the following culture conditions: unstimulated T cell control (Panel B), stimulated T cells (Panel C), stimulated T cells + macrophages control (Panel D), stimulated T cells + macrophages SN (Panel E) and stimulated T cells + macrophages PE (Panel F).

PE-cultured macrophages have a robust suppressive phenotype

In order to investigate the robustness of the PE-induced suppressive effect of macrophages on T cell proliferation we attempted to rescue the T cell proliferation *in vitro* (see Figure 1, Supplemental Data). As macrophages are known for their potential to metabolize pivotal nutrients, e.g. tryptophan [24], we added 10% NHS to the 10% PE culture condition to investigate whether addition of essential amino acids could enhance the T cell proliferation. However, addition of NHS did not result in an increase of T cell proliferation indicating that depletion of nutrients doesn't play a role in the observed suppressive macrophage phenotype. In addition, reduction of the PE concentration to 5% did not result in a recovery of T cell proliferation. Furthermore, the addition of IFN-γand LPS (M1 culture condition) to PE did not result in a recovery of T cell proliferation. These data combined illustrate the vigor of the suppressive macrophage phenotype induced by PE.

PGE2 is a potential mediator of suppressive macrophage function in PE

Given the clear functional difference between macrophages cultured in the presence of PE or tumor cell SN we investigated the presence and level of selected cytokines and the prostanoid prostaglandin E2 (PGE2) with known potential to induce a M1 or M2 macrophage phenotype [25]. We performed a magnetic bead-based multiplex assay and ELISA on the 6 PEs and SNs used for the macrophage cultures (Figure 3). In short, these data show a distinct similarity in cytokine profile between tumor cell line SNs and the PEs from which these tumor cells were originally derived. IL-6 and TGF-α, both associated with a M2 phenotype of TAMs [26], were at the highest level amongst the measured cytokines in the PEs and SNs. In addition, the pleiotropic cytokine VEGF was measured at relatively high levels. IL-12, TNF-α and IFN-γ are associated with a M1 phenotype skewed milieu. IFN-γ was undetectable and TNF-α could only be measured at low levels, but a relatively high concentration of IL-12 was found. The classical type 2 immune response cytokines IL-10, IL-13 and IL-4 could all be detected in PE at relatively low levels. Although the MPM cell lines were capable of producing IL-17, this cytokine was not detected in the PEs.

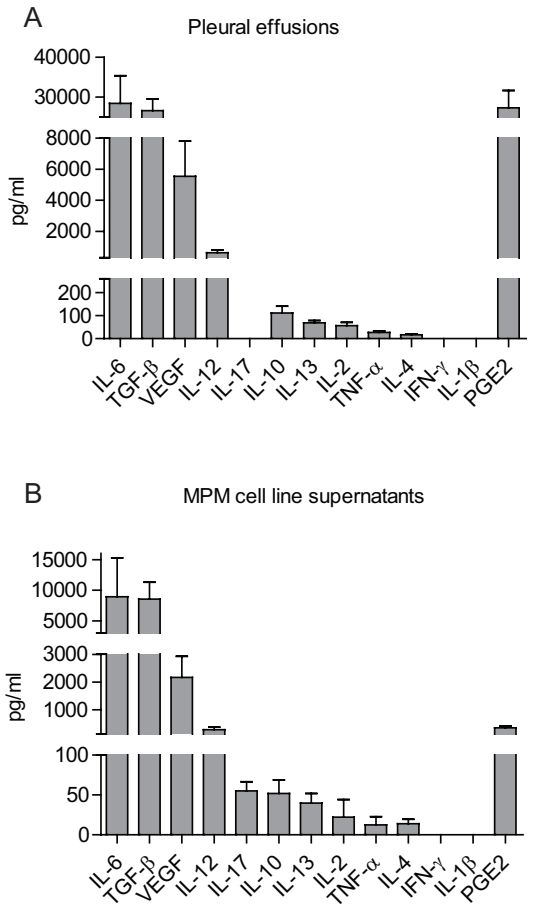

Figure 3 Concentrations of selected cytokines in PE supernatants and corresponding MPM cell lines supernatants (both n=6). Cytokine levels were measured using a magnetic bead-based multiplex assay. The prostanoid PGE2 was measured using an ELISA. The depicted concentrations of PE supernatants and MPM cell line supernatants are undiluted.

The measured cytokines in PE are partially or directly tumor cell-derived as the cytokines in supernatants of 6 corresponding MPM cell lines show a similar pattern compared to the PEs from which the MPM cell lines were developed. Given the marked difference in functional suppressive capacity, these cytokines are not likely to play a distinctive role in the suppressive effect of PE. In general, the measured cytokines were present at an approximately 3-fold increased level in PE compared to the SN. In contrast, PGE2 was present at a 80-fold higher level in PE compared to the SN. In addition, PGE2 levels in PE corresponded with the macrophage-induced T cell suppression (see Figure 2, Supplementary Data), which could not

be observed for any of the measured cytokines (data not shown). Furthermore, addition of synthetic PGE2 to the co-culture in the presence of MPM supernatant (PGE2 concentration 0.3 ng/ml) at the average concentration that was measured in the PEs (30 ng/ml) resulted in a 50.4% reduction of T cell proliferation (see Figure 2, Supplementary Data). These data indicate that PGE2 plays a role in the observed induction of an immunosuppressive phenotype of macrophages by PE.

Patient characteristics

In order to investigate the *in vivo* relevance of the previous *in vitro* findings, the presence and phenotype of TAMs and T cells was investigated in the PE of 30 MPM patients prior to treatment or pleurodesis. Patient characteristics are described in Table 1. The mean survival of the patients after diagnosis was 13.4 months (±7.5 months). The majority of the patients were male and most tumors were of the epithelioid type. All patients presented with considerable amounts of PE.

Patient characteristics

Age (mean ± SD)	68.4 ± 8.0	30 (100)
Sex	Male	29 (96.7)
	Female	1 (3.3)
Survival (months, mean ± SD)	13.4 ± 7.8	30 (100)
Histology	Epithelioid	26 (86.7)
	Sarcomatoid	2 (6.7)
	Biphasic	2 (6.7)
Pleural effusion		
Volume (ml, mean ± SD)	1353 ± 600	30 (100)

Macrophages and T cells in PE of MPM patients

Using flow cytometry, the presence and phenotype of T cell and TAM subsets was investigated in the PE of 30 MPM patients. TAM phenotype was determined according to the expression of CD163 (scavenger receptor, M2 marker) and/or CD206 (mannose receptor, M2 marker). Figure 3 of the Supplemental Data shows an example of the flow cytometric analysis of TAMs. The majority of the TAMs in PE expressed either CD206 or both CD206 and CD163. Because both markers are frequently used as M2 markers and macrophage marker expression is known to be heterogeneous, we classified TAMs that express either marker or both as M2. With a mean of 46.8% (±32.2%) of total alive cells, TAMs were the

most prevalent of the measured immune cells in PE of MPM patients, however the inter-patient variation was considerable (Figure 4A). In addition, T cell subsets were detected with clear patient-to-patient variability; in general, CD4 T cells were more prevalent than CD8 T cells (mean CD3 T cells 26.7±27.9%, CD4 T cells 15.4±18.8%, CD8 T cells 8.5±8.8%). Furthermore, the presence of regulatory T cells (Tregs) in PE of MPM patients was confirmed (mean 6.7±7.4% of CD4 T cells) [13,27].

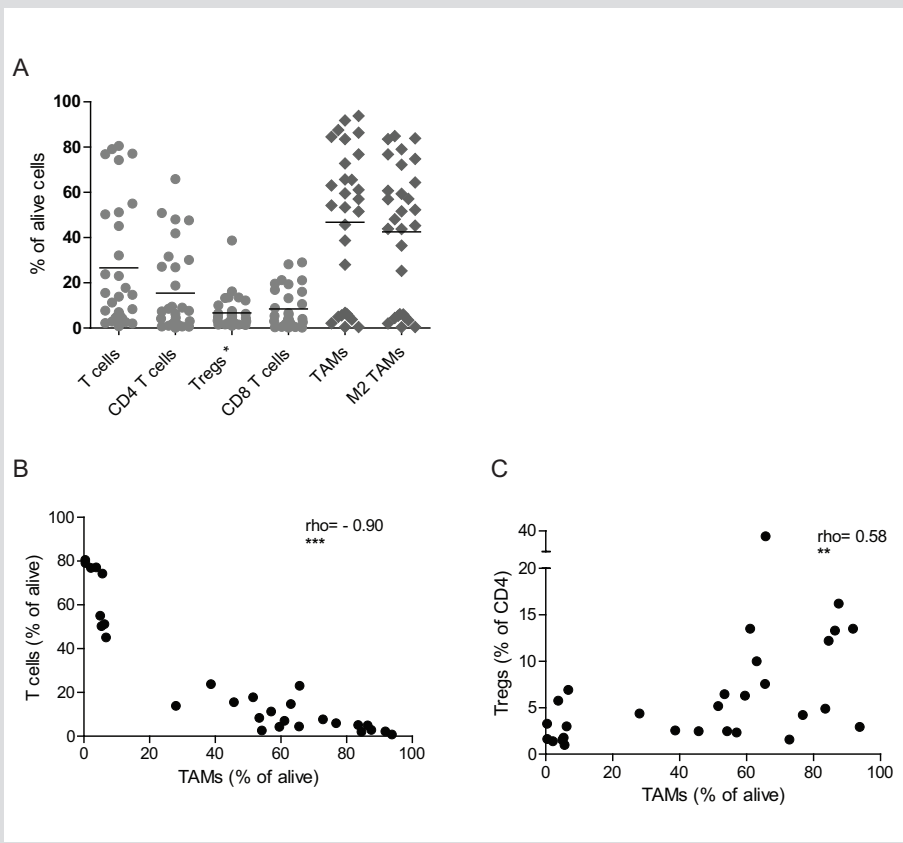

Figure 4 T cell subsets and TAMs in PE of 30 MPM patients.
Tregs are depicted as percentage of CD4 T cells. All other cell populations are depicted as percentages of total alive cells (Panel A). Tregs were classified as CD4+CD25+CD127-FoxP3+ cells. TAMs are CD45+CD14+CD68+, M2TAMs are CD206+, CD163+ or CD206+CD163+ TAMs. Panel B shows the correlation between all TAMs and T cells in the PE of MPM patients. T cells are all CD3+ cells, TAMs are all CD45+CD14+CD68+ cells. Spearman's rho -0.90, $p<0.001$. Panel C shows the correlation between all TAMs and Tregs, calculated as a percentage of CD3+CD4+ positive cells. Tregs were classified as CD4+CD25+CD127-FoxP3+ cells.

Based on the *in vitro* suppressive effect of macrophages on T cells in the presence of PE, we investigated the correlation between these cell types in the PE of 30 MPM patients. We found a negative correlation between TAMs and all T cells in these PEs (Figure 4B, rho -0.90, $p<0.001$), both CD4 T cells and CD8 T cells contributed to this correlation (rho -0.89, $p<0.001$ and rho -0.85, $p<0.001$ respectively). Because we confirmed that TAMs in PE are mainly of the M2 phenotype, the same correlations could be found when they are calculated with M2 TAMs instead of TAMs. Furthermore, Tregs (% of CD4) showed a positive correlation with TAMs (Figure 4C, rho 0.58, $p<0.01$), indicating the co-regulation of immunosuppressive cell types. In order to confirm the specificity of the correlation between TAMs and T cells, total B cells (CD19+) were also measured in the 30 PEs (mean 7.9±7.8 % of alive cells). There was no significant correlation found between B cells and TAMs (rho -0.06, $p=0.75$). Furthermore, PGE2 levels were measured in the 30 PEs but no correlation could be found between the PGE2 concentration and the TAMs or T cells present in the PEs (data not shown), indicating the multifactorial etiology of cellular trafficking in the pleural space.

DISCUSSION

In the present study, we investigated the immunosuppressive properties of PE from MPM patients and its influence on macrophage phenotype and interaction with T cells *in vitro* and *in vivo*. We demonstrated that in PE, cytokines associated with an immunosuppressive environment are abundantly present. Macrophages cultured in the presence of PE from MPM patients exhibited a M2 phenotype and suppressive function *in vitro*. PGE2 was identified as a potential responsible factor for the induction of the suppressive macrophage phenotype in PE. Furthermore, we confirmed that TAMs in PE of MPM patients are predominantly of the M2 phenotype and show a negative correlation with T cells *in vivo*.

It should be appreciated that the results obtained in this study are restricted to the pleural effusion and cannot be regarded as similar to the tumor microenvironment itself. From our data, it cannot be concluded that tumor-infiltrating macrophages have similar effects. We hypothesize, as can be derived from our data, that the pleural effusion itself may be one of the immunosuppressive mechanisms induced by the tumor in a complex way.

We have demonstrated earlier that MPM cell line supernatants are capable of inducing macrophages with the M2 phenotype [21]. The current study showed that the PEs, which were the original source of the MPM cell lines, induce a much stronger functional M2 phenotype to suppress T cell proliferation. In addition, both MPM cell line supernatants and PEs contained comparable patterns of a selected number of cytokines, suggesting that these cytokines could be tumor cell derived. However, the functional properties of the macrophages cultured in the presence of MPM cell line supernatants or PEs differed substantially. Macrophages cultured in the presence of PE suppressed T cell proliferation whereas MPM cell line conditioned media did not exert this effect. The presence of some of the measured cytokines, e.g. IL-6 and TGF-β, confirms the immunosuppressive character of the intrapleural compartment because they are known for their M2-skewing effect and association with a worse clinical outcome [25, 28-31]. However, our results indicate that these cytokines are not responsible for the suppressive function of macrophages in the current study. In contrast, we identified the prostanoid PGE2 as a potential inducer of the suppressive effect of PE on macrophages. PGE2 is known to be a potent regulator of immune responses and especially suppresses type 1 immunity, which has made PGE2 a popular target of interest in cancer immunotherapy [32, 33]. We have shown earlier that inhibition of the PGE2 synthesizing enzyme COX-2 resulted in an improved effect of dendritic cell immunotherapy in a mesothelioma mouse model [34]. This beneficial effect was ascribed to the reduction of MDSC levels. In addition, Zelenay et al. recently demonstrated that PGE2-dependent suppression of myeloid cell activation is an important mechanism of tumor immune escape [35].

Our current results demonstrate that PGE2 is present at high levels in the pleural effusion of MPM patients and could play a role in the immunosuppressive environment of this compartment through induction of a suppressive macrophage phenotype. Although PGE2

is a known inducer of the immunosuppressive phenotype in macrophages, there are other soluble factors present in the pleural space (e.g. M-CSF and CCL2), which can regulate the recruitment of monocytes and subsequent differentiation to macrophages. After maturation, we hypothesize that PGE2 produced by MPM cells is an important factor to induce the immunosuppressive phenotype which results in an autocrine positive feedback loop as macrophages themselves are also capable of PGE2 production. This mechanism provides additional rationale for the implementation of COX-2 inhibitors in tumors with known high levels of PGE2 and M2 macrophages in their microenvironment.

By demonstrating a negative correlation between TAMs and T cells in PE of MPM patients we have provided an indication that the *in vitro* suppressive effect of macrophages in the presence of PE also plays a role *in vivo*. The positive correlation between TAMs and Tregs further illustrates the immunosuppressive environment of PE in MPM patients. In this paper, we classified patient-derived TAMs that expressed either CD163 and/or CD206 as M2 macrophages. Although expression of these markers does not demonstrate any functional properties, CD163 and CD206 are widely used as pivotal human M2 markers [36].

Recently, Scherpereel *et al* showed that there is a defect in the recruitment of CD8 T cells in malignant PE of various cancer patients [27]. Our current data demonstrate a potential role for TAMs regarding this T cell inhibition and therefore identify TAMs as a pivotal target to improve the immunosuppressive environment in MPM.

Our study provides important insights regarding the use of the pleural cavity as a compartment to administer (immuno)therapies. Inherent to the fact that mostly epitheloid mesothelioma is associated with pleural effusions we have restricted our study on this subtype. The advantage of local delivery in close proximity of the tumor may add to an increased efficacy and may increase the dosages delivered to the tumor while limiting the toxicity to other organs. For instance, it was found that intrapleural dosing of cisplatin was feasible with a very high tissue penetration and a low serum concentration [37]. The intrapleural administration of immunotherapies to treat pleural malignancies has been an approach of interest for many years, however this method has remained experimental so far [38]. Sterman *et al* have achieved clinical responses in some patients after administration of intrapleural adenoviral-mediated interferon gene transfer [39-41]. Recently, Adusumilli *et al* showed promising results regarding the intrapleural administration of chimeric antigen receptor (CAR) T cell therapy in a mouse model of pleural malignancy [42]. This preclinical success will soon be followed by a phase I clinical trial. The immunosuppressive character of the soluble and cellular components in PE of MPM patients demonstrated in this study is an important factor to take into account when applying intrapleural immunotherapies. We propose that the characterization and targeting of the local immunosuppressive mechanisms, e.g. through the use of COX-inhibition, could greatly enhance the potential of immunotherapy in mesothelioma. Based on the current study the drainage of PE, when high levels of TAMs are present, prior to intrapleural administration of immunotherapy seems beneficiary in order to achieve optimal immune stimulation.

In conclusion, immunotherapeutic strategies that exploit the anti-tumor potential of the immune system are emerging for the treatment of malignant pleural mesothelioma. However, the immunosuppressive environment created by the tumor hampers the potential of these immunotherapies. The current study demonstrates that pleural effusion is an important immunosuppressive compartment in MPM and that TAMs play a pivotal role in hampering the anti-tumor immune response.

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SUPPLEMENTARY TABLES

Table 1 Primer sequences

Gene	Forward primer	Reverse primer
β-actin	CTGTGGCATCCACGAAACTA	AGTACTTGCGCTCAGGAGGA
CD68	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC
CD163	GCGGGAGAGTGGAAGTGAAAG	GTTACAAATCACAGAGACCGCT
IL-10	TCAAACTCACTCATGGCTTTGT	GCTGTCATCGATTTCTTCCC
CD80	AAACTCGCATCTACTGGCAAA	GGTTCTTGTACTCGGGCCATA
PD-L1	TATGGTGGTGCCGACTACAA	TGCTTGTCCAGATGACTTCG
HLA-DR	AGTCCCTGTGCTAGGATTTTTCA	ACATAAACTCGCCTGATTGGTC

Table 2 Flow Cytometry antibodies

Marker	Fluorochrome	Clone	Company
CD3ε	APC-Cy7	UCHT1	eBioscience
CD4	FITC	RPA-T4	BD Biosciences
CD8a	APC-eFluor450	RPA-T8	eBioscience
CD25	PE-Cy7	M-A251	BD Biosciences
CD127	PE	M21	BD Biosciences
FoxP3	APC	PCH101	eBioscience
CD14	PE Texas Red	TuK4	Invitrogen
CD16	Pacific Blue	3G8	BD Biosciences
CD68	Biotin	clone Y1/82A	Biolegend
	Streptavidin APC-Cy7		Biolegend
CD163	PE	GH1/61	eBioscience
CD206	PerCP-Cy5.5	19.2	eBioscience
CD11c	APC	S-HCL-3	BD Biosciences
HLA-DR	PE-Cy7	L243	BD Biosciences
CD45	FITC	HI30	eBioscience

SUPPLEMENTARY FIGURES

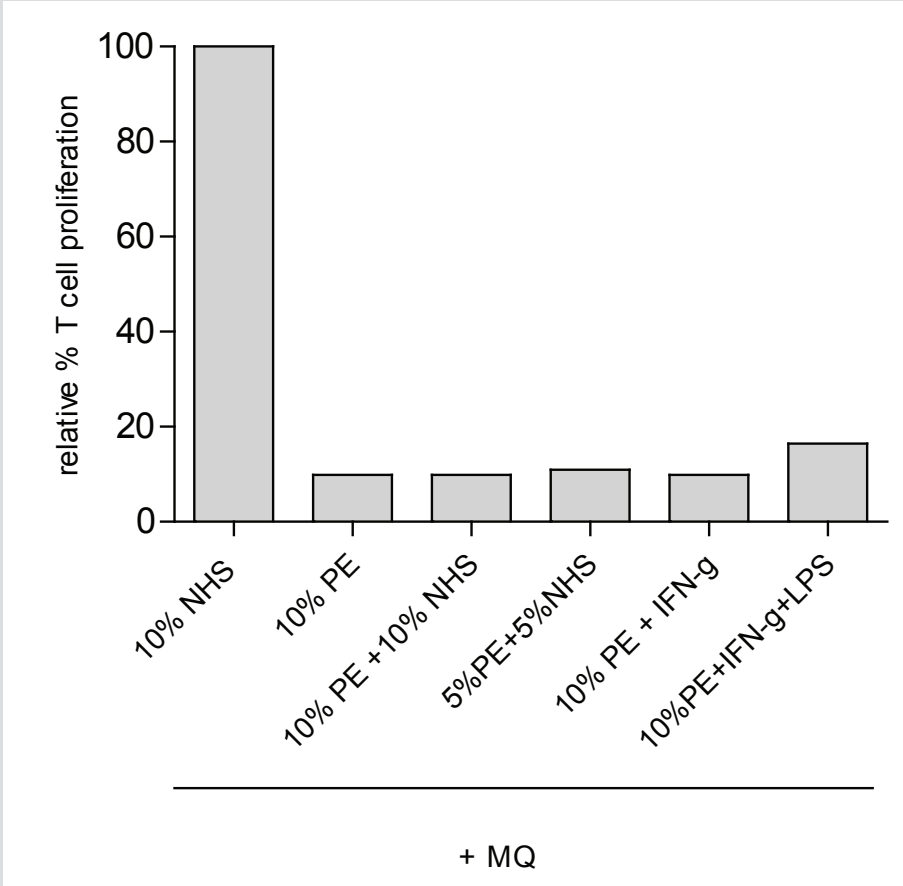

Figure S1 Macrophages cultured in the presence of PE display a robust suppressive phenotype. All depicted conditions are co-cultures of macrophages and T cells. One PE with distinct suppressive properties was chosen to perform this assay. Proliferation of T cells during co-culture with macrophages in the presence of 10% NHS was used as the reference value (set to 100%), all other conditions are calculated relatively to this proliferation. Addition of 10% PE to the co-culture resulted in a profound reduction of T cell proliferation, which could not be rescued by the addition of NHS, or M1-skewing factors IFN-γ and/or LPS.

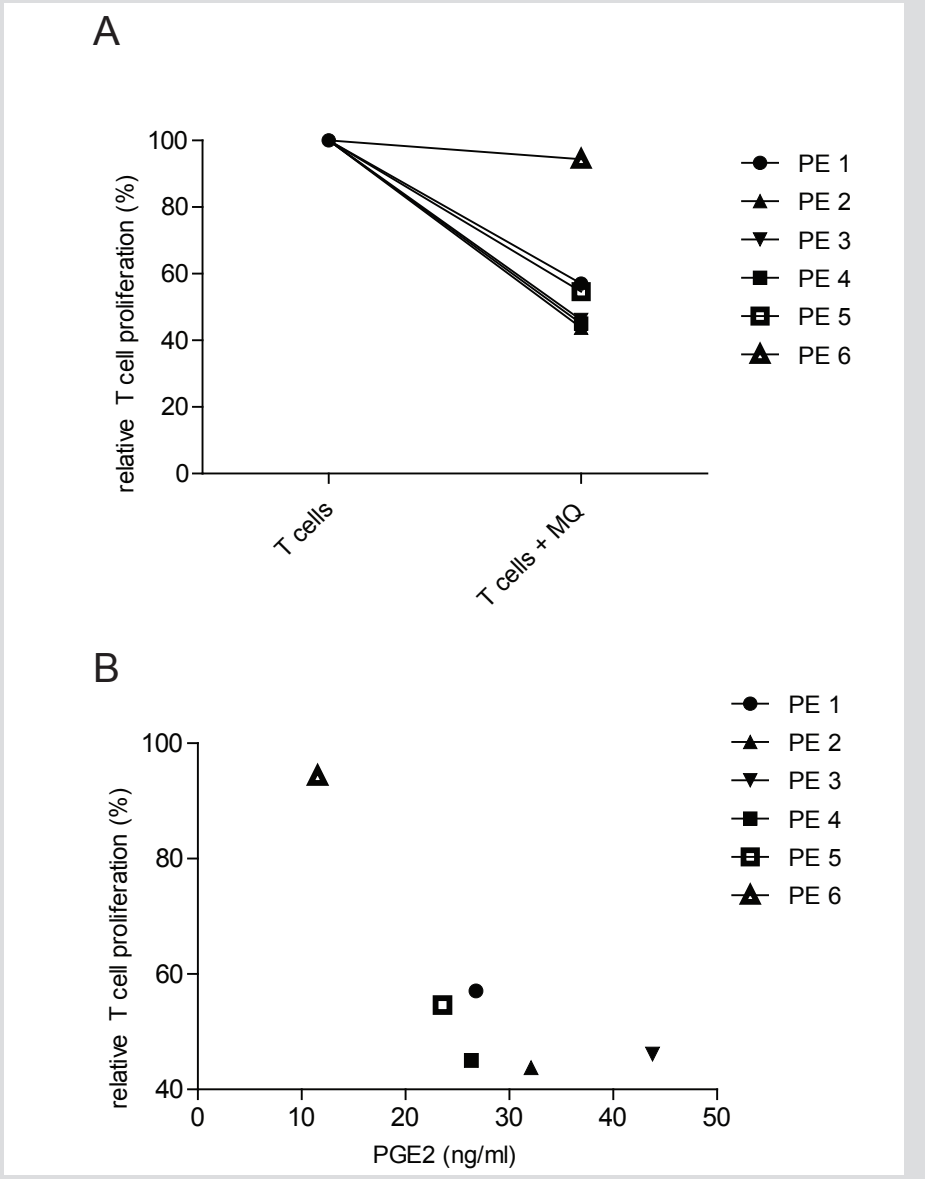

Figure S2 Prostaglandin E2 (PGE2) could be responsible for the PE-induced suppressive function of macrophages. Panel A shows the suppressive effect per tested PE. PE 6 displays a markedly reduced suppressive effect on macrophages, compared to the other 5 PEs (panel A). When correlating the relative T cell proliferation during co-culture with PGE2 concentrations (panel B), PE 6 also contained the lowest PGE2 concentration. This result illustrates the potential role of PGE2 in the suppressive effect of PE on macrophages. Furthermore, addition of synthetic PGE2 at 30 ng/ml (average PE concentration) to one of the tumor cell line supernatants with an average PGE2 concentration (0.3 ng/ml, MQ control condition) resulted in reduction of T cell proliferation of 50.4% during co-culture with macrophages (MQ PGE2 condition, panel C).

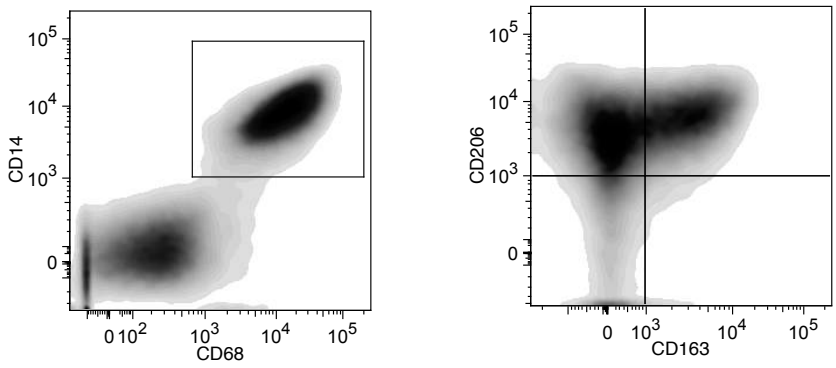

Figure S3 Flow cytometric analysis of TAMs in PE. TAMs were characterized as CD45+CD14+CD68+ cells. These TAMs were further classified depending on their CD206 and CD163 expression. All TAMs which expressed either CD206, CD163 or both, were classified as M2 TAMs.

Chapter 5

Precision immunotherapy; dynamics in the cellular profile of pleural effusions in malignant mesothelioma patients

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ABSTRACT

Objectives: Clinical studies have proven the potential of immunotherapy in malignancies. To increase efficacy, a prerequisite is that treatment is tailored, so precision immune-oncology is the logical next step. In order to tailor treatment, characterization of the patient's tumor environment is key. Pleural effusion (PE) often accompanies malignant pleural mesothelioma (MPM) and is an important part of the MPM environment. Furthermore, the composition of PE is used as surrogate for the tumor. In this study, we provide an insight in the dynamics of the MPM environment through characterization of PE composition over time and show that the immunological characteristics of PE do not necessarily mirror those of the tumor.

Materials and Methods: From 5 MPM patients, PE and tumor biopsies were acquired at the same time point. From one of these patients multiple PEs were obtained. PEs were acquired performing thoracocenteses and total cell amounts were determined. Immunohistochemistry was performed to quantify immune cell composition (T cells, macrophages) and tumor cells in PE derived cytospins and tumor biopsies.

Results: The PE amount and (immune) cellular composition varied considerably over time between multiple (n=10) thoracocenteses. These dynamics could in part be attributed to the treatment regimen consisting of standard chemotherapy and dendritic cell (DC)-based immunotherapy. In addition, the presence of T cells and macrophages in PE did not necessarily mirror the infiltration of these immune cells within tumor biopsies in 4 out of 5 patients.

Conclusions: In this proof-of-concept study with limited sample size, we demonstrate that the composition of PE is dynamic and influenced by treatment. Furthermore, the immune cell composition of PE does not automatically reflect the properties of tumor tissue. This has major consequences when applying precision immunotherapy based on PE findings in patients. Furthermore, it implies a regulated trafficking of immune regulating cells within the tumor environment.

Keywords: malignant mesothelioma, pleural effusion, immunotherapy, tumor environment

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a highly aggressive tumor mainly caused by the inhalation of asbestos fibers. MPM can develop from both the visceral pleura and the parietal pleura. The occurrence of pleural effusion is associated with approximately 70% of the MPM patients, especially in MPM of the epithelioid subtype [1]. Pleural effusion accumulates in the pleural space when influx of effusion outweighs efflux. Increased production occurs due to excessive plasma leakage through hyperpermeable intratumoral vessels. In addition, blockade of the pleuropulmonary lymphatics by tumor cells results in reduced absorption [2]. Build-up of pleural effusion can result in profound shortness of breath and deterioration of quality of life. Although effusion cytology for diagnostic purposes in MPM is controversial and not recommended in the ESMO Clinical Practice Guidelines for malignant pleural mesothelioma, drainage is commonly performed to relief symptoms [3]. Pleural effusion of mesothelioma patients can comprise different cell types and soluble factors, which can be derived directly from the tumor, its environment and/or from the vasculature. Immune cells like T cell subsets (e.g. CD8 T cells and regulatory T cells) and tumor-associated macrophage subsets (anti-tumor M1 or pro-tumor M2 TAMs) are present in most malignant pleural effusions [4-7].

As immunotherapy is gaining ground in many different tumor types, various immunotherapeutic approaches are also being investigated in clinical studies in mesothelioma patients [8-12]. In order to optimize the efficacy of immunotherapy in mesothelioma, a tailor-made approach is warranted.

Given the close proximity of pleural effusion to the pleural tumor, it is an important part of the mesothelioma environment and often used as a surrogate marker for the tumor tissue. Furthermore, the pleural cavity can be an attractive site to locally administer (immuno) therapies. In this study, we investigate the robustness of the pleural effusion composition and whether it reflects the pleural tumor in mesothelioma.

MATERIALS AND METHODS

Patient material

Five MPM patients were selected from whom tumor biopsies and pleural effusion cytospins were derived at the same time point. The biopsies and effusions were acquired during VATS surgical biopsy procedures, prior to any treatment. All patients were diagnosed with MPM of the epithelioid subtype by the Dutch National Mesothelioma Panel. Pleural effusion was collected in sterile tubes or bags without anticoagulant. Pleural cells were pelleted from pleural effusions using centrifugation at 400G for 10 minutes. Ficoll density gradient centrifugation was applied to pleural effusions with evident blood contamination to separate the red blood cells from the leucocytes. Tumor biopsies were embedded in Tissue-Tek II OCT-compound (Miles, Naperville, IL, USA), snap frozen in liquid nitrogen and stored at -80°C. Tissue sections (6 µm) were cut using a HM-560 cryostat (Microm, Heidelberg, Germany). Cytospin preparations were made of an optimally diluted PE cell suspension (Shandon Cytospin 4, Thermo Electron Corporation, Massachusetts, United States). This study is a retrospective analysis of data, all patient materials were acquired between 2010 and 2013.

Immunohistochemistry

Cytospins and tumor biopsies were stained with CD8 (Dako, Glostrup, Denmark) and the commonly used M2 macrophage marker CD163 (eBioscience, San Diego, USA). The cytospin series of the patient followed in time were stained for calretinin, CD68 and CD3 (Dako). Antibodies were incubated for 1 hour and detected using the RAM - APAAP method (Dako). Naphtol-AS-MX-phosphate (0.30 mg/ml, Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) and new fuchsine (160 mg/ml in 2M HCl; Chroma-Gesellschaft, Köngen, Germany) were used as substrate. An isotype-matched antibody was used as control. The percentage of CD8- or CD163- positive cells in representative areas of the tumor biopsies was determined as described earlier [13,14]. In the cytospins, in three representative areas the amount of positively stained cells among a total of 100 cells was counted and we defined the average value of three counts as the percentage of positive cells in the cytospin. Tumor slides and cytospins were independently evaluated by L.L. and K.B.

RESULTS

Longitudinal pleural effusion follow-up

One patient underwent 10 thoracocenteses to relieve dyspnea symptoms in the course of one year. The maximum pleural effusion amount was drained until the flow through the drain stopped. During this year the patient was treated with four courses of cisplatin and pemetrexed and as a maintenance treatment with three courses of dendritic cell-based immunotherapy and pleurodesis attempts with talc [15]. Treatment responses were evaluated according to the Modified RECIST criteria [16, 17]. Since no measurable lesion was present when the diagnosis was made, initiation of chemotherapeutic treatment was delayed until a measurable lesion had developed. Chemotherapy induced a partial response (PR) and immunotherapy was followed by a stable disease state (SD), which lasted one year. The thoracocenteses were performed prior to a pleurodesis procedure with talc slurry (fig. 1 – week 50 time point) which did not result in pleurodesis and ultimately the placement of an indwelling PleurX® catheter. In order to gain insight in the dynamics of pleural effusion formation and composition we evaluated the cellular amount and the proportion of tumor cells and T cells in time. In figure 1A the amounts of pleural effusion in time are depicted. This amount varied between 400ml and 1600ml. In addition to the amount of pleural effusion, the total cellular amount varied considerably (fig. 1B). The total cellular amount decreased markedly after chemotherapy and immunotherapy. In concordance with the total cellular amount, the percentage of cells positive for the frequently used mesothelioma tumor cell marker calretinin (fig. 1C) decreased after chemotherapy. Tumor cell amounts in pleural effusion also depicted clinical response; progression of disease was associated with an increase and partial response with a decrease in tumor cells. After immunotherapy the percentage of calretinin positive cells was not affected. In addition to tumor cells, the dynamics of CD3 positive T cells were studied (fig. 1D). Over time the cellular compartment consisted of 3% to 20% of T cells. The relative amount of T cells in the pleural effusion of this patient was diminished both after chemotherapy and immunotherapy (fig. 1D). There was no evident correlation between the amount of T cells and disease state. In addition to T cells, CD68 positive macrophages were measured, this subset was not influenced by treatment regimen or disease state and their presence in the pleural effusion of this patient was constitutively high (data not shown). Over the course of one year the pleural effusion amount and (immune) cellular composition varied considerably in this patient. These dynamics can partly be explained by the treatment regimen; however apparent fluctuations beyond the treatment timeframes are visible. Since the final pleural effusion sample (fig. 1 – week 54 time point) was acquired after a pleurodesis procedure with talc slurry (performed at week 50 time point), the presence of talc in the intrapleural space could have influenced the immune infiltrate, however we found no changes in T cell or macrophage amounts.

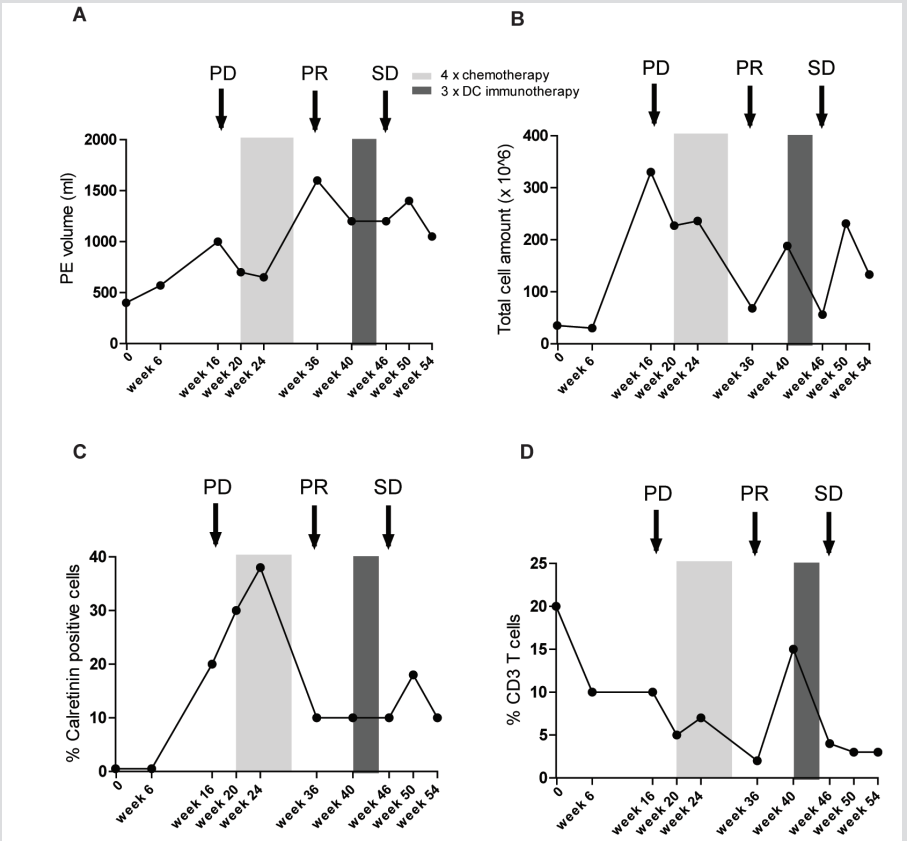

Figure 1 Dynamics in pleural effusion amount and cellular composition in an MPM patient over one year from diagnosis onwards. Abbreviations: PE=pleural effusion, PD=progression of disease, PR=partial response, SD=stable disease. CT-scan evaluated disease states are depicted above the graphs. The grey bars depict the period during which the patient received 4 courses of chemotherapy (cisplatin/pemetrexed, light grey bar) and 3 courses of DC immunotherapy (dark grey bar). Ten subsequent thoracocenteses were performed in order to relieve dyspnea. Panel A shows the variation in pleural effusion amount. Panel B shows the variation in total cellular amount. Panel C shows the percentage of calretinin positive tumor cells. Panel D shows the variation in relative amount of CD3 positive T cells over time.

Pleural effusion vs. tumor

In order to further clarify the interactions between pleural effusion cells and the pleural tumor a study was performed to investigate the infiltration of CD8 T cells and M2 TAMs in tumor biopsies and accompanying pleural effusions which were acquired at the same time point, before the start of any treatment. None of the investigated pleural effusions were contaminated with blood and no further purification was necessary. An example of the immunohistochemical staining of patient number 5 is shown in Figure 2.

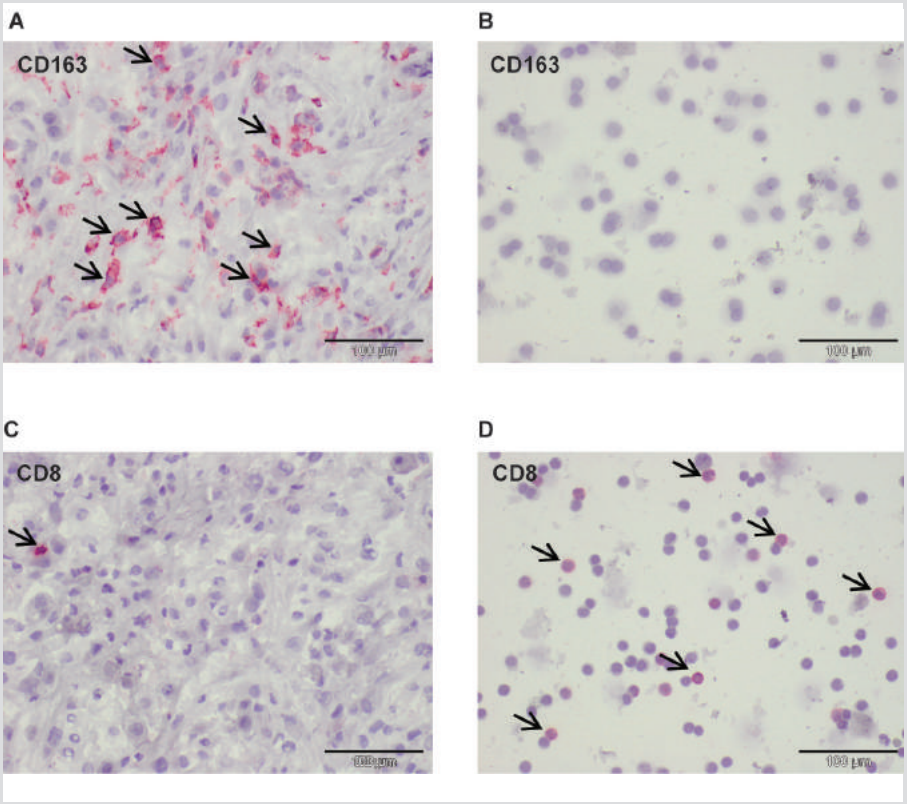

Figure 2 Immunohistochemical staining of MPM biopsy and pleural effusion cytospin of patient number 5. Arrows indicate cells positive for either CD163 (Panel A and B), or CD8 (Panel C and D). Panel A and B show staining of M2 macrophage marker CD163 in the tumor biopsy (panel A) and pleural effusion cytospin (panel B). Panel C and D show staining of CD8 in the tumor biopsy and pleural effusion cytospin respectively.

This study among five patients showed that there can be a discrepancy between the distribution of CD8 T cells and M2 TAMs in pleural effusion and the presence of these immune cells in the tumor tissue (fig. 3). In patient 3 and 5 no M2 TAMs were detected in the PE while the accompanying tumor biopsies contained a substantial amount of M2 TAMs (fig. 3A). Patient 1, 2, and 5 had high levels of CD8 T cells in their pleural effusion but CD8 T cells were virtually absent in the corresponding tumor biopsies (fig. 3B). Furthermore, M2 TAMs were predominantly present in the tumor biopsies while CD8 T cells were mostly present in the pleural effusions.

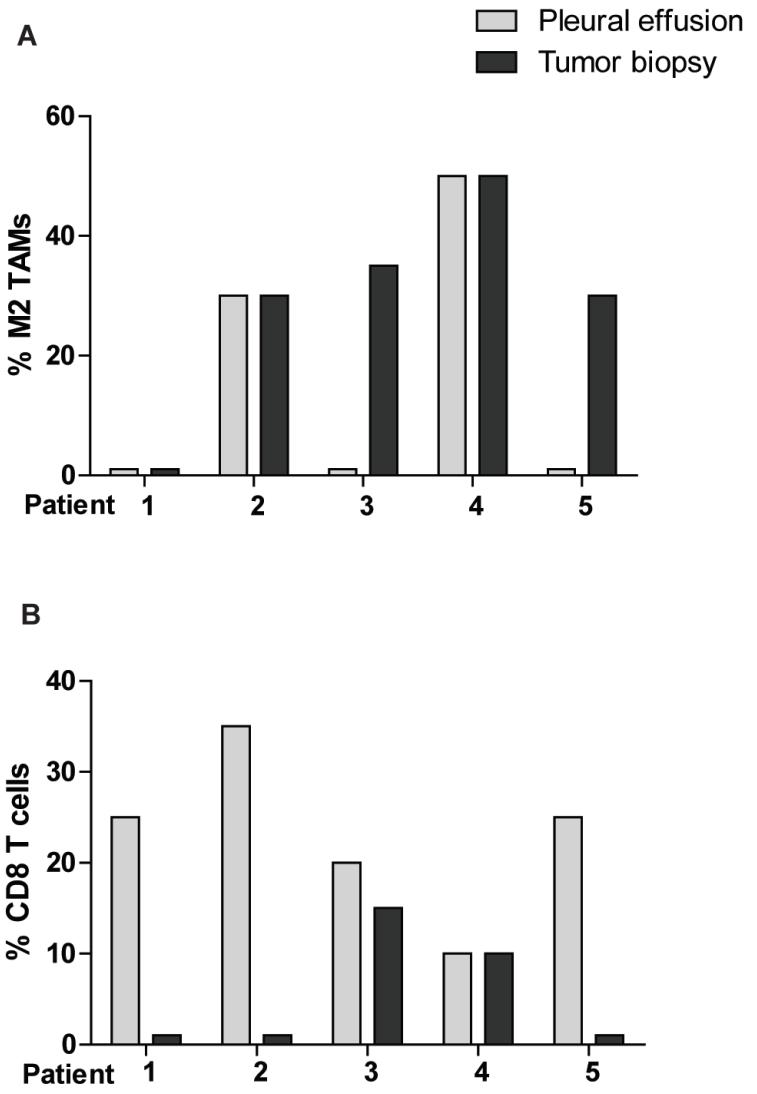

Figure 3 M2 TAM amounts and CD8 T cell amounts in pleural effusions do not mirror the content of the tumor. Of five MPM patients tumor biopsies were available which were acquired at the same time points as the accompanying pleural effusions. M2 TAMs (CD163) and CD8 T cells (CD8) were identified via immunohistochemical staining of cytospins derived from pleural effusions and tumor biopsy slides. Panel A shows the differences in presence of M2 TAMs between pleural effusions and tumor biopsies, Panel B shows these differences for CD8 T cells. Results are depicted as percentages of positively stained cells within the total tumor slide or cytospin.

DISCUSSION

The current study shows for the first time that the cellular composition of pleural effusion in MPM patients is dynamic and influenced by treatment response. In addition, the immune cell composition of pleural effusion does not necessarily reflect the properties of the tumor. Pleural effusion is often studied as an easy-access reflection of the properties of the pleural tumor. As therapies are becoming more individualized and precise, a thorough characterization of the local milieu is warranted. Because of the limited patient number, the current study should be regarded a proof-of-concept for future studies.

We presented a patient who underwent 10 thoracocenteses in the course of one year to relieve dyspnea symptoms, on the patients' request a pleurodesis procedure with talc slurry was postponed until the treatment regimen was completed. In this patient we showed that the cellular composition is influenced by response to therapy. The amount of tumor cells found in PE correlated with the response to treatment. The composition of immune cells is more complex and cannot be explained from this single patient experiment. The main conclusion to be drawn is that timing, disease status, and treatment may all have an influence on the cellular content of pleural effusion. Furthermore, we showed that the immune composition of pleural effusion can differ greatly from the intratumoral composition. We constitutively found higher levels of CD8 T cells in the pleural effusions compared to the tumor biopsies, which could indicate that the tumor has a protective mechanism against the influx of these immune cells with anti-tumor effects. McCoy et al. showed that higher proportions of proliferating CD8 T cells in peripheral blood were associated with poor survival in patients with advanced thoracic malignancies [18]. This finding supports our hypothesis that high levels of pro-inflammatory immune cells in the periphery (e.g. pleural effusion, blood), could be a sign of potent defense against an anti-tumor immune response in the tumor itself. In contrast to CD8 T cells, anti-inflammatory M2 TAMs showed the opposite trend with higher levels in the tumor biopsies compared to pleural effusions. M2 TAMs are well known for their potential to counter act the anti-tumor immune reponse [19-22]. The found discrepancy between the presence of CD8 T cells and M2 TAMs in pleural effusion and tumor biopsies demonstrates the potential controlled trafficking of immune cells within the mesothelioma environment. Unfortunately, in the current study there were no matched blood samples available from the included patients. It would be of great interest to simultaneously measure immune populations in tumor, pleural effusion and peripheral blood in larger patient cohorts to further investigate the trafficking and interactions of immune cells between different compartments. In this study we have chosen to focus on T cells and TAMs because these immune cells are prevalent and clinically relevant in MPM. Furthermore we have shown earlier that the influx of CD8 T cells and phenotype of TAMs in MPM biopsies can predict survival and local tumor outgrowth [13, 14].

Conclusions

Based on our current study and earlier findings we propose the following model. Depending on the tumor stage and treatment regimen, both the MPM tumor and the accompanying pleural effusion can be infiltrated by different pro- and anti-tumor immune cells (Figure 4). We found that the immune infiltrate in the tumor may not be mimicked in the pleural effusion, and propose that pleural effusion provides a dynamic insight in the tumor-host interactions. Therefore, in order to further develop precision immunotherapy in MPM, pleural effusion should be considered as a separate immunological compartment from the pleural tumor. This finding should be taken into account in order to properly tailor immunotherapy to the individual MPM patient. Our proposed model has to be confirmed in larger MPM patient cohorts, however the current findings indicate that precise and longitudinal characterization of the pleural effusion microenvironment in MPM patients reveals local tumor-host interactions essential to further optimize immunotherapy in MPM.

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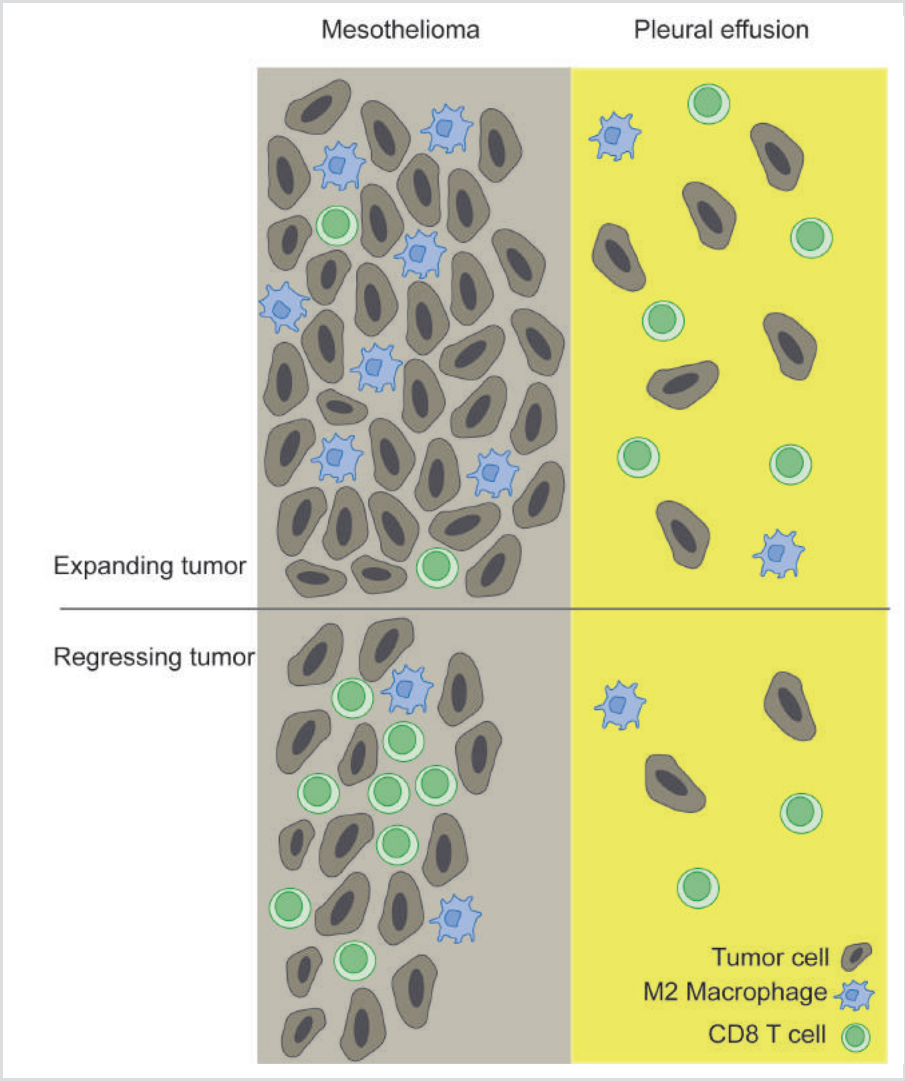

Figure 4 Proposed model for dynamics of the mesothelioma and accompanying pleural effusion milieu. An expanding mesothelioma tumor will consist of relatively high amounts of M2 macrophages which can locally induce an immunosuppressive environment. In addition, low amounts of tumor-infiltrating CD8 T cells are present. In the pleural effusion of the expanding tumor higher amounts of CD8 T cells are present. The relative amount of macrophages is higher in the tumor compared to the pleural effusion, both in the expanding and in the regressing tumor. Furthermore, the regressing tumor contains relatively high amounts of infiltrating CD8 T cells, and less CD8 T cells and tumor cells in the pleural effusion.

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Chapter 6

Combination therapy with a CD40-agonist and dendritic cell immunotherapy has additive effects in a murine mesothelioma model

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ABSTRACT

Background: The potential of immunotherapy in mesothelioma has recently been demonstrated in multiple (pre)clinical studies. The success of immunotherapy relies on the induction of an anti-tumor immune response which has to overcome the local immunosuppressive environment in established tumors. Tumor-associated macrophages (TAMs) are an important part of the suppressive environment in mesothelioma and reprogramming these TAMs towards a more pro-inflammatory phenotype using a CD40 agonistic antibody has shown promising results in multiple solid tumors. Dendritic cell (DC) immunotherapy has been shown to elicit anti-tumor T cell responses and is currently being studied in mesothelioma patients at our institution. We hypothesize that the combination treatment with a CD40-agonist and DC therapy has synergistic effects and the aim of the current study is to investigate the efficacy of this combinatorial approach.

Methods: Wildtype Balb/c mice were injected intraperitoneally (i.p.) with the AB1 murine mesothelioma cell line. Different treatment regimens were compared as follows: untreated control group (n=6), monotherapy with CD40-agonist (FGK4.5 monoclonal antibody, n=5), monotherapy with DC immunotherapy (n=5) and combination therapy of DC immunotherapy followed by treatment with the CD40-agonist (n=5). Three days after completion of the treatment regimens, blood was drawn and analyzed using flow cytometry to investigate peripheral immune activation. All mice were monitored and sacrificed when showing signs of severe illness.

Results: Blood analysis revealed that peripheral monocytes of the CD40-agonist group and the combination therapy group showed an increase in expression of MHC-II and PD-L1 compared to the mice in the control group and the DC immunotherapy group. In addition, the combination therapy induced a profound increase in effector CD8 T-cells and proliferating CD8 T-cells compared to the monotherapies. The survival analysis at day 90 post tumor cell injection demonstrates a 17% survival of the control group, 60% survival of the DC therapy group, 80% survival of the CD40 group and 100% survival of the combination therapy.

Conclusion: Combination therapy of DC immunotherapy and a CD40-agonistic antibody induces additive immune activation in the peripheral blood of mesothelioma-bearing mice compared to the monotherapies. The presented data demonstrate the potential of the combination of cellular immunotherapy and targeting of the local tumor microenvironment in mesothelioma.

INTRODUCTION

CD40 is a member of the TNF receptor superfamily and is broadly expressed by e.g. antigen-presenting cells (APCs; dendritic cells, B cells, monocytes, macrophages), platelets and endothelium[1]. Furthermore, CD40 is expressed by most B cell lymphomas and a substantial number of epithelial malignancies. CD40-CD40L interactions are central to the generation of T cell dependent, humoral and cytotoxic T cell responses [2].

The expression of CD40 on a broad range of malignancies and its central immunostimulatory role has made CD40 a popular immunotherapeutic target [3]. The discovery of the potential of agonistic CD40 antibodies to mimic the signal of CD40L initiated the first preclinical studies investigating the potential of these antibodies to induce effective anti-tumor immune responses [4-7].

Although historically most attention has been focused on the role of the CD40-CD40L interaction on lymphocytes, recently the importance of CD40 activation on macrophages in the tumor environment has been discovered. It has been shown that CD40 stimulation enhances the capacity of APCs to activate tumor-specific T cells and T cell–independent immunity by activating tumoricidal tumor-associated macrophages [8]. The group of Beatty et al showed that in a mouse model of pancreatic ductal carcinoma, the regression of tumors required the presence of macrophages, and not T cells. This specific role for TAMs in the therapeutic effect of CD40 agonists has led to our hypothesis that CD40 activation could also be beneficial in the mesothelioma environment, which is known to be dominated by macrophages [9]. Furthermore, because we propose that the combination of re-education of the local environment with cellular immunotherapy is an effective strategy to induce an anti-tumor immune response, we conducted a pilot study to investigate the safety and efficacy of combining a CD40 agonistic antibody with dendritic cell (DC-) immunotherapy in a murine mesothelioma model.

MATERIALS AND METHODS

AB1 tumor cell culture and tumor lysate

The AB1 cell line was derived from tumors induced by crocidolite asbestos injected intraperitoneally into a BALB/c mouse [10]. AB1 tumor cells were cultured in AB1 TCM (RPMI 1640, 5% FBS, and gentamicin (Gibco/Invitrogen Breda, the Netherlands) in T20, T75 or T175 tissue culture flasks (Thermo Scientific) in in a humidified atmosphere at 37°C and 5% CO_2, in air. When cells grew until a density between 60-80% confluence was reached and cells were treated with trypsin (0.05% trypsin, 0.53 mM EDTA in phosphate buffered saline (PBS, all GIBCO/Invitrogen) and replated in a concentration depending on previous cell density. For tumor lysate, cells were harvested, counted and dissolved in PBS to be further processed for lysate. The cell suspension was frozen in liquid nitrogen and disrupted by four cycles of freeze-thawing followed by sonication for 4 × 10 seconds with an amplitude of 10 microns, using a Soniprep 150 ultrasonic disintegrator equipped with a microtip (Sanyo Gallenkamp BV, Breda, The Netherlands) on ice. Cell lysate was aliquoted and stored at −80°C.

Dendritic cell cultures

Dendritic cells were cultured in Petri dishes from bone marrow cells of healthy 8-week old female BALB/c mice for 10 days in the presence of DC TCM (RPMI 1640 + Glutamax 5% FBS, 0,75% gentamicin (Gibco/invitrogen Breda, the Netherlands), 50μM β-Mercaptoethanol) and 20ng/ml GM-CSF (recombinant) in a humidified atmosphere at 37°C and 5% CO_2, in air. DC-TCM + GM-CSF was refreshed every 3 days. On day 9 one plate of DCs was harvested and cells were counted to determine the amount of cells per plate. Next, AB1 tumor cell lysate was added to the plates in a concentration of 2-3 tumor cells/DC. 8 Hours later, CpG (1668 phos, Invitrogen, Breda, the Netherlands) was added to the plates in a concentration of 1 μM/plate. On day 10, DCs were harvested and purified by lympholyte mammal density gradient centrifugation (lympholyte-M, Cedarlane, Hornby, ON, Canada) to lose excess lysate. DCs were counted and analyzed with flow cytometry to check for maturation status of the DCs.

Injection of mice, administration of CD40 antibody and monitoring

Experiments were approved by the local Ethical Committee for Animal Welfare and complied with the Guidelines for the Welfare of Animals in Experimental Neoplasia by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) and by the Code of Practice of the Dutch Veterinarian Inspection. 8-Week old female WT BALB/c mice were injected intraperitoneally (i.p.) with 0.5x10^6 AB1 tumor cells (harvested at a cell density of 50-80% confluence) dissolved in 250 μl PBS. A 25G needle was used to inject the tumor cells in the left lower abdominal quadrant while mice were fixated.

100 μg (5 mg/kg) of the mouse monoclonal CD40-agonistic antibody (FGK 4.5. BioXCell) or

the IgG2a isotype control (kindly provided by L. Boon, Bioceros, Utrecht, The Netherlands) was dissolved in PBS in a final volume of 250µl and injected similar to the DCs. Both antibodies were endotoxin-free. Mice were monitored every 1-2 days until 90 days when the experiment ended. When mice reached a humane endpoint (severe symptoms of distress e.g. hunched back, ruffled fur, no grooming, cachexia, anemia or jaundice, tumor breakthrough through the abdominal wall) they were euthanized by CO_2 asphyxiation. Hereafter, mice were exsanguinated by heart puncture and blood was collected in an EDTA tube. Next, the abdominal wall was incised and the tumor nodules were located and extracted. The spleen was also excised for further experiments. All solid tissues were weighed on a microbalance and further processed for flow cytometry, immunohistochemistry and storage to used later.

Flow Cytometry

Cells were put in a 96-well plate and washed with FACS buffer (0.05% NaN_3, 2% BSA in PBS) and Cells were stained with 50µl antibody mix containing 1:300 2.4G2 antibody (Fcγ II/III receptor blocking antibody; kindly provided by L. Boon, Bioceros, Utrecht, The Netherlands) for extracellular markers, 30 minutes at 4°C in the dark in FACS buffer (in Brilliant Violet buffer (BD) when a mix contained >1 BV marker), and for 15 minutes in 50µl PBS with Amcyan (Invitrogen) for live/dead staining. For intracellular staining, cells were fixated for 30 minutes using fix/perm buffer (eBioscience) followed by 60 minutes permeabilization in permeabilization buffer (eBioscience). Subsequently, cells were stained in 50µl antibody mix containing intracellular antibodies. Cells were washed in permeabilization buffer and in FACS buffer before being measured.

Anti-mouse antibodies directed against the following extracellular markers were used for flow-cytometry: F4/80, MHCII, CD40, CD115, CD11b, CD335, CD3e, CD8a and CD19 (eBioscience), LyC6, CD11c, CD80, PD-L1, Ly6G, PD-1 and CD25 (BD biosciences), CD206 (AbD Serotec), CD45 (Abcam) and KLRG1 (Biolegend). For intracellular staining anti-mouse antibodies were used directed against Foxp3 and Ki-67 (eBioscience). Data acquisition was performed by flow cytometry (LSR II; BD Biosciences) and data analysis was performed with FlowJo software (Tree Star, Inc.)

RESULTS

First, the effect of the CD40-agonistic antibody FGK4.5 in a mesothelioma mouse model was assessed. Because major side-effects have been reported (mainly auto-immunity) with the use of CD40-agonistic antibodies in mice, we performed a dose-escalation study to determine the safest applicable dose. 8 week old female BALB/c mice were injected intraperitoneally (i.p.) with increasing concentrations of FGK4.5. Although the mice injected with the highest concentration of the antibody (5 μg/kg) experienced symptoms of discomfort in the days following injection, alterations in peritoneal macrophage phenotype and T-cell proliferation were most pronounced at this concentration (fig.1A, B). Peritoneal macrophages displayed a more pro-inflammatory phenotype characterized by higher MHC-II and lower CD206 expression compared to PBS treated mice. The 5 μg/kg antibody treated group showed more Ki-67 positive proliferating CD4 and CD8 T-cells in blood compared to the lower dosages treatment groups. Spleen size and weight increased dose-dependently following 3 injections of the CD40-agonistic antibody once every 48 hours (fig. 1C, D) but did not cause apparent symptoms in the mice.

To test whether CD40-ligation with FGK4.5 was beneficial as monotherapy or additive or synergistic when combined with DC-therapy, we injected wildtype BALB/c mice with AB1 tumor cells i.p. on day 1 followed by DC-therapy or PBS on day 10 (fig. 2A). DCs were cultured in 10 days from WT BALB/c bone marrow cells in TCM supplemented with GM-CSF as previously described by Hegmans et al. Cells were loaded with AB1-tumor cell lysate on day 9 and matured with CpG to be harvested and injected i.p. on day 10. DCs were mature as demonstrated by the increased expression of MHCII, CD80, PDL1 and CD40 on the cell surface (data not shown). We previously established that treating mice with DC-therapy 10 days following tumor injection is suboptimal, and results in the majority of mice dying from the tumor. Treating mice with this treatment schedule allows the assessment of a possible treatment benefit of combination therapy over monotherapy. Following injection, DCs were allowed to migrate to the lymph nodes and 48 hours later, the CD40-agonistic antibody (FGK4.5) was administered i.p. (5 mg/kg) or an isotype antibody for the control group. Mice were inspected every 1-2 days during therapy for any possible symptoms of the treatments or the tumor load. Mice were euthanized when they showed signs of severe illness or at the end of the experiment 90 days after tumor cell injection. A blood sample was taken from all mice 48 hours after the last injection of the CD40 antibody (day 16, fig. 2A). The combination of DC-therapy with an anti-CD40 agonistic antibody resulted in the complete survival of all mice for the entire 90 day duration of the experiment, in contrast to the untreated group where all but one animal died from end-stage mesothelioma (fig. 2B). Monotherapy of the CD40-agonist or mere DC-therapy also increased survival in mesothelioma bearing mice compared to giving no treatment. Although this experiment was not designed to investigate

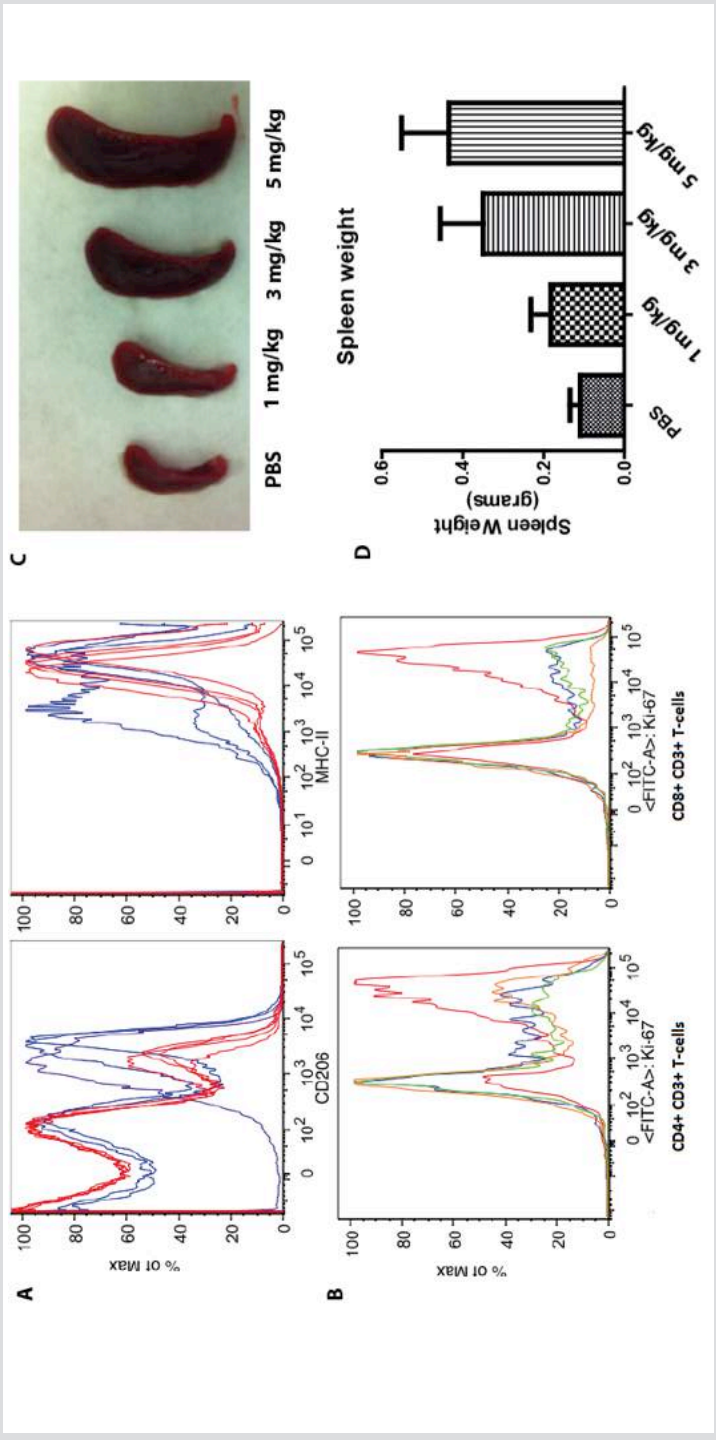
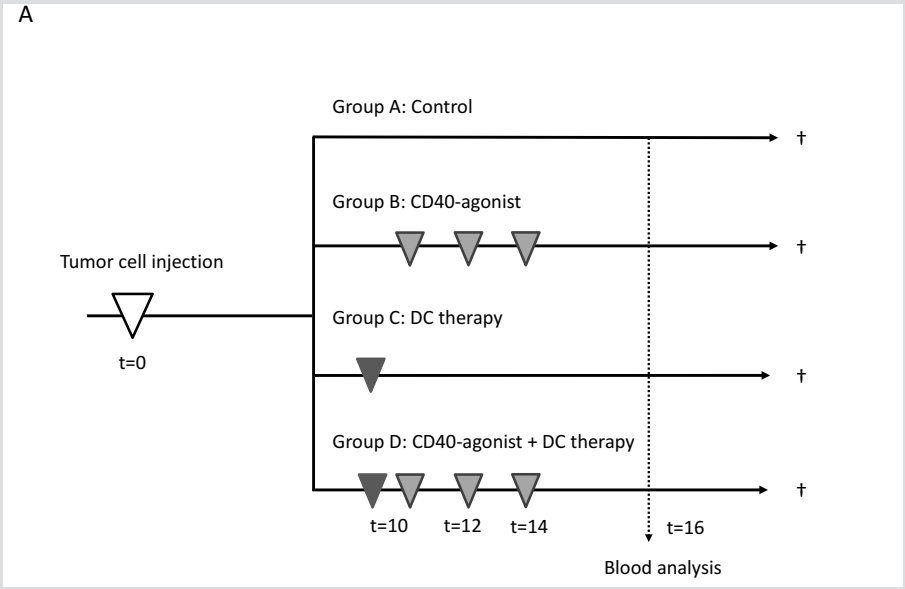


Figure 1 The effects of a CD40-agonistic antibody on peritoneal macrophages and circulating T-cells in healthy mice using different concentrations of antibody. 8-week-old female BALB/c mice were intraperitoneally injected with a CD40-agonistic antibody (FGK4.5) in 1, 3 and 5 mg/kg (20, 60 and 100 µg/kg respectively) or with PBS every 48 hours for 5 days (a total of 3 injections). Mice were euthanized 5 days later and spleen, blood and peritoneal lavage were obtained. (A) Peritoneal F4/80+ macrophages were obtained following lavage of the abdominal cavity and stained by flow cytometry for M2 marker CD206 and M1 marker MHCII. (Red = CD40-agonist treated mice (5 mg/kg), blue = PBS treated mice). (B) CD8+ T cells and CD4+ T cells in blood were intracellularly stained for Ki-67 (orange = PBS, green = 1 mg/kg, blue = 3 mg/kg, red = 5 mg/kg). (C, D) Spleens were extracted and macroscopically inspected and weighed.

the mechanisms underlying the treatment benefit of CD40-agonist/DC-therapy treated mice, we analyzed the blood of mice following treatment on day 16 of the experiment to look for signs of enhanced immune responses.

CD40-activation 'matured' myeloid cells in the blood as evidenced by the higher expression of MHCII, PDL1 and CD80 (not shown) on monocytes, suggesting a M1-like differentiation pattern (fig. 3). B-cell proliferation was also significantly enhanced by CD40 ligation *in vivo* (data not shown). Nonetheless, B-cell numbers in blood were reduced compared to control conditions, possibly due to the CD40-induced splenic accumulation of these cells and concomitant splenomegaly observed earlier (fig. 1C).
Although T cell populations varied only marginally between different conditions, the T cell phenotype was evidently altered (fig. 3). Both CD8+ cytotoxic T cells and CD4+ helper T cells (not shown) were highly proliferating as demonstrated by the increased proportion of cells positive for Ki-67. An increased fraction of cells CD8+ T cells became positive for KLRG1, a marker for (short-lived) effector T cells or terminally differentiated cells, indicating altered dynamics in CD8+ T-cell memory formation following CD40-agonistic treatment and DC-therapy. The combination of both DC-therapy and the CD40 agonistic antibody resulted in an additive increase of the above-mentioned parameters both in monocytes and in T lymphocytes.



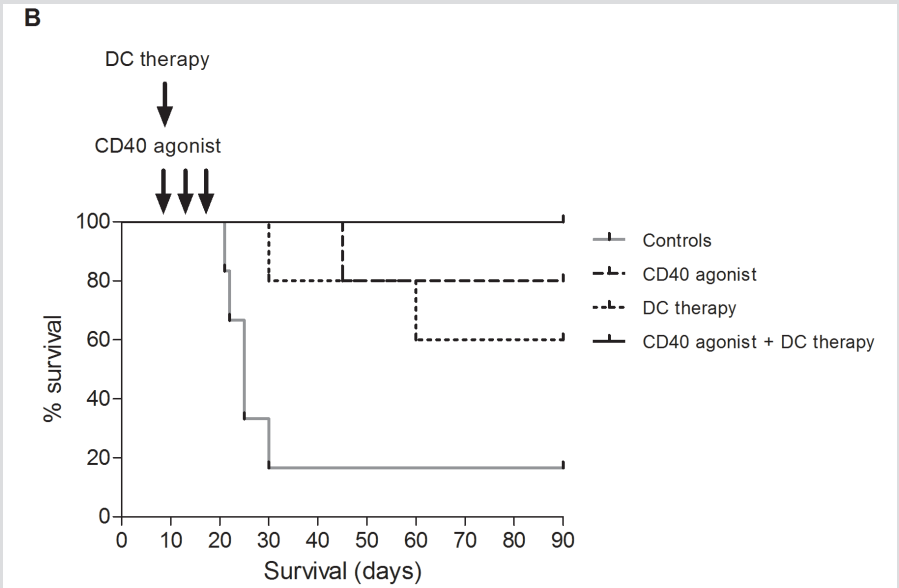


Figure 2 A Treatment schedule: 8 week-old female WT BALB/c mice were injected i.p. with 0.5x10^6 AB1 tumor cells on t=0 followed by i.p. injection of 2.5x10^6 tumor-lysate loaded DCs for treatment groups C (n=5) and D (n=5) or with PBS for group A (n=6) and B (n=5). 8 Hours later, mice from groups B and D received 5mg/kg CD40-agonist (FGK4.5) whereas mice from groups A and C were injected with an IgG2a isotype control. Antibody treatment was repeated twice in the following days and mice were monitored for a total of 90 days to assess survival. Panel B demonstrates the Kaplan Meier curve showing the survival of the different treatment groups for the duration of the experiment (90 days).

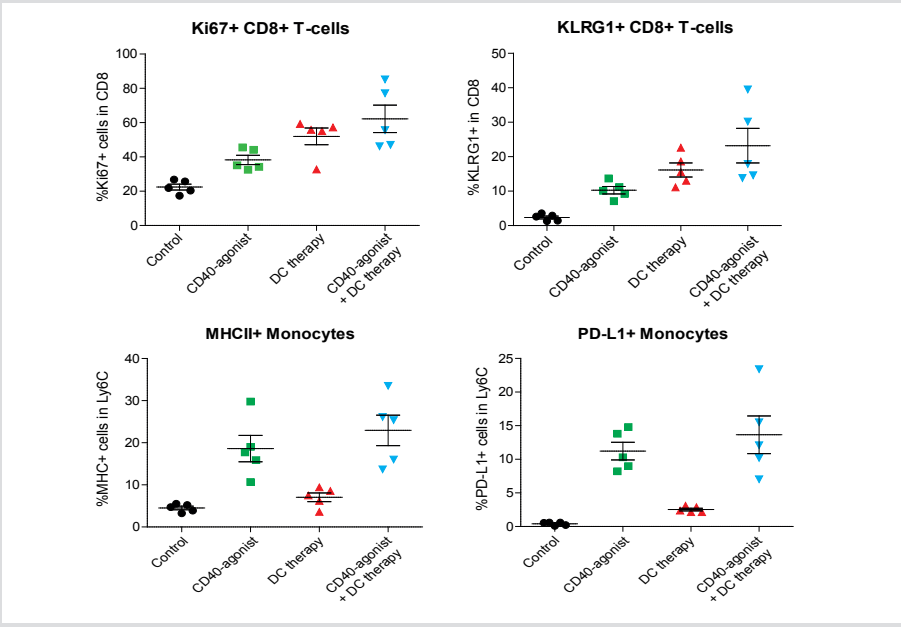


Figure 3 Leukocyte populations in blood during therapy in mesothelioma bearing mice untreated (control) or treated with a CD40-agonist (FGK4.5), DC-therapy or the combination. Cells were gated after exclusion of doublets, dead cells (Amcyan +) and CD45- cells. Monocytes were gated from CD45+ lineage (CD3+ CD19+) negative, CD11b+ LyC6high Ly6-. CD8+ T cells were gated from CD45+ CD3+ lymphocytes. The proportions of CD8+ T cells positive for Ki67 and KLRG1 were assessed, as were the frequencies of MHCII+ and PDL1+ monocytes. All data presented as mean ± SEM.

DISCUSSION

This pilot study demonstrates that the combination of a CD40 agonistic antibody and DC-immunotherapy is feasible in a mesothelioma mouse model. Furthermore, the combination therapy induced additive systemic immune activation compared to the monotherapies. Although the survival difference was not statistically significant compared to the monotherapies, the combination therapy did result in 100% survival, which is promising. Although this pilot study was undertaken based on the hypothesis that a CD40 agonistic antibody could induce a more pro-inflammatory macrophage phenotype in the tumor environment, the current study was designed to investigate the feasibility and effect on survival of the combination of a CD40 agonistic antibody and DC immunotherapy. Therefore, the effect of the antibody on local macrophage phenotype will have to be the subject of future studies. However, the current pilot study did demonstrate that combining a CD40 agonistic antibody with cellular immunotherapy is safe and feasible in mesothelioma mouse models. Furthermore, treatment with a CD40-agonistic antibody in mesothelioma bearing mice enhances survival and alters both myeloid and lymphoid cell populations in the blood following therapy. CD40 is widely expressed on many different cells both in secondary lymphoid organs, in the blood and in the TME [3]. Our tumor cells did not express CD40 *in vitro* or *in vivo* (data not shown) which has been the case for other tumor types [11]. Although we hypothesized that CD40-ligation on TAMs would skew their phenotype and result in better T-cell responses locally in the tumor, the effects of systemic CD40-ligation are most likely diverse in nature. The importance of CD4+ helper T cells for CD8+ T cell function in cancer has long been appreciated and found to be relevant for immunotherapy as T cell therapy greatly benefited from infusion of both CD4+ and CD8+ T cells [4,12]. Dendritic cells express CD40 which enables them to become 'licensed' by CD4+ T cells during CD8+ T cell priming and hence induce stronger effector CD8+ T cell responses. DC-licensing has been found to be primarily important in settings of limited inflammation (e.g. cancer) when additional signals are mandatory for effective T cell priming to occur [13]. Furthermore, different CD8+ T cells are generated when primed in a setting of abundant inflammatory signals such as CD40-signalling [14-16]. When priming occurs during inflammatory settings, short lived effector cells (KLRG1+ CD127-) are formed at the expense of CD8+ memory T cells. The fact that KLRG1+ CD8+ T cells were increased in the blood of mice effectively treated with combination immunotherapy, suggests a potential role of these cells in mediating tumor regressions and prolonging survival. Whether the observed T cells are tumor specific could not be assessed in this model. The survival data and phenotypic analysis of blood suggests an additive effect between treatment with a CD40 agonistic antibody and DC immunotherapy. Which specific alterations in cell function and phenotype underlie this beneficial effect remains to be investigated.

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Chapter 7

Depletion of tumor associated macrophages alone is insufficient to restore anti-tumor immunity but improves cell-based immunotherapy in preclinical models of mesothelioma

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Submitted for publication

ABSTRACT

New immunotherapeutic strategies are needed to (re-)sensitize tumors to the destructive power of the host immune system in all patients. Malignant mesothelioma is a cancer characterized by a poor prognosis, resistance to conventional therapies and a prominent tumor associated macrophage (TAM) infiltrate linked to immune suppression, angiogenesis and tumor aggressiveness. TAM depletion using the M-CSFR-inhibitor PLX3397 could potentially reactivate anti-tumor immunity.

We show, that M-CSFR-inhibition effectively reduced TAMs, circulating non-classical monocytes, neo-angiogenesis and ascites in mesothelioma mouse models, but without improving survival. Combined with DC-therapy, however, survival was synergistically enhanced with a concomitant decrease in TAMs and enhanced CD8+ T-cell frequency and functionality. Finally, combination therapy treated mice were protected from tumor rechallenge and displayed superior T-cell memory responses.

These data indicate that decreasing local immune suppression in the tumor without providing proper immune activation does not result in improved survival but in combination, generates robust and durable antitumor immunity.

INTRODUCTION

With the implementation of checkpoint inhibition therapy for several malignancies, immunotherapy has evolved to become an effective strategy in the treatment of advanced cancer [1]. However, it has become apparent that although a subgroup of advanced disease stage patients benefits and shows prolonged overall survival to these therapies, the majority of patient tumors does not respond or eventually relapses. Efforts have been made to characterize the mechanisms behind immunotherapy efficacy, leading to the distinction between inflamed, immune sensitive tumors, and non-inflamed tumors where there is immunological exclusion or ignorance [2-4]. PD-1 blocking antibodies appear to be most effective in patients with a pre-existing immune cell infiltrate in the tumor, which can be functionally enhanced by this form of immunotherapy [5]. The next breakthrough in cancer immunotherapy will be finding ways to sensitize non-inflamed and resistant inflamed tumors to therapy, thereby increasing response rates and survival in advanced stage cancer patients [2,3].

Malignant mesothelioma (MM) is a cancer with a dismal prognosis that has not improved significantly in the last decades with the broad implementation of conventional cancer treatment modalities [6]. MM is often characterized by a prominent stromal component, dominated by the presence of macrophages [7,8]. These tumor associated macrophages (TAMs) often display an 'alternatively activated' ('M2') immune inhibitory phenotype characterized by the production of interleukin 10 (IL-10) and surface expression of CD206 and inhibitory molecules such as PD-L1 [9]. Furthermore, TAMs have been shown to be critical regulators of angiogenesis and they are closely linked to local tumor outgrowth and pleural fluid mediated immune suppression in MM patients [10-12].

TAMs are known to be highly dependent on macrophage-colony stimulating factor (M-CSF) for their survival, proliferation, and recruitment towards tumors [9,13]. In addition, M-CSF promotes an M2 phenotype [14,15] and its levels in the tumor and circulation are correlated with poor survival in several solid tumor types [16]. For these reasons different approaches to inhibit the M-CSF/M-CSFR pathway have been designed in order to deplete TAMs [17-19]. Broadly, these molecules can be subdivided into antibodies targeting M-CSF (sparing IL-34-M-CSFR signaling), the M-CSFR, and small molecule tyrosine kinase (TK) inhibitors with targeting downstream receptor signaling with different specificities for M-CSFR, c-kit and FLT3 [16]. The advantage of TK-inhibitors over antibody targeted therapies is their capability of targeting both murine and human M-CSFR-signaling improving translatability across species, inhibition of autocrine M-CSF/M-CSFR-signaling, and the lack of rebound monocytopoeiesis after cessation of therapy due to intact receptor-mediated internalization of M-CSF which is TK-independent [13]. PLX3397 is a clinically tested M-CSF-receptor (M-CSFR) and c-kit tyrosine kinase inhibitor shown to be relatively safe and effective in reducing TAMs in several solid tumor types [19,20]. Whether PLX3397 is effective in depleting TAMs in mesothelioma, and possibly capable of restoring anti-tumor immunity in these tumors in currently unknown.

Cell-based immunotherapies such as dendritic cell (DC)- and T-cell based therapies circumvent aberrant antigen presentation and the formation of ineffective immune responses in cancer, possibly explaining their efficacy over conventional tumor vaccines [21]. We have previously demonstrated that DC-therapy is a safe and effective way to generate functional anti-tumor immunity and clinical responses in MM patients [22,23]. We hypothesize that TAM mediated immune suppression could limit DC-therapy efficacy and that depletion of TAMs would improve response rates and response durability in MM models.

We show that PLX3397 is effective in depleting TAMs from mesothelioma but that monotherapy does not improve survival. Combining M-CSFR-inhibition with DC-therapy to induce effective anti-tumor immune responses improved survival in our mouse models with mice being protected from disease following tumor rechallenge. This therapeutic synergy may prove to be an alternative strategy to improve response rates and survival with immunotherapy.

METHODS

Mesothelioma Mouse Models

Female 8-12 week old BALB/c (H-2d) mice (Envigo, Zeist, The Netherlands) and CBA/J mice (Janvier, Hannover, Germany) were housed under specific pathogen-free conditions at the animal care facility of the Erasmus MC, Rotterdam. Experiments were approved by the local Ethical Committee for Animal Welfare. The AB1 cell and AC29 mesothelioma cell lines were kindly provided by Professor Bruce W.S. Robinson of the Queen Elizabeth II Medical Centre, Nedlands, Australia. The cell line was derived from tumors induced by crocidolite asbestos injected intraperitoneally into CBA/J and BALB/c mice [26]. Tumor cells were cultured in RPMI 1640 medium containing 25 mM HEPES, Glutamax, 50 g/ml gentamicin, and 5% (v/v) fetal bovine serum (FBS) (all obtained from Gibco) in a humidified atmosphere and at 5% CO_2, in air. For culture, either T25, T75 or CellSTACKs (Corning Life Sciences) were used to reach appropriate tumor cell frequencies for injection. AB1 and AC29 cells were passaged once or twice a week to a new flask by treatment with 0.05% trypsin, 0.53 mM EDTA in phosphate buffered saline (PBS, all Gibco). The cell line was regularly tested and remained negative for mycoplasma contamination. At the start of the experiment, CBA/J or BALB/c mice were intraperitoneally injected with either 20x10^6 AC29 cells or 0.5x10^6 AB1 cells respectively, dissolved in PBS, or with PBS as control in a volume of 200µl using 25G needle syringes. Mice were inspected daily for signs of disease and a Body Condition Score (BCS) was assessed. When mice reached their predefined humane endpoint (in all cases due to high tumor burden) they were euthanized by CO_2-mediated asphyxiation, and relevant tissues including blood, spleen, tumor and ascites were collected.

Tumor Lysate Production

AB1 cell line-derived tumor lysate was prepared from cells suspended per ml PBS. The cell suspension was frozen in liquid nitrogen and disrupted by four cycles of freeze-thawing followed by sonication for 3x 10 seconds with an amplitude of 10 microns, using a Soniprep 150 ultrasonic disintegrator equipped with a microtip (Sanyo Gallenkamp BV, Breda, The Netherlands) on ice. Cell lysate was aliquoted and stored at -80°C.

Culture Conditions of Bone Marrow-Derived DC Used for Vaccination

DCs were generated following an adapted protocol (Lutz et al. 1999) as previously described [24]. Both femurs, tibiae and fibulae were collected from healthy control mice (8-10 weeks old) and were crushed in a pastel and mortar. After filtration over a 100 µm nylon mesh cell strainer (BD Biosciences), bone-marrow derived cells were lysed and seeded in 100-mm Petri dishes (day 0) and cultured in 10 ml DC Culture Medium [DC-CM]: RPMI 1640 containing glutamax-I (Gibco) supplemented with 5% (v/v) FBS, 50 M -mercaptoethanol (Sigma-Aldrich), 50 g/ml gentamicin (Invitrogen), and 20 ng/ml recombinant murine gran-

ulocyte macrophage-colony-stimulating factor [GM-CSF, kindly provided by Professor B. Lambrecht, VIB Ghent, Belgium]. Cells were cultured at in a humidified atmosphere at 5% CO_2, in air. At day 3, 10 ml of fresh DC-CM was added. On day 6, 10 ml of each plate was replaced with 10 ml of fresh DC-CM. After 9 days of culture, AB1 cell lysate was added to the DC cultures, to the equivalent of three AB1 cell-equivalents per DC. After 8 hours, 10 g/ml CpG (ISS-ODN 1668, Invitrogen) was added to the culture to allow complete maturation while incubated overnight. The next day, DCs were harvested by gentle pipetting and purified by Lympholyte-Mammal (Cedarlane) density gradient centrifugation, the interphase washed three times in PBS and resuspended at a concentration of viable cells. The quality of the DC preparation was determined by cell-counting, morphologic analysis and cell surface marker expression by flow cytometry, as previously described [24].

DC-culture with PLX3397

Mature, lysate pulsed-DCs were generated as demonstrated above and cultured for 48 hours in GM-CSF DC-TCM and varying concentrations of PLX3397 (provided by Plexxikon inc. as part of a material transfer agreement) or the vehicle (Dimethyl sulfoxide, DMSO, Sigma-Aldrich) in 6-well plates. PLX3397 was reconstituted in DMSO to reach a stock concentration of 20mM, which was then diluted to a concentration range of 0.1 to 10µM in DC-TCM. After 48 hours, cells were gently pipetted from the 6-well plate and analyzed for viability and surface expression of immune markers using multi-color flow-cytometry.

Treatment with Tumor Lysate-Pulsed DCs and/or PLX3397

On day 0, BALB/c or CBA/J mice (4 groups each consisting of 6-8 mice) were inoculated intraperitoneally with AB1 or AC29 tumor cells, respectively. On day 10, mice were treated with either 2-3x10^6 DCs dissolved in 200µl PBS, or PBS alone. Also, depending on the treatment arm, mice were fed *ad libitum* PLX3397-containing chow or control chow of equal nutritional value and consistency until the end of the experiment or prior to rechallenge. On day 15 post-tumor cell injection and prior + after rechallenge, blood was extracted via the tail vein while mice were fixated. During the remainder of the follow-up period, mice were examined daily for evidence of illness caused by overt tumor growth. Mice were killed if profoundly ill, according to UKCCCR regulations and body condition score, and were scored as a death in survival analysis. No mice had to be censored for survival analysis.

Immunohistochemistry (IHC) on Tumor Material

Tumor biopsies were embedded in Tissue-Tek II optimum cutting temperature medium (Miles, Naperville, IL, USA), snap-frozen, and stored at at -80˚C . Tissue sections (6 m) were cut on a cryostat (Cryostar NX70, Thermo-Fisher Scientific). Frozen sections were warmed to RT, fixed with acetone for 10 minutes and rinsed in PBS. Sections were incubated in peroxidase blocking solution (0.1% H2O2 and 0.1% sodium azide in PBS) for 30 minutes

and rinsed with PBS. Slides were placed in a semi-automatic stainer (Sequenza) and incubated in 1:10 diluted normal Goat serum (CLB, Amsterdam, Netherlands) for 10 min and subsequently for 60 min with the diluted primary Abs, followed by rinsing in PBS for 5 min and incubation for 30 min with diluted secondary Abs. Double-immunostaining was carried out using PE-conjugated rat anti-mouse CD8 (1:20, clone MEC13.3, BD Biosciences), with FITC-conjugated CD206 (1:80, clone mr5d3, Bio-Rad) and FITC-conjugated F4/80 (1:10, clone BM8, eBioscience) with CD31 antibodies (1:10, clone MEC13.3, BD Biosciences). Binding of antibody was detected using alkaline phosphatase- (AP-) or peroxidase- conjugated goat anti-rat (Sigma-Aldrich) and Naphtol-AS-MX-phosphate (0.30 mg·mL^{-1}; Sigma-Aldrich) + new fuchsine (160 mg·mL^{-1} in 2 M HCl; Chroma-Gesellschaft, Köngen, Germany) or AEC (0.1 M NaAc + 1% AEC stock [100mg AEC in 10ml DMF], Vectorlabs), respectively, were used as substrate. The specificity was checked using a protein concentration-matched non-relevant rat antibody and PBS. Finally, the sections were rinsed in distilled water and mounted in vecta mount (Vector). Slides were scanned using a Nanozoomer 2.0 HT (Hamamatsu).

Quantification of IHC Images

Scanned IHC-slides were viewed using NDP-viewing software (Hamamatsu) at 20x and 40x magnification and regions of interest were captured and imported into ImageJ software (NIH). Colors were separated, thresholds were installed to select for positive cells and these were depicted as percentage of total area. For each sample, 5 random areas (including tumor rim and center) at 20x magnification were selected, analyzed and averaged. CD8-positive cells were well demarcated and counted (average of 5 random tumor areas per sample) to be expressed as cells/mm^2.

Preparation of Single Cell Suspensions from Tissues

Single cell suspensions were generated from the spleens, blood and tumors of mice from each group. All tissues were either weighed in a microbalance (Shimadzu) in case of tumors and spleens, or volume determined for blood. Briefly, spleens were aseptically removed and mechanically dispersed over 100 µm nylon mesh cell strainer (BD Biosciences) followed by erythrocyte lysis using osmotic lysis buffer (8.3% NH_4Cl, 1% $KHCO_3$, and 0.04% Na_2EDTA in Milli-Q). Blood was collected in EDTA tubes (Microvette CB300, Sarstedt) and subsequently lysed. Tumors were collected, and dissociated using a validated tumor dissociation system (Miltenyi Biotec). Cells suspensions were filtered through a 100 µm nylon mesh cell strainer (BD Biosciences) and counted in trypane blue with a hemocytometer using the Burker-Turk method.

Flow Cytometry

For measurements of cytokine production in lymphoid cells by flow cytometry, cells were re-stimulated for 4 h at 37°C using PMA and ionomycin supplemented with GolgiStop (BD

Biosciences). For assessing cytokine production by myeloid cells, cells were only subjected to 4 hours incubation with Golgistop. For cell surface marker staining, cells were washed with FACS-wash (0.05% NaN_3, 2% BSA in PBS) and Fc II/III receptor blocking was performed using anti-mouse 2.4G2 antibody (1:300; kindly provided by L. Boon, Bioceros, Utrecht, The Netherlands). After the blocking procedure, properly diluted antibodies (supplementary table 1) for cell surface staining were added into each sample and placed on ice for 30 minutes protected from light. Cells were washed in FACS-wash followed by a PBS wash, and then stained for viability using fixable LIVE/DEAD aqua cell stain (Thermo-Fisher Scientific, 1:200). After two additional washes with FACS-wash, cells either measured (in case of only surface staining) or in case of intracellular staining; fixated, permeabilized and stained using Fix/Perm buffer (in case of nuclear protein staining including Foxp3 and Ki-67, eBiosience) or 4% PFA and 0.5% saponin (in case of cytokine stainings, Sigma-Aldrich). Antibodies were stained for 30 minutes in case of the PFA/Saponin protocol and 60 minutes for the intranuclear staining protocol, on ice in the dark. A fixed number of counting beads (Polysciences Inc.) was added prior to data acquisition to determine the absolute amount of cells. Data were acquired using an LSR II flow cytometer (BD) equipped with three lasers and FACSDiva software (BD) and analyzed by FlowJo (Tree Star Inc., USA) software V10.1.

Statistical Analysis

Data are expressed as medians with interquartile range. Comparisons between groups were made using the Mann-Whitney U-test for independent samples, or the Wilcoxon signed rank test in case of paired samples. When correlations were depicted, Spearman's rank correlation test was performed to test for statistical significance. A two-tailed value of $p<0.05$ was considered statistically significant. Survival data were plotted as Kaplan-Meier survival curves, using the log-rank test to determine statistical significance. Data was analyzed using Graphpad Prism software (Graphpad, V5.01)

RESULTS

DC-therapy synergizes with M-CSFR-inhibition in orthotopic mesothelioma mouse models

In the past we have shown that applying DC-therapy in syngeneic and orthotopic mesothelioma mouse models is an effective and translational system for assessing anti-tumor T-cell responses and evaluating treatment efficacy [24,25]. In both the BALB/c model (using the AB1 mesothelioma cell line) and the CBA/J model (AC29 cell line) tumor cells are injected intraperitoneally (i.p.). Compared to the BALB/c model, the CBA/J has a more pronounced TAM-dependent phenotype (including higher expression of M-CSF, data not shown) and mice develop ascites, reflecting disease heterogeneity [26-28]. The M-CSFR-inhibitor PLX3397 was used to target TAMs in our models. To ensure that there was no direct effect of PLX3397 on the murine mesothelioma cell lines, expression of both receptors was determined using flow cytometry and tissue microarray analysis, which showed negligible levels of both molecules (data not shown). Also, to exclude any direct effects of PLX3397 on the DCs administered, maturated tumor-lysate pulsed DCs were cultured *in vitro* with increasing levels of PLX3397 or vehicle alone and cell viability and membrane expression of several relevant surface markers was assessed using multicolor flow cytometry (Fig. S1). As previously reported by others, GM-CSF-cultured cells are comprised of a heterogeneous population of dendritic cells and macrophages, which we could also identify in our system (Fig. S1A) [29]. There was no major effect of PLX3397 on DC viability (Fig. S1A) or surface expression of MHC-II, CD86, PD-L1, M-CSFR, and c-kit (data not shown). There was a minor decrease in viability beyond therapeutic concentrations (>3μM), which occurred in the macrophage fraction of the heterogeneous cell population (data not shown). This lack of DC-sensitivity towards PLX3397 could be explained by the rapid down-regulation of M-CSFR surface expression following maturation using unmethylated CpG (Fig. S1B-C).

Mice were treated at day 10 when solid tumors had established i.p. and at the site of injection [24,30] and the M-CSFR-inhibitor PLX3397 was administered in chow at the day of DC-injection and continued for duration of the experiment unless indicated otherwise (Fig. 1A-B). PLX3397 monotherapy did not improve survival in both mesothelioma tumor models (Fig. 1C-D). DC-therapy prolonged survival only in the AC29 tumor model. Combination therapy, however, significantly enhanced survival in both tumor models (Fig. 1C-D).

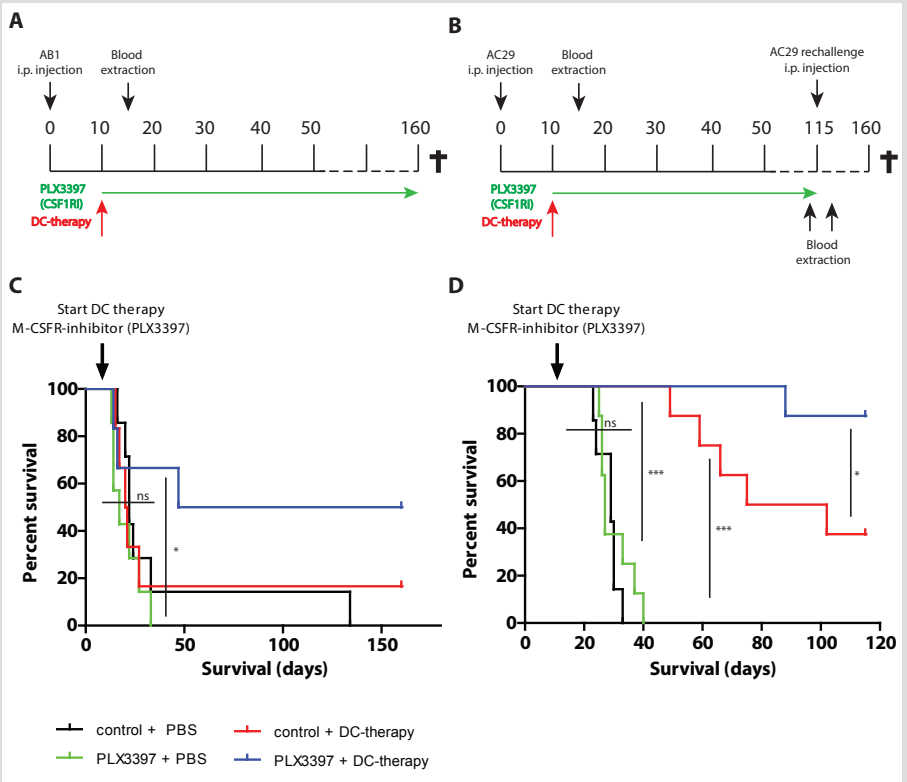

Figure 1 Efficacy of DC-therapy is synergistically enhanced by combination therapy with TAM-depletion in mesothelioma mouse models.
(A-B) Wildtype, female BALB/c (n=6/arm) and CBA/J (n=8/arm) mice were intraperitoneally injected with either 0.5x10^6 AB1 or 20x10^6 AC29 syngeneic tumor cells, respectively. Mice were then treated once on day 10 with either intraperitoneal administration of PBS or mature, autologous tumor lysate loaded dendritic cells. Concurrently, mice were started on control or PLX3397-containing chow until the end of the experiment or until rechallenge. Blood was extracted on t=15 and before and after rechallenge in case of CBA/J mice. Animals were monitored daily for the duration of 160 days and euthanized in case of severe illness.
(C-D) Kaplan-Meier curves of the survival experiments in CBA/J (AC29) and BALB/c (AB1) mesothelioma tumor models. Statistical significance was determined using the Log-rank test with $p<0.05$ being statistically significant. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant

M-CSFR-inhibition causes a decrease in CD206+ F4/80+ TAMs, blood vessel density and ascites.
No gross macroscopic changes in tumor volume between untreated and M-CSFR-inhibitor treated mice was found, reflecting the lack of a difference in survival between these two groups (data not shown). To assess whether M-CSFR-inhibition was effective in depleting TAMs, we compared tumors of the more TAM-dependent CBA/J mice treated with or without PLX3397 using immunohistochemistry (IHC). Microscopically, there was an evident decrease in F4/80+ TAMs, CD31+ endothelial cells and CD206+ (M2) cells in treated mice

compared to control mice (Fig. 2A-G). It has been recently shown that TAMs are crucial for tumor angiogenesis and concomitant ascites production in metastatic ovarian carcinoma[31]. In line with these findings we found a strong correlation between CD31+ endothelial and TAM areas in these tumors and a decrease in ascites volume in the M-CSFR-inhibitor treated mice (Fig. 2D-E). Tumors of untreated mice already contained a CD8+ T-cell infiltrate which was not further enhanced following TAM-depletion (Fig. 2H).

DC-therapy induces CD8+ T-cell responses and decreases Tregs whereas M-CSFR-inhibition specifically depletes non-classical monocytes in blood during therapy

We next sought to investigate the potential mechanisms that lead to enhanced survival in DC-therapy only and combination immunotherapy arms. To observe the presence of functional anti-tumor immune responses underpinning prolonged survival in these mice, we extracted blood 5 days after start of treatment and analyzed the circulating immune compartment using multicolor flow cytometry (Fig. S2) DC-therapy produced a significant increase in CD8+ T cells with CD4+ T-helper cells remaining at a constant level, whereas proliferation was increased in both T-cell subsets (Fig. 3A-D, S3A). T-regulatory cells (Tregs) were decreased following DC-therapy and this was further amplified by the addition of PLX3397 mediated M-CSFR-inhibition, resulting in an improved CD8+ T-cell/Treg-ratio (Fig. 3C, Fig. S3B). Besides proliferation, CD8+ T cells from DC-therapy treated mice were predominantly of an effector phenotype, illustrated by the expression of Killer-cell lectin like receptor G1 (KLRG1) and CD4-CD8 double positivity (Fig. 3D-F) [32,33]. These CD4-CD8 double positive cells were also highest in proliferation in all treatment arms as demonstrated by a higher fraction of cells being Ki-67-positive (Fig. S3C-D).

Whereas DC-therapy primarily influenced lymphocyte dynamics and phenotype, PLX3397 therapy predominantly affected myeloid subsets (Fig. 4). Granulocytes and total monocytes were increased by DC-therapy but only granulocytes were expanded by PLX3397 as mono- and combination therapy (Fig. 4A-B). Monocytes can be subdivided into classical Ly6Chi- and non-classical Ly6Clow monocytes with each subset having different functions and migration patterns in blood [34,35]. Dissecting monocyte subsets in our model revealed near complete depletion of non-classical (Ly6Clow) monocytes in the PLX3397 treated arms, whereas classical (ly6Chi) monocytes increased (Fig. 4C-D). Interestingly, non-classical monocytes were highest in PD-L1 expression compared to their Ly6Chi classical counterparts, which was also significantly decreased by M-CSFR-inhibition (Fig.4E). Classical monocytes reached equal numbers and levels of PD-L1 expression in blood of DC-therapy and combination therapy treated mice (Fig. 4D,F). Overall, the observed synergy between therapies was illustrated by an improved CD8+ T-cell phenotype which was primarily DC-therapy mediated, and a decrease in predominantly PD-L1+ non-classical monocytes due to M-CSFR-inhibition. Similar patterns in blood immune cell dynamics could be discerned in the AB1 tumor model, however, numbers of mice were limited at day 5 after start of treatment (Fig. S4).

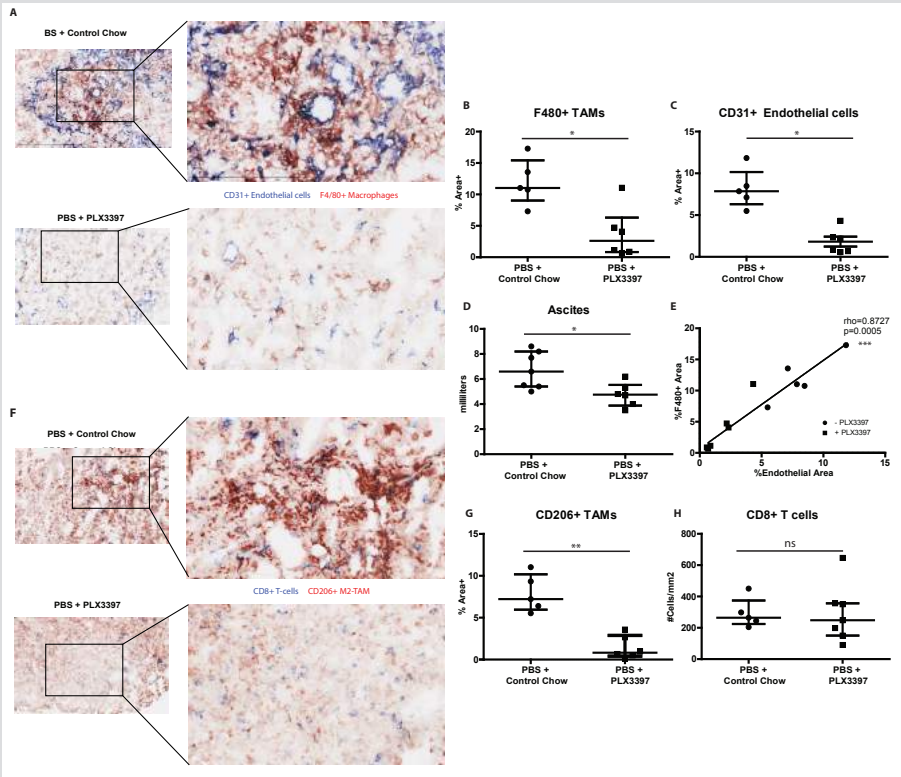

Figure 2 TAM depletion causes a decrease in ascites production in CBA/j mice accompanied by a decrease in CD206+ F4/80+ macrophages and blood vessel density.

(A) Tumors of untreated and PLX3397 only treated mice were extracted and tissue sections were double stained for tumor associated macrophages (TAM), (F4/80, *red*) and endothelial cells (CD31, *blue*) using immunohistochemistry (IHC). Tissues are displayed at a 20X magnification (error bar length is 400μm) with a further close-up at 40X magnification (error bar length is 200μm).

(B-C) F4/80+ TAMs and CD31+ endothelial cells were quantified in both groups using ImageJ software on 5 independent tumor sections (including tumor center and rim) at 20X magnification and averaged to be expressed as percentage of total area.

(D) When mice were sacrificed at end stage disease, ascites was aspirated and the volume was measured and expressed as milliliters.

(E) TAM- and endothelial cell density was correlated in untreated (*circles*) and PLX3397-treated (*squares*) mice.

(F) Tissue sections were double stained for CD206-positivity (M2-TAM marker, *red*) and CD8 (cytotoxic T cells, *blue*) using IHC.

(G) CD206+ positive cells were quantified similar to (B-C) and expressed as percentage of total area.

(H) CD8+ T cells were counted (5 individual areas per tumor and averaged) and expressed as cells per cubic millimeter of tumor area (mm^2).

All data are displayed as dot plots including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test with $p<0.05$ being statistically significant. The Spearman's Rank Correlation coefficient (rho) was determined in case of Fig. 2E. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, TAM= tumor associated macrophage.

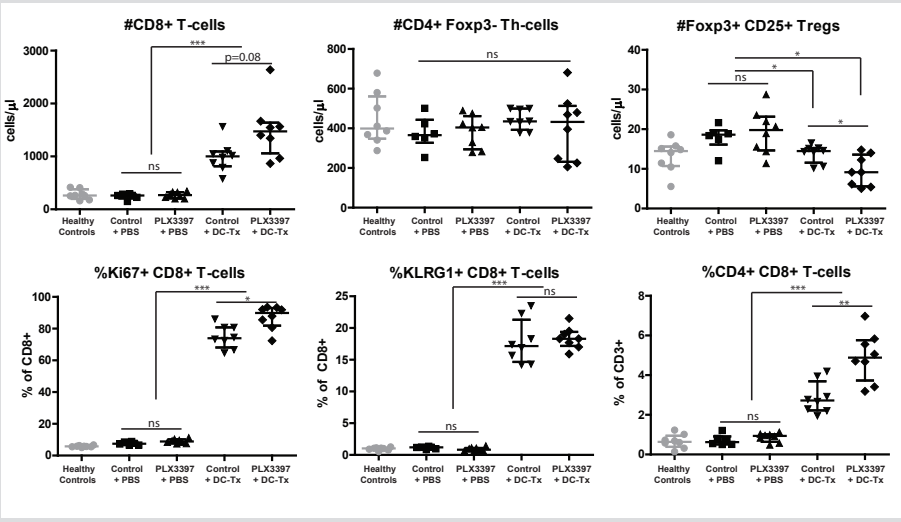

Figure 3 CD8+ T-cell proliferation and effector phenotype are further enhanced when combining TAM-depletion with DC-therapy whereas Tregs decrease in blood during therapy.

(A-C) Blood was extracted 5 days after start of treatment (day 15 after tumor cell injection) from all CBA/J mice (n=8/group) and was analyzed by multicolor flow cytometry. Counting beads were used to quantify populations as cells per µl blood. Immune cell-subsets were characterized as displayed in supplementary figure S2A.

(D-F) CD8+ T cells were further analyzed for percentage of proliferating (Ki67+ in D), and effector (KLRG1+, in E or CD4+, in F) cells.

These data are representative of two independent experiments. All data is displayed as dot plots with including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test with $p<0.05$ being statistically significant. Healthy controls were measured to depict cell frequencies and phenotypes in the non-tumor bearing host, but were not included in further statistical testing. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, Th cells= T-helper cells, Tregs= T regulatory cells.

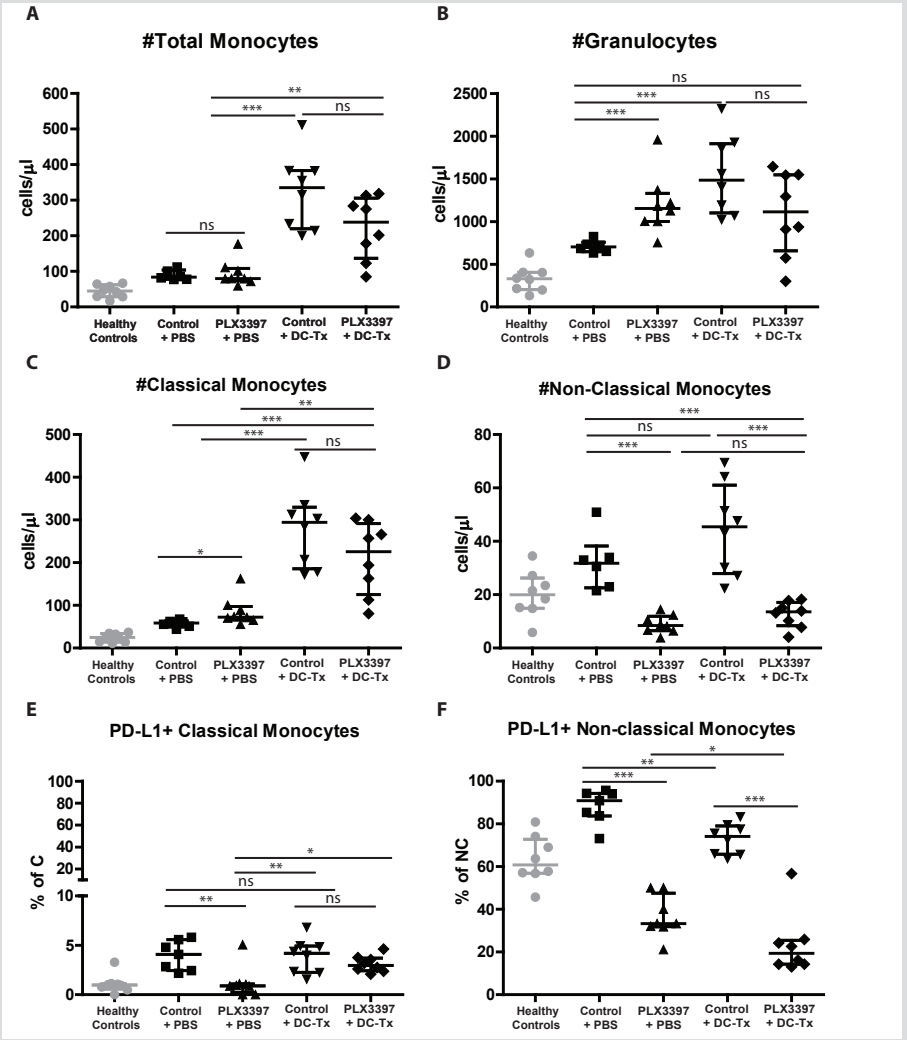

Figure 4 Non-classical monocytes are specifically depleted following M-CSFR-inhibition and these cells are highest in PD-L1 expression in blood of mice during therapy. **(A-B)** Total monocytes (A) and granulocytes (B) in treated tumor bearing CBA/J mice were measured in parallel with the lymphoid cell subsets depicted in Fig. 3. **(C-D)** Monocytes were further classified into classical (Ly6Chigh) and non-classical (Ly6Clow/-) monocytes and expressed as number of cells per µl blood.

(E-F) PD-L1 positivity was determined on both monocyte subsets and expressed as percentage of PD-L1-positive cells in each subset.

These data are indicative of two independent experiments. All data is displayed as dot plots with including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test with $p<0.05$ being statistically significant. Healthy controls were measured to depict cell frequencies and phenotypes in the non-tumor bearing host, but were not included in further statistical testing. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, PD-L1= programmed death- ligand 1.

Tumors of combination therapy treated mice exhibit a favorable tumor microenvironment characterized by low IL-10+ TAMs and increased functional CD8+ T cells during therapy

Circulating immune cell subsets could be manipulated early after DC-therapy and M-CSFR-inhibition but whether the tumor microenvironment (TME) was equally affected during treatment was unclear. Therefore, we sacrificed mice at day 15 to examine the TME for the effects of treatment and to relate possible changes in immune cell composition to those observed in spleen and blood. Five days after commencing therapy there was already a considerable tumor burden which was at that point still comparable between the different treatment groups (Fig. 5A). TME immune composition at day 15, however, markedly differed between treatment groups in line with interim analysis in blood and end stage disease tumors of diseased mice (Fig. S5A). M-CSFR-inhibition diminished both IL-10+ (M2) and IL-10- (M1) TAMs as monotherapy and in combination with DC-therapy (Fig. 5B). Confirming earlier results, the number of CD8+ TILs was not altered following PLX3397 monotherapy, and these TILs displayed an exhausted phenotype [36], defined as PD-1 positive, lymphocyte-activation gene 3 (LAG-3) positive, and interferon-gamma (IFN-γ) negative (Fig. 5C). DC-therapy in contrast, enhanced the number of non-exhausted CD8+ T-cells in the tumor (Fig. 5C).

As circulating non-classical monocytes and TAMs were preferentially depleted by PLX3397, we assessed whether these cell types were correlated to each other. Non-classical monocytes correlated with gross TAMs whereas classical monocytes did not (Fig. 5D). This correlation became more significant when non-classical monocytes were related to IL-10+ TAMs in contrast to the classical monocytes (Fig. 5E). We also further characterized non-classical monocytes as being higher in IL-10 and PD-L1 expression, whereas these cells were lower in surface MHC-II, further establishing the immune suppressive phenotype of these cells (Fig. 5F). In contrast to tumor burden at day 15, tumor induced splenomegaly was abolished in PLX3397 treated mice, which was strongly linked to the depletion of splenic macrophages (Fig. S5B-F). Similar findings regarding macrophage- and monocyte depletion were made in the spleens of mice during treatment, but in contrast to the TAMs, splenic macrophages were strongly correlated to classical monocytes (Fig. S5G-H).

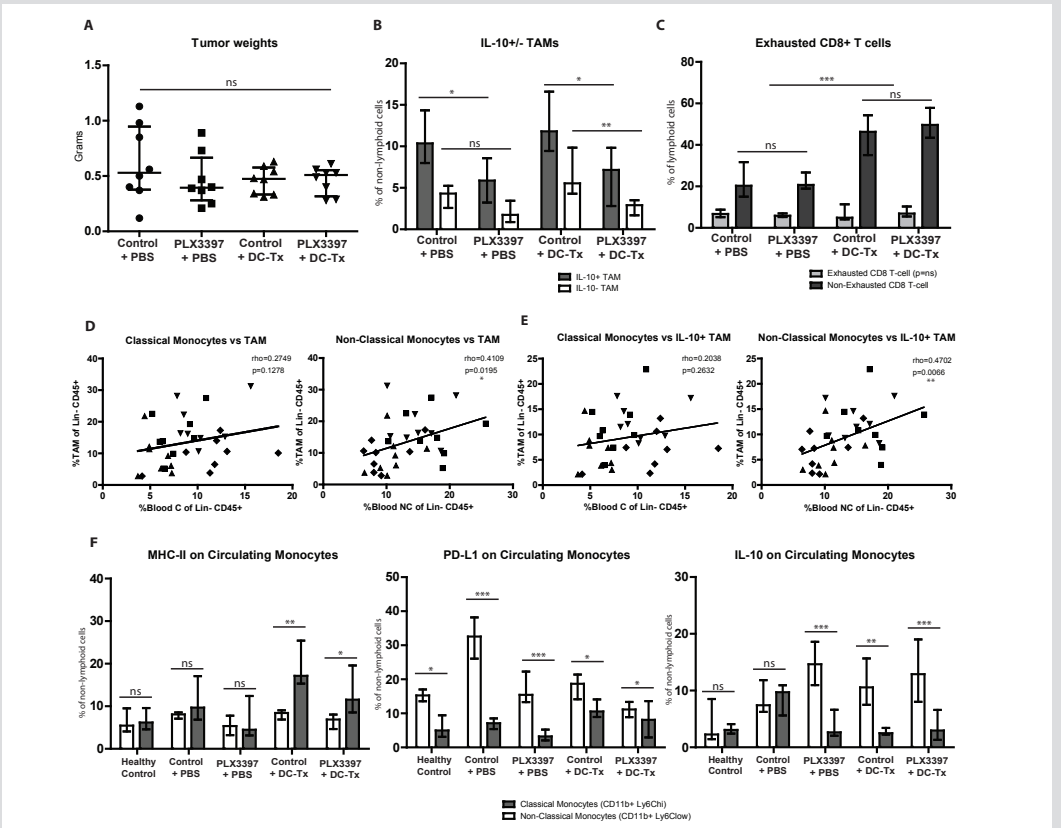

Figure 5 M-CSFR-inhibition decreases tumor-associated macrophages in mesothelioma whereas DC-therapy alters CD8+ TIL phenotype in mice during treatment. Identical to previous experiments, CBA/J mice were intraperitoneally inoculated with tumor cells and treated on day 10 with either DC-therapy or control PBS, and/or continuous PLX3397- or control treatment. Only now, mice were sacrificed on t=15 days to examine the tumor microenvironment, blood and spleen in the different treatment groups.
(A) Tumors were extracted from the peritoneal cavity and weighed.
(B) Tumors were dissociated using a commercially available kit generating single cell suspensions that were stained and analyzed by flow cytometry. To assess IL-10 production by TAMs, cells were pre-treated for 4 hours with Golgistop, followed by membrane and intracellular staining and sample acquisition. TAMs were divided into IL-10 positive and negative and denoted as percentage of non-lymphoid cells, to correct for changes in lymphoid cells due to treatment.
(C) Similar to (B), CD8+ tumor infiltrating lymphocytes (TILs) were identified after 4 hours of *in vitro* stimulation but now, cells were also stimulated using phorbol myristate acetate (PMA) and ionomycin with Golgistop followed by antibody staining and analysis. The distinction was made between 'exhausted' (Program Death 1+ [PD-1], Lymphocyte-activating-gene 3+, [LAG3+] & Interferon-gamma-[IFN-γ]) and 'non-exhausted' (PD-1-, LAG3-, IFN-γ+) CD8+ TILs. Cells were depicted as percentage of total lymphoid cells, to correct for changes in the myeloid compartment due to treatment
(D-E) Blood classical- and non-classical monocytes (as percentage of non-lymphoid cells) were correlated to total TAMs (D) or IL-10+ TAMs (E).
(F) Circulating classical- and non-classical monocytes were further characterized by assessing surface expression of PD-L1, MHC-II and IL-10 by flow cytometry. All data is displayed as dot plots or bar graphs including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test or in case of correlations using the Spearman's Rank Correlation test with $p<0.05$ being statistically significant. Healthy controls were measured to depict cell frequencies and phenotypes in the non-tumor bearing host, but were not included in further statistical testing.
*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, TAM= tumor associated macrophage, MHC-II= major histocompatibility complex-II, IL-10= interleukin 10.

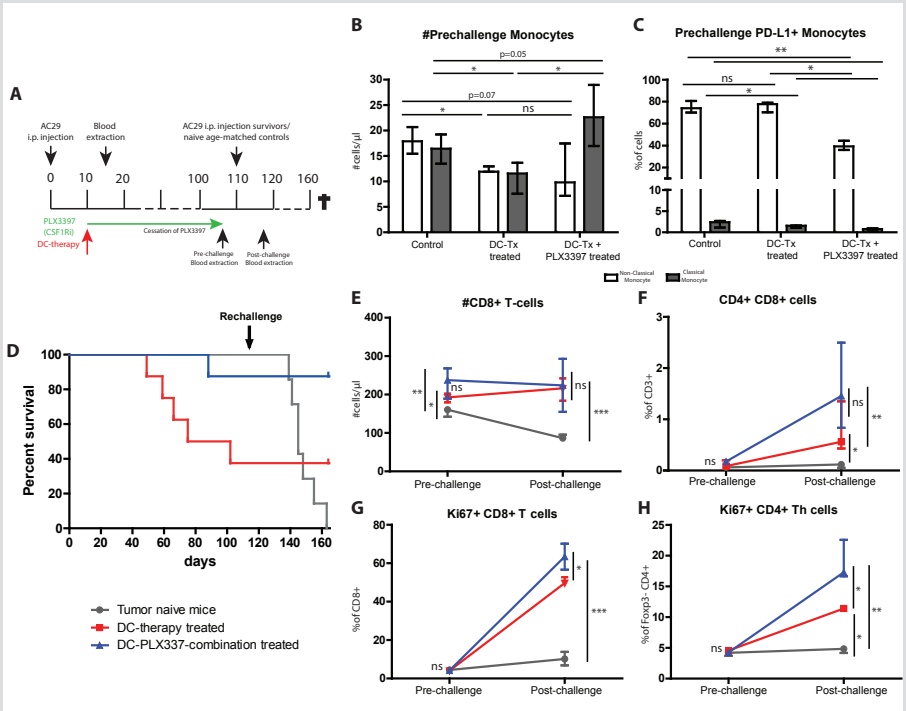

Figure 6 Mice treated with DC-therapy and/or M-CSFR-inhibition were protected from tumor-rechallenge with combination therapy treated mice displaying superior recall responses. **(A)** Surviving mice from Figure 1 and age-matched tumor naïve mice were rechallenged with 20x10^6 tumor cells intraperitoneally and were subsequently monitored for signs of illness. Three days prior to rechallenge, PLX3397-treatment in the combination therapy treated mice was terminated and mice were put on control chow for the further duration of the experiment. Also, blood was extracted at that time point, and again eight days after rechallenge. **(B-C)** Monocyte subsets and related PD-L1 expression was assessed by flow cytometry in blood prior to rechallenge.

(D) Kaplan-Meier curve of survival following rechallenge. For DC-only *(red)* and combination therapy treated mice *(blue)*, curves were identical to the KM-curve in figure 1 but on day t=110, mice were rechallenged to observe whether treated mice were protected from cancer recurrence. Tumor naïve littermates *(grey)* were injected with tumor cells in parallel to ensure tumor pathogenicity and correct for age-dependent issues.

(E-H) Several T-cell features were analyzed in DC-only, DC+PLX3397 combination and untreated mice before and after rechallenge.

All data is displayed as bar graph or line graphs including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test with $p<0.05$ being statistically significant. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, PD-L1= programmed death-ligand 1.

Mice treated with DC-therapy and/or M-CSFR-inhibition were protected from tumor-rechallenge with combination therapy treated mice displaying superior recall responses

To test whether surviving mice were free of tumor and protected against a second tumor encounter, they were re-challenged with the same dose of mesothelioma cells in parallel

with age-matched tumor naïve mice (Fig. 6A). Prior to rechallenge, PLX3397 treatment was stopped and blood was extracted before and after tumor cell injection. Before rechallenge, combination therapy-treated mice exhibited increased levels of classical monocytes compared to DC-monotherapy treated mice, whereas non-classical monocyte levels were comparable (Fig. 6B). While PD-L1 expression was negligible on classical monocytes, monocyte frequency was significantly lower in mice treated with both therapies (Fig. 6C). After rechallenge, all tumor naïve mice reached their humane endpoint due to high tumor burden whereas DC-therapy treated and combination therapy treated mice remained disease free (Fig. 6D). Following cessation of treatment and tumor rechallenge, monocytes and related PD-L1 levels in combination therapy treated mice reached those of DC only treated mice (data not shown). CD8+ T-cell levels were higher in protected mice before and after challenge compared to control mice, and CD8/CD4 double positive cells were similarly elevated after rechallenge (Fig. 6E-F). The same increase in Ki-67-positivity during the primary immune response was also evident in CD8+ T cells and CD4+ T-helper cells of combination therapy treated mice during the recall-response, indicating superior CD8+ T-cell memory in these mice (Fig. 6G-H).

Taken together, these findings demonstrate that targeting the TME and simultaneously directing the immune system to combat cancer can lead to durable responses in mesothelioma bearing mice.

DISCUSSION

We have shown that combination therapy using two safe anti-cancer treatment strategies being DC-therapy and M-CSFR-inhibition (PLX3397) results in improved overall survival and superior immune reactivity towards the tumor. Whereas TAM-depletion in its self was insufficient to increase and improve anti-tumor T-cell responses, DC-monotherapy improved survival in mice but still most mice progressed after treatment, similar to a fraction of patients that develop resistance immunotherapy [22,23,37]. When we analyzed these tumors to look for mechanisms of therapeutic resistance, there were dense CD206+ F4/80+ TAM infiltrates similar to untreated mice (*unpublished observations*). TAMs are a major leukocyte subset in the stroma of mesothelioma and other cancers and capable of potently suppressing endogenous or treatment-induced anti-tumor T-cell responses [9,12]. Depletion of this immune suppressive cell type may thus render previously immune resistant or escaped tumors sensitive to checkpoint inhibition [38], chemo- and radiotherapy [39-41], and cellular therapies such as adoptive T-cell transfer [42,43] and now also DC-therapy. Therapies such as chemo(radio)therapy and PD-1 checkpoint inhibitors, however, come with significant side effects and appear to be most effective in boosting a pre-existing anti-tumor T cell response [3,4]. DC-therapy effectively induces novel anti-tumor immune responses, which can be further sustained and improved by targeting the generally immune suppressive TME [24,44].

M-CSFR-inhibition has been shown to effectively deplete TAMs in multiple tumor models but its effects on survival as monotherapy or combined with other therapies differ considerably between systems. For example, when M-CSFR-inhibition was combined with a tumor vaccine or with certain chemotherapeutic drugs, efficacy of these therapies was abrogated, questioning the role of TAMs in tumor behavior and response to these therapies [18,45,46]. In our models, M-CSFR-inhibitor monotherapy was insufficient to prolong survival on its own despite gross changes in stromal composition. These differences in drug efficacy may be explained by TAM-dependency of certain therapies, mechanism of M-CSFR-inhibition, timing of therapy and tumor stage and type [46]. Using the M-CSFR-inhibitor BLZ945 as monotherapy, Pyonteck *et al.* showed significant improvement of survival in glioblastoma bearing mice [18]. Although resistance to monotherapy ensued in the majority of mice, tumors regressed due to skewing rather than depletion of M2 TAMs. They then showed that this skewing was dependent of glioblastoma cell-derived factors including granulocyte-macrophage colony stimulating factor (GM-CSF) [18,47]. GM-CSF is often used to culture pro-inflammatory M1 macrophages and DCs whereas M-CSF cultured macrophages display a pronounced M2 phenotype [14,29,48]. In other tumor types such as mesothelioma, both patient and mice produce considerable amounts of M-CSF, but only sparsely produce GM-CSF [49] (*unpublished observations*) being a possible explanation for the depletion, rather than skewing of TAMs in our models.

Although there were CD8+ TILs present in mesothelioma tumors of untreated mice, these cells did not increase in frequency or functionality following TAM-depletion and displayed an

exhausted, dysfunctional phenotype. By inducing an immune response using DC-therapy in late stage disease, survival was unaltered in BALB/c mice, or prolonged in the CBA/J model with most mice showing disease progression/recurrence during follow-up. These responses were only durable in the majority of mice when DC-therapy was applied in a TAM-deficient tumor environment. We could detect this synergy between therapies in blood T cells of mice during therapy and following rechallenge, but not explicitly in the tumor on day 15. This may be a time-dependent process (e.g. T-cell persistence in the tumor) or a still unknown mechanism responsible for treatment combination efficacy. However, as (IL-10+) TAMs were effectively depleted and tumor vasculature was normalized following M-CSFR-treatment, T-cell functionality and persistence likely benefited [50].

Despite the fact that we could not fully exclude an indirect effect of TAMs on CD8+ T cells via DCs, as was shown for breast cancer by Ruffell and colleagues [51], we did not observe intratumoral DCs in our murine mesothelioma models (data not shown). Supportive of these observations is the decrease or lack of circulating DCs and loss of DC immune stimulatory capacity in mesothelioma patients, which correlated with a worse survival [52]. This highlights the limited functionality of endogenous anti-tumor immune induction and acquisition of a functional TIL pool in these tumors, and advocates the use of immunotherapeutic strategies that broaden and improve novel anti-tumor immune reactions such as DC-therapy [53,54].

Uncertainty remains about the ontogeny of TAMs in solid tumors. While it is appreciated that classical (Ly6Chi) monocytes are known to be the main precursors of inflammatory DCs and macrophages including TAMs, incongruences exist regarding the sequence of events that precludes M2-TAM development [55-57]. Seminal studies by Geissmann and Yona *et al*. have shown that classical monocytes are obligatory precursors of non-classical monocytes, and that the functions and migration patterns of both cell types differ, with non-classical monocytes being crucial for endothelial and tissue integrity [34,35,58]. Others have also previously described non-classical monocytes to be particularly dependent on M-CSF for their survival [13,59]. The fact that non-classical monocytes and TAMs share a similar immune suppressive phenotype and are most sensitive to M-CSFR-inhibition suggests a likely relationship between these cell types. Future research will have to further delineate the contribution of non-classical monocytes to TAMs in cancer.

The spleen has been reported to be a major contributor of macrophage and neutrophil precursors to tumors [60]. Coincidentally, we observed a dramatic decrease in spleen weight in M-CSFR-inhibitor treated mice (which was closely linked to the depletion of splenic macrophages) but not in total spleen cell numbers (data not shown). Although we cannot exclude the possibility that depletion of TAM precursors in the spleen by M-CSFR-inhibition negatively impacted the number of macrophages in the tumor, there was no clear correlation between the two cell populations (data not shown) which is in line with more recent data confirming the spleen to be a minor contributor of myeloid cells in tumors [61].

In conclusion, we have demonstrated that therapeutic efficacy of M-CSFR inhibition as monotherapy is limited in mesothelioma, and that this efficacy likely depends on the tumor type and remaining tumor-derived molecules produced. TAM-depletion combined with an effective DC-mediated anti-tumor T-cell response is capable of producing durable tumor responses and functional anti-tumor immunity.

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SUPPLEMENTARY FIGURES

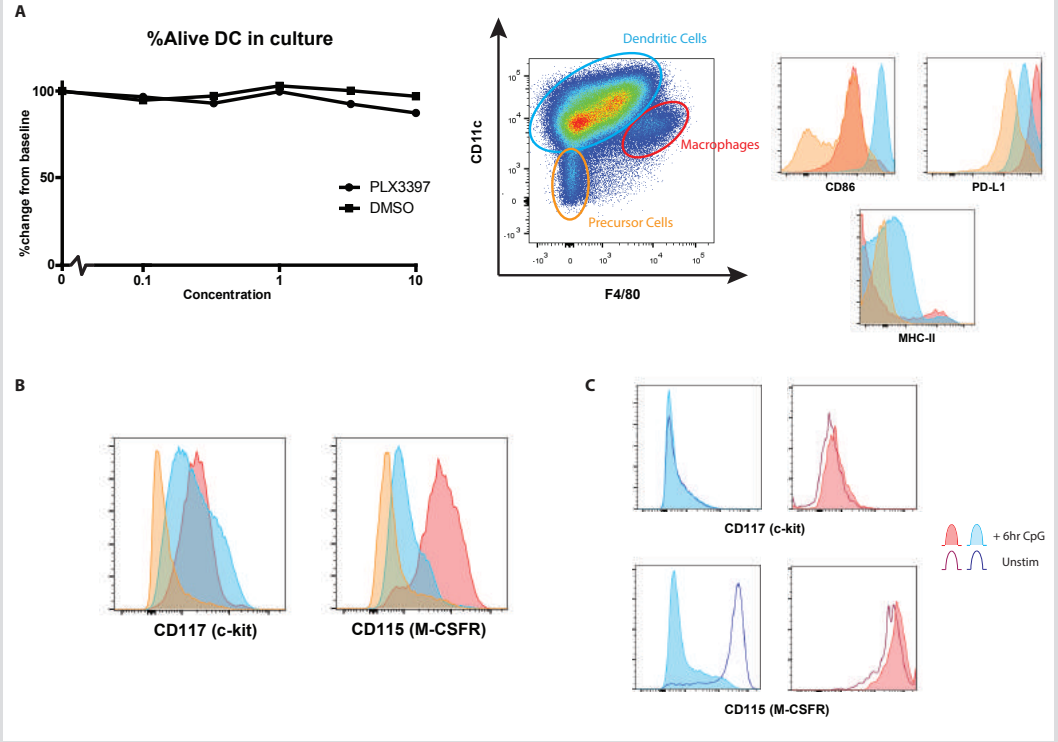

Figure S1 GM-CSF cultured dendritic cells are minimally affected by PLX3397. Mature, lysate loaded DCs were cultured for 48 hours with varying concentrations (no adds, 0.1µM, 0.33 µM, 1.0 µM, 3.33 µM, 10.0 µM) of PLX3397 or the vehicle DMSO to assess effects of the inhibitor on DC-survival and phenotype.
(A) DC-viability was assessed using a live/dead marker and measured by flow cytometry. For all conditions, 1x10^6 cells were added to each well and viability was expressed as percentage from baseline (being DC with no adds after 48 hours). In the upper middle panel, the populations derived from the GM-CSF-based culture are depicted based on differential expression of CD11c and F4/80, with DCs being CD11c high-F4/80 low-intermediate *(blue)*, macrophages being CD11c intermediate – F4/80 high *(red)*, and precursor cells *(orange)* being double negative for both markers. In the upper right panel: differential expression of CD86, PD-L1 and MHC-II on the different cell populations after 48 hours of culture without adds.
(B) expression of CD115 (M-CSF-receptor) and CD117 (c-kit) on the cell populations in (A) after 48 hours of culture without adds.
(C) Dynamics in CD115 and CD117 expression in (unstimulated) DCs *(blue)* or macrophages *(red)* either unstimulated and immature *(clear)* or after six hours of stimulation with unmethylated CpG motifs *(filled)*.

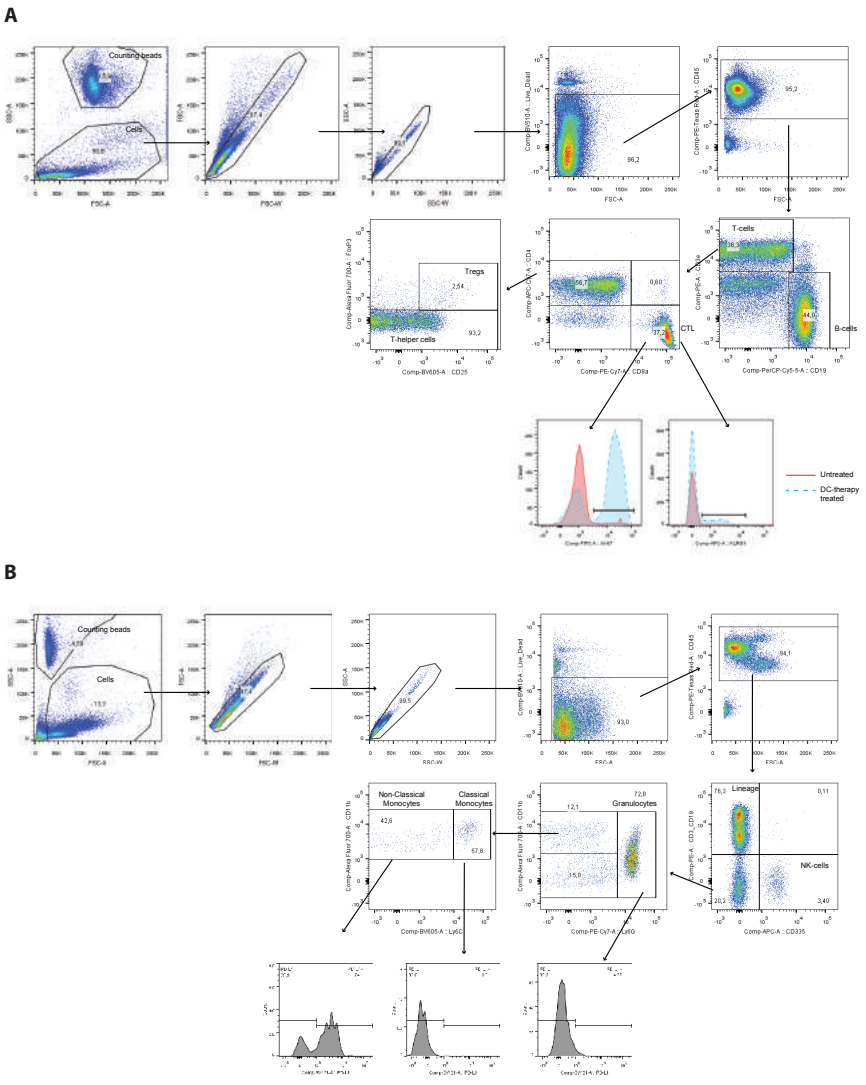

Figure S2 Gating of immune cell subsets in the blood of mice during therapy. **(A)** Immune cell subsets were defined based on markers derived from literature and measured by multicolor flow cytometry
(B) Cells were derived from lysed blood, tumor and spleen derived single cell suspensions. Counting beads, doublets and dead cells were gated out leaving viable single cells for subsequent immunophenotyping. For the analysis of myeloid cells, gating was preceded by removal of lymphocytes and NK-cells using a lineage mix (CD3+, CD19+ CD49b+).

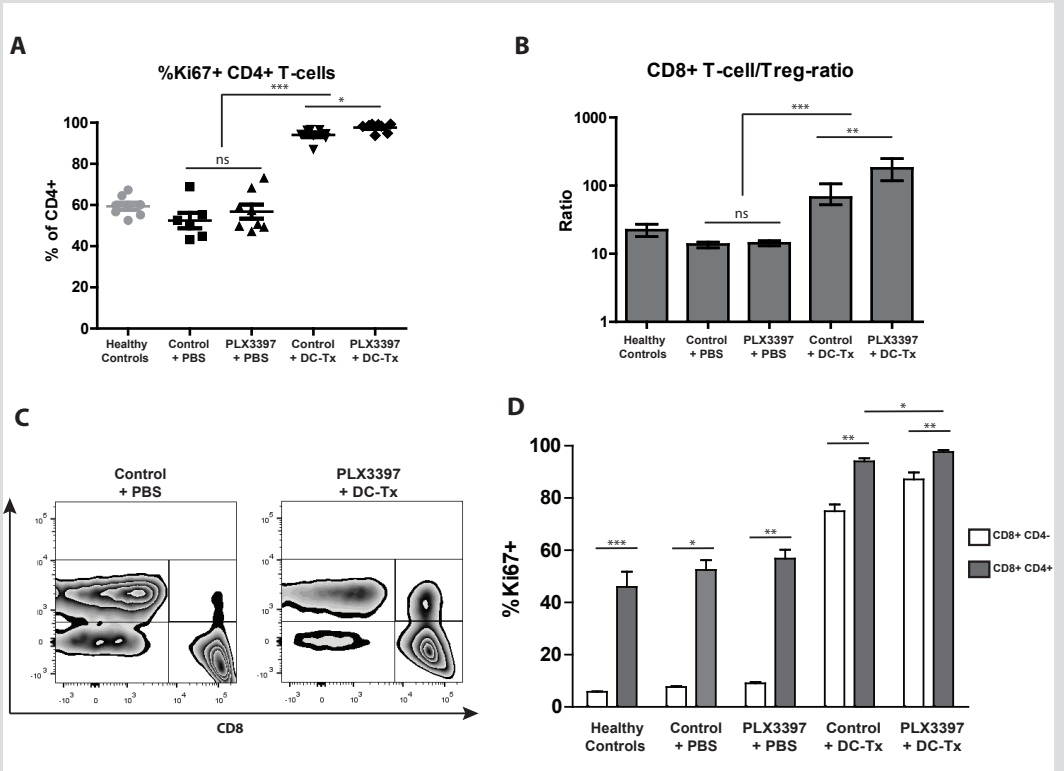

Figure S3 CD4/CD8 Double positive T-cells are most abundant after DC-TAM-depletion combination therapy and highest in proliferation marker Ki-67 compared to single positive CD8+ T cells. **(A)** Similar to Figure 3D, proliferation was assessed in circulating CD4+ T helper cells on day 5 following treatment using the intracellular marker Ki-67. Proliferating cells are depicted as percentage of total CD4+ cells.

(B) The ratio of absolute CD8+ T cells and T regulatory cells in blood on day 5 following start of treatment was calculated and depicted on a log axis.

(C-D) CD4+ CD8+ double positive cells were assessed in all treatment groups but were especially evident in the combination therapy treated group. In the right panel, Ki-67-positivity in the parent population is depicted in CD4+ CD8+ double positive *(grey)* and CD8+ single positive cells *(white)*.

Data is displayed as dot plot or bar graph including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test with $p<0.05$ being statistically significant. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, Treg = T-regulatory cell

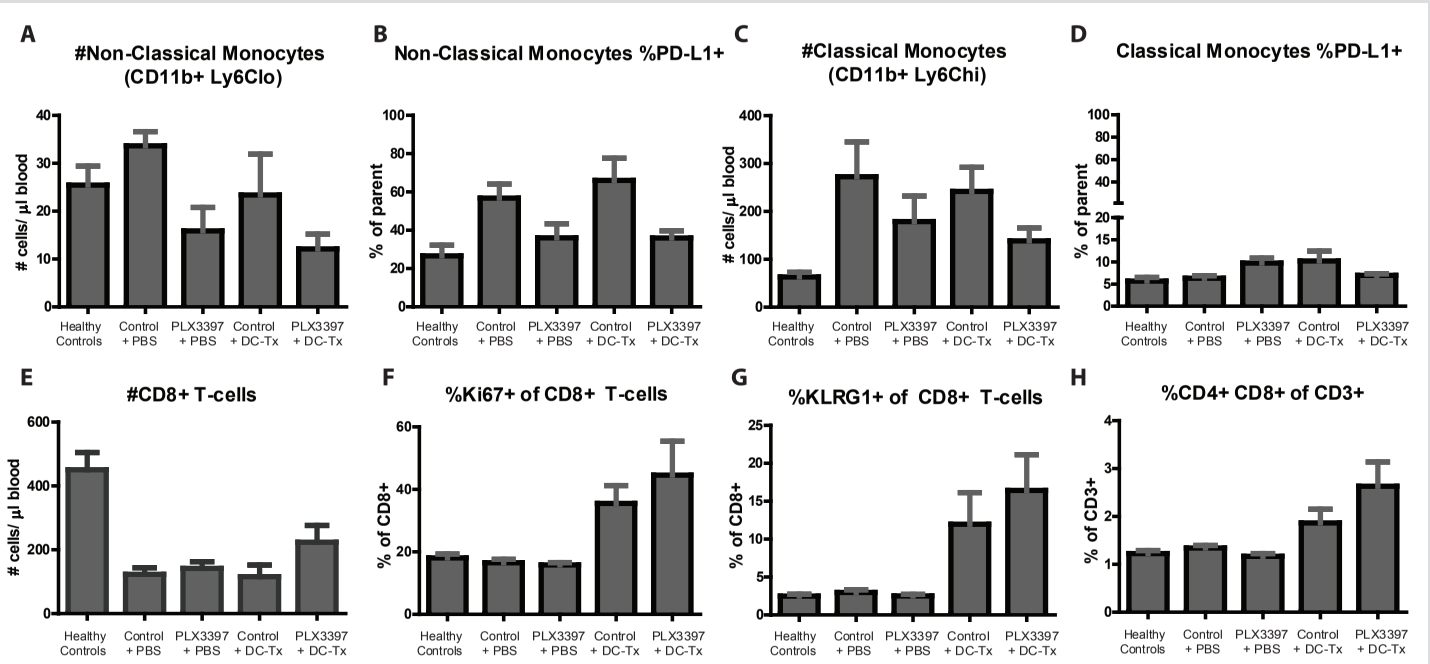

Figure S4 Similar patterns in myeloid and lymphoid cell dynamics in response to treatment were made in the balb/c AB1 tumor model compared to the CBA/j AC29 model. Blood was extracted from BALB/c mice (n=4-5 at t=15, at start of experiment n=6) five days after start of therapy (t=15 post-tumor cell injection) and measured using flow cytometry. The same populations were identified and quantified as in Figure 3 and 4. All data is displayed as bar graph including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test with $p<0.05$ being statistically significant. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, PD-L1= programmed death-ligand 1.

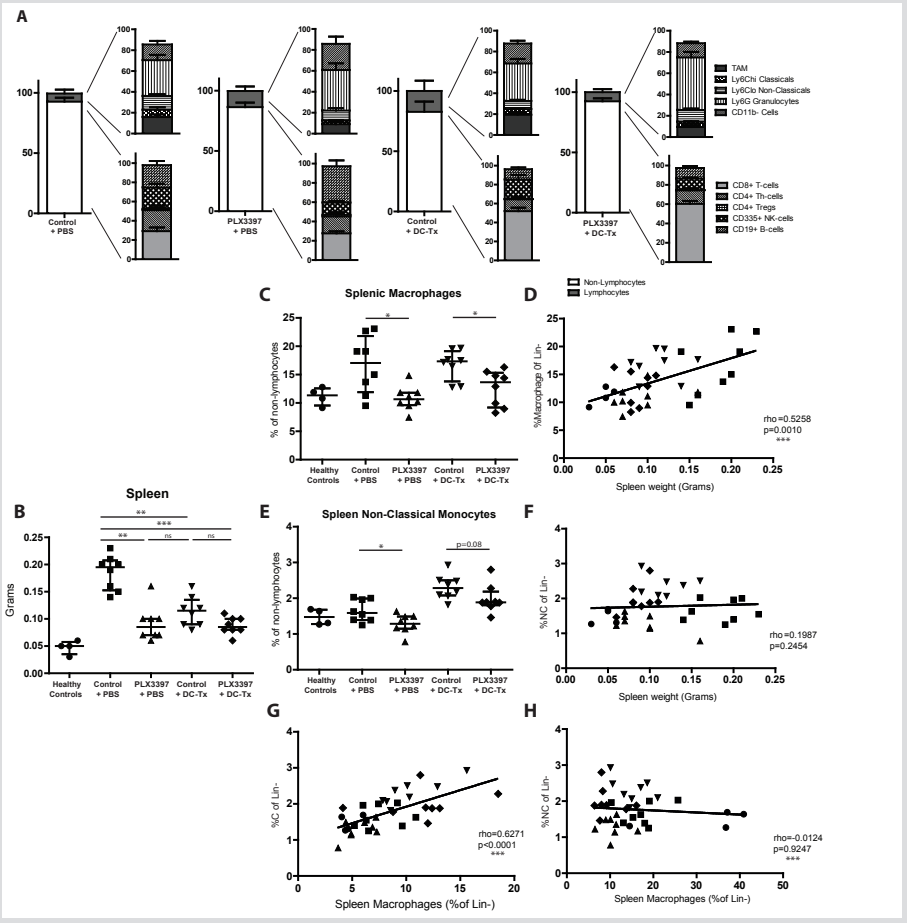

Figure S5 The TME spleen immune contexture in response to treatment on day 15 post tumor injection. Tumor bearing CBA/J mice were sacrificed on t=15 days to examine the tumor microenvironment, blood and spleen in the different treatment groups. **(A)** The TME of all mice in the 4 treatment groups was dissected by multicolor flow cytometry into lymphoid cells and myeloid cells, with further subset characterization per cell type. Cells are depicted as percentage of alive leukocytes (CD45+), followed by subset characterization that was depicted as percentage of lymphoid (CD3+/CD19+/CD335+) or myeloid (CD11b+) cells. **(B)** After sacrifice of mice on t=15, spleens were extracted and weighed. **(C, E)** Splenic macrophages and non-classical (NC) monocytes were determined using flow cytometry and depicted as percentage of non-lymphoid leukocytes. **(D, F)** Correlations were made between splenic non-classical monocytes or macrophages and spleen weight. **(G-H)** Correlations were made between classical monocytes or non-classical monocytes in blood versus splenic macrophages. All data is displayed as dot plots or bar graphs including the median and error bars indicating interquartile range. Statistical significance was determined using the Mann-Whitney U test or in case of correlations using the Spearman's Rank Correlation test with $p<0.05$ being statistically significant. Healthy controls were measured to depict cell frequencies and phenotypes in the non-tumor bearing host, but were not included in further statistical testing. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant, DC-Tx= DC-therapy, TAM= tumor associated macrophage, NK-cell = Natural Killer cell, Th cell= T helper cell, Treg= T regulatory cell.

Chapter 8

Biomarkers for immune checkpoint inhibitors

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LETTER TO THE EDITOR

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We read with interest Luana Calabrò and colleagues' phase 2 study [1], which investigated the efficacy, safety, and immunological activity of tremelimumab, an anti-CTLA4 monoclonal antibody, in patients with advanced malignant mesothelioma [1,2]. They noted disease control in nine (31%) of the 29 enrolled patients and proposed CD4-positive, ICOS-positive T cells as a predictive marker of treatment outcome. Although these results are promising we believe that studies concerning biomarkers for immune checkpoint inhibitors should take another direction.

Different checkpoint targeting agents have been developed and investigated to overcome the inhibitory signaling pathways that switch off T cells. Besides treatments that act on CTLA4, drugs targeting the programmed death (PD) protein 1 or its ligands PD-L1 and PD-L2, LAG3, HAVCR2, IDO1, CD276, and VTCN1 have now been tested for a wide variety of different cancers [3]. However, in view of the low response rates and potential serious side effects, the development of robust predictive biomarkers is needed.

The authors made a clear effort to identify lymphocytic subsets as biomarkers of response. Intriguingly, the increase in peripheral CD4-positive, ICOS-positive T cells 30 days after treatment was shown to be a potential predictive marker of tremelimumab treatment outcomes in patients with mesothelioma. An increase in peripheral CD4-positive, ICOS-positive T cells after treatment with tremelimumab provides information regarding the T cell activation induced by CTLA4 blockade. Differences in these T cell counts after treatment imply differences in the proportion of immunosuppression attributable to CTLA4 expression. However in the present approach the underlying cause of differences in peripheral T cell activation after treatment with tremelimumab remains unclear and this result can only be obtained after the start of therapy. The investigators did investigate several lymphocytic subgroups at baseline, however the selected populations are non-specific and are not related to the treatment approach. It would have been very informative if the investigators had reported data regarding CTLA4 expression in the tumour or peripheral blood before treatment to gain insights into the mechanisms of treatment response.

Without a doubt, biomarkers that can predict response to these checkpoint inhibitors before use are desirable. The part played by different immunosuppressive mechanisms can change during treatment and disease progression dependent on cellular interactions and local signals, clearly affecting the efficacy of any immunotherapy [4]. Proper biomarkers for the different immune checkpoint inhibitors enable the selection of the appropriate inhibitors for an optimized, patient-tailored treatment [5]. The search for these biomarkers is now warranted by the encouraging clinical activity of tremelimumab in patients even with chemotherapy-resistant advanced malignant mesothelioma.

The authors have no conflicts of interest to declare.

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Chapter 9

Checkpoint blockade in lung cancer and mesothelioma

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ABSTRACT

In the past decade, immunotherapy has emerged as a new treatment modality in cancer. The most success has been achieved with the class of checkpoint inhibitors; antibodies which unleash the anti-tumor immune response. Following the success in melanoma, numerous clinical trials are being conducted investigating checkpoint inhibitors in lung cancer and mesothelioma. The programmed death protein 1-protein death ligand 1/2 (PD-1 – PD-L1/2) pathway and cytotoxic T-lymphocyte associated protein 4 (CTLA4) are currently the most studied immunotherapeutic targets in these malignancies. In non-small cell lung cancer (NSCLC), anti-programmed death receptor -1 (PD-1) antibodies have become part of the approved treatment arsenal. In small cell lung cancer (SCLC) and mesothelioma the efficacy of checkpoint inhibition has not yet been proven. In this concise clinical review, an overview of the landmark clinical trials investigating checkpoint blockade in lung cancer and mesothelioma is provided. Since response rates are around 20% in the majority of clinical trials, there is much room for improvement. Predictive biomarkers are therefore essential to fully develop the potential of checkpoint inhibitors. To increase efficacy, multiple clinical trials investigating the combination of CTLA4 inhibitors and PD-1/PD-L1 blockade in lung cancer and mesothelioma are being conducted or underway. Given the potential benefit of immunotherapy in these devastating diseases, implementation of current and new knowledge in trial designs and interpretation of results is essential to move forward.

INTRODUCTION

Lung cancer and mesothelioma are malignancies with uniformly poor outcome as the majority of patients are diagnosed with advanced, non-curable disease. These patients have traditionally been treated with cytotoxic chemotherapy with modest increases in survival [1-3]. In advanced non-small cell lung cancer (NSCLC), platinum-based combination chemotherapy followed by maintenance treatment with single-agent chemotherapy and/or an anti-angiogenic agent remains the cornerstone of therapy in the majority of patients, increasing both progression-free survival (PFS) and overall survival (OS) by several months [4-6]. In second-line, NSCLC patients have historically been treated with docetaxel with or without anti-angiogenic agents, again offering modest survival benefits [7]. The discovery of targetable genetic alterations, which are present in a minority of patients with NSCLC, has improved outcomes, but these patients also eventually relapse [8,9].

Similarly, in extensive stage disease small cell lung cancer (ED-SCLC), platinum-based combination chemotherapy remains the treatment of choice. Despite high objective response rates with chemotherapy, most patients with SCLC relapse within the first year after initial treatment [10,11]. Standard second-line therapy in SCLC consists of salvage chemotherapy with limited efficacy [12,13].

In malignant pleural mesothelioma (MPM), platinum/multi-targeted anti-folate combination, offering a 3 month overall benefit, is the only FDA approved treatment [14]. No second line treatments for MPM have been approved for standard-of-care treatment.

Immunotherapy has, however, emerged as a potential new treatment modality in lung cancer and mesothelioma, with the aim to induce anti-tumor immunity with durable clinical responses. Recent progress has been made with the class of checkpoint inhibitors: antibodies directed against inhibitory receptors and ligands which can be co-opted by tumor cells or stromal cells [15-17]. Encouraged by the FDA-approval of the CTLA4 blocking antibody ipilimumab in metastatic melanoma, clinical trials have been conducted in NSCLC and to a lesser extent in SCLC and mesothelioma [18-21]. This concise clinical review provides an overview of the landmark clinical studies investigating checkpoint blockade in lung cancer and mesothelioma, and focuses on the hurdles necessary to overcome in order to successfully implement this class of drugs in these notoriously treatment-resistant malignancies.

PD-1 and PD-L1 blockade in lung cancer and mesothelioma

PD-1 blocking antibodies

The programmed death protein 1-protein death ligand 1/2 (PD-1 – PD-L1/2) pathway is currently the most studied immunotherapeutic target in lung cancer. Table 1 summarizes results of the landmark clinical trials investigating monotherapy with checkpoint blocking antibodies. Antigen-experienced effector T cells express PD-1 upon activation and are consecutively downregulated after PD-1 binds to one of its ligands, PD-L1 or PD-L2, expressed

on various antigen-presenting cells (APCs), stromal cells, and/or tumor cells. Since tumor cells are capable of upregulating PD-L1, the PD-1 checkpoint is active in the tumor microenvironment [15]. The PD-1 blocking antibody nivolumab was the first checkpoint inhibitor to receive FDA-approval for the treatment of advanced NSCLC, demonstrating an increase in OS in patients who had disease progression after platinum-based chemotherapy in two phase 3 randomized clinical trials, comparing nivolumab and docetaxel in squamous and non-squamous NSCLC respectively [22, 23]. The incidence of grade 3-4 adverse events (AEs) was lower in the nivolumab arm compared to docetaxel in both trials.

The most common reported immune-related toxicities were hypothyroidism, colitis, pneumonitis, nephritis and rash. Although presence of PD-L1 expression in the tumor was not an inclusion criterion, responses to nivolumab did improve with increasing levels of PD-L1 expression.

After the approval as a second-line treatment for advanced NSCLC patients, the potential of nivolumab as first-line treatment is now being studied. CheckMate 026 is a phase 3 randomized clinical trial investigating the treatment of advanced, treatment-naive squamous and non-squamous NSCLC patients. Only patients with PD-L1 positive tumors (>1% positivity on tumor cells) were included, the primary outcome PFS was assessed in patients with ≥5% PD-L1 expression. Initial results were recently presented at the ESMO 2016 meeting, and although not all outcome measures are currently available, there was no improvement in PFS in advanced NSCLC patients treated in first-line with nivolumab monotherapy versus platinum-based chemotherapy [24]. This may be ascribed to the chosen inclusion criteria, mainly the cut-off level of >5% PD-L1 positivity, which may not enrich the population sufficiently to outperform chemotherapy in the first line setting. In addition, nivolumab is currently being tested in the neoadjuvant setting in early-stage, resectable NSCLC patients, and promising preliminary results were presented at the ESMO 2016 meeting, with 40% of patients (6 of 16) achieving major pathologic responses (<10% residual viable tumor, NCT02259621) [25]. Nivolumab is currently also being investigated as a first-line treatment for advanced NSCLC patients in combination with the CTLA4-blocking antibody ipilimumab in the CheckMate 227 trial (NCT02477826).

Pembrolizumab is another PD-1 blocking antibody that has been FDA approved as second-line therapy for advanced NSCLC patients and as first-line therapy of patients with NSCLC whose tumors have ≥50% expression of PD-L1. The KEYNOTE-001 phase 1 trial demonstrated the safety of pembrolizumab, and showed a positive correlation between outcomes and PD-L1 expression [26]. The randomized phase 2b study, KEYNOTE-010, showed an OS benefit in patients with advanced NSCLC who were treated in second-line with pembrolizumab (2 dose levels) compared to docetaxel [27]. Grade 3-5 AEs were observed in 13% of the patients on the pembrolizumab 2mg/kg arm, 16% of those in the pembrolizumab 10mg/kg arm, and in 35% of subjects in the docetaxel arm. One of the inclusion criteria of KEYNOTE-010 was ≥1% expression of PD-L1 on tumor biopsies, and consequently, unlike

nivolumab, pembrolizumab was registered in combination with a companion diagnostic PD-L1 immunohistochemical test documenting a ≥1% PD-L1 positivity in tumor biopsies [26]. The use of archival tissue samples appeared justified since the demonstrable clinical benefit was irrespective of whether archival or new tumor samples were used.

The phase 3 study KEYNOTE-024 was positive for its primary endpoint PFS in previously untreated advanced NSCLC patients with ≥50% PD-L1 expression on tumor cells and without an *EGFR* mutation or *ALK* translocation. Patients were randomized to treatment with either pembrolizumab or the investigator's choice of platinum-based chemotherapy [28]. Grade 3-5 AEs occurred in 26.6% of the pembrolizumab arm compared to 53.3% of the chemotherapy arm. On the basis of the second interim analysis, the trial was stopped and patients in the chemotherapy group were offered pembrolizumab. Pembrolizumab is now the first immunotherapy that is FDA-approved for first-line use in advanced NSCLC. The use is restricted to patients with ≥50% PD-L1 expression on tumor cells.

In addition, the KEYNOTE-042 phase 3 trial in which first-line pembrolizumab is compared to standard chemotherapy in advanced NSCLC patients with a lower PD-L1 expression threshold of ≥1% is currently ongoing, with a planned subgroup analysis in different PD-L1 levels (NCT02220894). Pembrolizumab in combination with, carboplatin/pemetrexed (CP) chemotherapy demonstrated increased PFS compared to CP alone in cohort G of the KEYNOTE-021 study [29].

In SCLC, there are no randomized data available regarding the efficacy of PD-1/PDL-1 checkpoint inhibitors. The phase 1/2 study CheckMate 032, in which patients with advanced SCLC were treated with nivolumab or nivolumab plus ipilimumab, showed promising preliminary results, however these have to be confirmed in a randomized trial [30]. The KEYNOTE-028 study, a phase 1b study of pembrolizumab in advanced PD-L1 positive solid tumors, included an advanced SCLC cohort. Of the 135 screened SCLC patients, 37 (27%) had PD-L1 positive tumors and 17 patients were treated with pembrolizumab [31]. The ORR was 35% in this small patient group and final clinical outcomes are pending at this time point.

In progressive mesothelioma patients, nivolumab monotherapy is currently being investigated in the phase 2 single-arm NivoMes study (NCT02497508) without a PD-L1 expression selection criterion. At the World Conference of Lung Cancer (WCLC) 2016, the results after 24 weeks of treatment were presented demonstrating disease control (partial response and stable disease) in 33% of 34 treated patients [32]. In the mesothelioma cohort of the phase 1b KEYNOTE-028 study, 84 patients were screened, 38 (45%) had PD-L1 positive (≥1%) tumors and 25 patients were treated with pembrolizumab [33]. As presented at the WCLC 2016, the ORR was 20% [34]. Furthermore the interim analysis of a phase 2 trial of pembrolizumab in 34 progressive mesothelioma patients presented at the WCLC 2016 demonstrated a response rate of 21% (12% in PD-L1 negative patients and 27% in patients with ≥1% PD-L1 positive tumors) [35].

Table 1 Overview of landmark phase 2/3 randomized studies and pivotal phase 1b-2 studies of checkpoint blocking antibodies in lung cancer and mesothelioma

		Compound	Study	Author	Phase	Line	Histology	Control	N
NSCLC	*anti PD-1*	Nivolumab	CheckMate 057	Borghaei et al	3	2nd	nonsquamous	Docetaxel	582
			CheckMate 017	Brahmer et al	3	2nd	squamous	Docetaxel	272
			CheckMate 026	Socinski et al (ESMO 2016)	3	1st	all	IC	423
		Pembrolizumab 2mg/kg	KEYNOTE-010	Herbst et al	2b	2nd	all	Docetaxel	688
		Pembrolizumab 10mg/kg	KEYNOTE-010	Herbst et al	2b	2nd	all	Docetaxel	689
		Pembrolizumab	KEYNOTE-024	Reck et al	3	1st	all	IC	305
		Pembrolizumab + CP	KEYNOTE-021G	Langer et al	2	1st	nonsquamous	CP	123
	anti PD-L1	Atezolizumab	POPLAR	Fehrenbacher et al	2	2nd	all	Docetaxel	287
		Atezolizumab	BIRCH	Besse et al and Garassino et al	2	1st-3rd	all	NA	659
		Atezolizumab	OAK	Barlesi et al (ESMO 2016)	3	2nd-3rd	all	Docetaxel	850
		Durvalumab	ATLANTIC‡	Garassino et al (WCLC 2016)	2	≥3	all	NA	307
		Avelumab	JAVELIN	Verschraegen et al (ASCO 2016)	1b	1st	all	NA	145
	anti CTLA4	Ipilimumab (phased)+chemo		Lynch et al	2	1st	all	Paclitaxel + carbo	204
		Tremelimumab		Zatloukal et al	2	2nd	all	Best supportive care	84
SCLC	*anti PD-1*	Pembrolizumab	KEYNOTE-028	Ott et al (ASCO 2015)	1b	2nd	small cell	NA	17
	anti PD-L1	NA							
	anti CTLA4	Ipilimumab + chemo		Reck et al	3	1st	small cell	etoposide + platinum	1132
Mesothelioma	*anti PD-1*	Nivolumab	NivoMes	Baas et al (WCLC 2016)	2	2nd	all	NA	18
		Pembrolizumab	KEYNOTE-028	Alley et al (WCLC 2016)	1b	2nd	all	NA	25
		Pembrolizumab		Kindler et al. (WCLC 2016)	2	2nd	all	NA	35
	anti PD-L1	Avelumab	JAVELIN	Hassan et al (ASCO 2016)	1b	2nd	all	NA	53
	anti CTLA4	Tremelimumab	DETERMINE	Kindler et al (ASCO 2016)	2b	2nd/3rd	all	placebo	571

		Compound	Study	N	OS (mo)	HR, p	PFS (mo)	HR, p	ORR, p
NSCLC	*anti PD-1*	Nivolumab	CheckMate 057	582	12.2 vs 9.4	0.73, p=0.0015	2.3 vs 4.2	0.92, p=0.32	19% vs 12%, p=0.02
			CheckMate 017	272	9.2 vs 6.0	0.59, p<0.001	3.5 vs 2.8	0.62, p<0.001	20% vs 9%, p=0.008
			CheckMate 026	423	14.4 vs 13.2	1.02	4.2 vs 5.9	1.15, p=0.25	unavailable
		Pembrolizumab 2mg/kg	KEYNOTE-010	688	10.4 vs 8.5	0.71, p<0.001	3.9 vs 4.0	0.88, p=0.07	18% vs 9%, p=0.0005
		Pembrolizumab 10mg/kg	KEYNOTE-010	689	12.7 vs 8.5	0.61, P<0.001	4.0 vs 4.0	0.79, p=0.004	18% vs 9 %, p= 0.0002
		Pembrolizumab	KEYNOTE-024	305	80% vs 72%*	0.60, p=0.005	10.3 vs 6.0	0.50, p<0.001	45% vs 28% (no p available)
		Pembrolizumab + CP	KEYNOTE-021G	123	92% vs 92%*	p=0.39	13.0 vs 8.9	0.53, p=0.0010	55% vs 29%, p=0.0016
	anti PD-L1	Atezolizumab	POPLAR	287	12.6 vs 9.7	0.73, p=0.04	2.7 vs 3.0	0.94, ns	14.6% vs 14.7%, ns
		Atezolizumab	BIRCH	659	71-82%*	NA	2.8-5.5	NA	17-32%
		Atezolizumab	OAK	850	13.8 vs 9.6	0.73,p=0.0003	2.8 vs 4.0	0.95, ns	13.6% vs 13.4%, ns
		Durvalumab	ATLANTIC‡	307	9.3 - 10.9	NA	1.9-3.3	NA	7.5 - 30.9%
		Avelumab	JAVELIN	156	unavailable	unavailable	4.0	unavailable	22.4%
	anti CTLA4	Ipilimumab (phased)+chemo		204	12.2 vs 8.3	0.87, p=0.23	5.1 vs 4.2	0.96, p=0.02	32% vs 18%, ns
		Tremelimumab		84	unavailable		20.9% vs 14.3% †	unavailable	4.8% vs. 0%, ns
	anti PD-1	Pembrolizumab	KEYNOTE-028	17	unavailable		unavailable		35%
SCLC	*anti PD-L1*	NA							
	anti CTLA4	Ipilimumab + chemo		1132	11.0 vs 10.9	0.94, p=0.38	4.6 vs 4.4	0.85, ns	unavailable
	anti PD-1	Nivolumab	NivoMes	18	unavailable		unavailable		33% (disease control)
Mesothelioma		Pembrolizumab	KEYNOTE-028	25	18	NA	5.4	NA	20%
		Pembrolizumab		34	11.9	NA	6.2	NA	21%
	anti PD-L1	Avelumab	JAVELIN	53	unavailable	unavailable	3.9	unavailable	9.4% (unconfirmed)
	anti CTLA4	Tremelimumab	DETERMINE	571	7.7 vs 7.3	0.92, p=0.41	unavailable		unavailable

*CP: Carboplatin + Pemetrexed, IC: investigator's choice platinum doublet chemotherapy, NA: not applicable, * 6 months OS, † 3 months PFS,* ‡ cohort 2 and 3

PD-L1 blocking antibodies

In addition to PD-1 blocking antibodies, PD-L1 blocking antibodies are currently under development. The PD-L1 blocking antibody atezolizumab is being developed with a companion diagnostic scoring PD-L1 expression on tumor cells (TC) and/or tumor-infiltrating immune cells (IC) [36]. In the single-arm phase 2 BIRCH trial, treatment-naïve or pretreated advanced NSCLC patients with ≥5% PD-L1 expression received atezolizumab infusions, with the primary endpoint ORR of 17% in the pretreated patients - an updated analysis is awaited. Recent results of the patients treated in first-line demonstrated an ORR of 32% (TC group) and 24% (IC group) [37]. The phase 2 POPLAR study compared atezolizumab to docetaxel in platinum-pretreated patients with advanced NSCLC without PD-L1 preselection [38]. The study was positive for the primary endpoint, OS; an association was found between an increasing OS and higher PD-L1 expression level. Interestingly, both PD-L1 expression on tumor cells and tumor-infiltrating immune cells independently predicted an improved OS with atezolizumab. Grade 3-4 AEs were reported in 11% of patients in the atezolizumab arm compared to 39% of patients in the docetaxel arm.

Recently, the results of the phase 3 randomized OAK trial involving 1225 pre-treated advanced NSCLC patients unselected for PD-L1 expression were presented at the ESMO 2016 meeting [39]. In this study, the primary endpoint OS was significantly improved in the atezolizumab arm compared with the docetaxel arm, regardless of PD-L1 expression level. The clinical benefit was, however, more pronounced in patients with high PD-L1 expression level on tumor cells and/ or tumor-infiltrating immune cells. Grade 3-4 AEs occurred in 15% of azetolizumab and 43% of docetaxel treated patients. Subgroup analyses of the OAK trial presented at the WCLC 2016 confirmed the clinical benefit of atezolizumab in NSCLC, regardless of histology and in subgroups of never smokers and patients with brain metastases [40].

Durvalumab and avelumab are two additional PD-L1 blocking antibodies currently under investigation in multiple malignancies. In a phase 1/2 study, patients with advanced NSCLC were treated with durvalumab [41] -the ORR was 14% in the 149 enrolled patients evaluable for response. The single arm phase 2 ATLANTIC study investigated the efficacy of durvalumab in NSCLC patients who progressed after 2 or more lines of prior systemic treatments. The study demonstrated durable clinical responses, and the primary outcome of ORR increased with higher PD-L1 expression levels on tumor cells [42]. Furthermore, the role of durvalumab as adjuvant therapy is currently under study in a phase 3 randomized, placebo-controlled study in locally-advanced NSCLC patients following chemoradiation (PACIFIC, NCT02125461). In addition, there is an actively accruing clinical trial evaluating the efficacy of adjuvant durvalumab after complete resection in NSCLC patients (BR31, NCT02273375).

Avelumab is a PD-L1 blocking monoclonal antibody which is currently being tested in multiple clinical settings and cancer types (JAVELIN programme). At the ASCO 2016 and WCLC 2016 meetings, preliminary results of clinical studies of avelumab in both NSCLC and mesotheli-

oma were presented. Interim results from a phase 1b clinical trial of first-line avelumab in 156 advanced NSCLC patients demonstrated an ORR of 22.4% [43, 44]. Concomitantly, avelumab is being investigated as first-line treatment in advanced PD-L1 positive NSCLC patients in a phase 3 clinical trial (JAVELIN Lung 100). In a phase 1b study in advanced mesothelioma patients, second-line treatment with avelumab resulted in an ORR of 9.4% (14.3 % in PD-L1 positive (PD-L1 expression ≥5% as cut-off) and 8.0% in PD-L1 negative patients) [45]. To the best of our knowledge, there are no results of clinical trials available on the potential of PD-L1 blocking antibodies in SCLC.

Conclusions regarding PD-1/PD-L1 blockade in thoracic malignancies

It is now well appreciated that overall survival is prolonged with checkpoint inhibitors although long-term data are lacking, due to the limited time of follow-up of the clinical trials. One of the common findings among the studies is the fact that most of the long term surviving patients are those who had demonstrated a radiographic response to treatment with checkpoint inhibitors [46]. Response rates (and consequently prolonged OS) of the PD-1 and PD-L1 blocking antibodies in advanced NSCLC are remarkably similar, with ORR approximately 20% in nearly all clinical trials, leaving much room for improvement. Randomized clinical trials with various monoclonal antibodies directed against PD-1 and/or PD-L1 in lung cancer and mesothelioma are ongoing in different stages of the disease and lines of treatment. Although the clinical potential of these antibodies is undisputed, many questions remain to be answered regarding the selection of patients most likely to benefit from monotherapy or combination treatment and which combinations of treatment at which sequence of treatment should be given.

CTLA4 blockade in lung cancer and mesothelioma

Cytotoxic T-lymphocyte associated protein 4 (CTLA4) is upregulated on CD4 effector T cells upon activation and constitutively overexpressed on regulatory T cells (Tregs) [47]. CTLA4 functions upon ligation with CD80 or CD86 on APCs - e.g. dendritic cells - in the onset of the immune response during T cell priming and activation and enhances the immunosuppressive function of Tregs [48, 49]. Unlike the PD-1/PD-L1 checkpoint which functions in the tumor microenvironment, CTLA4 has a global impact on the immune system and functions primarily in the lymph nodes.

Compared to the PD-1-PD-L1 axis, clinical studies investigating CTLA4 blockade in lung cancer and mesothelioma are relatively scarce. The CTLA4 blocking IgG1 antibody ipilimumab was investigated as first-line therapy in combination with carboplatin/paclitaxel versus placebo plus chemotherapy in a phase 2 trial for advanced NSCLC patients [50]. There were three experimental arms in this study: chemotherapy plus placebo; and chemotherapy plus ipilimumab in a phased or concurrent regimen. None of the outcomes were significantly improved in patients enrolled in the concurrent chemoimmunotherapy arm, but phased

ipilimumab showed significant improvement in the primary endpoint, median PFS. The incidence of grade 3-4 AEs was 15% for the phased arm, 20% for the concurrent arm and 6% for the control arm. The phased ipilimumab schema is being actively evaluated further in an ongoing phase 3 study comparing chemotherapy vs phased ipilimumab and chemotherapy in squamous NSCLC (NCT02279732). In ongoing clinical trials of anti-CTLA-4 monoclonal antibodies in NSCLC, attention is mostly focused on the role of ipilimumab in a combinatorial approach with nivolumab.

The potential of ipilimumab has also been investigated as first-line treatment in advanced SCLC patients in a large phase 3 clinical trial, after an initial positive phase 2 study. This trial comparing chemotherapy with etoposide and platinum plus ipilimumab 10 mg/kg or placebo in a phased induction schedule followed by ipilimumab or placebo maintenance was negative for its primary endpoint, OS [51,52]. There are no data available regarding the efficacy of ipilimumab in mesothelioma patients.

The CTLA4 blocking IgG2 antibody tremelimumab has been investigated in NSCLC and mesothelioma, but not in SCLC. A phase 2 study randomized advanced NSCLC patients to second-line treatment with tremelimumab or best supportive care and was negative for its primary endpoint PFS at 3 months [53].

By far the largest clinical trial investigating immunotherapy in mesothelioma has been the DETERMINE trial; a phase 3 placebo-controlled study of tremelimumab in 571 advanced mesothelioma patients as second and third-line treatment. The results of this trial were presented during the 2016 ASCO meeting, and demonstrated that there was no statistically significant difference in median OS, despite promising results in the phase 2 trial [54 55]. A phase 2 study in which tremelimumab is combined with durvalumab in mesothelioma patients in second or third-line is currently ongoing in Italy (NCT01843374).

Thus far, in lung cancer and mesothelioma, CTLA4 blocking antibodies have not produced the clinical benefits seen in melanoma. Given the current absence of robust response biomarkers of CTLA4 blockade, the potential mechanisms behind these disappointing results are the subject of ongoing debate while ipilimumab and tremelimumab are moving forward in combination with PD-1/PD-L1 blockade in multiple clinical trials.

Biomarkers of response

Can PD-L1 expression predict efficacy of PD-1 blockade?

Topalian et al. recently provided an overview of the current potential biomarkers for PD-1- and CTLA4 blockade and provided guidance on how best to move forward in the quest for robust biomarkers [16]. The authors proposed that potential biomarkers should be identified based on the mechanism of action of the drug target. As the PD-1 checkpoint regulates local immune responses within the tumor microenvironment, local PD-L1 expression on both tumor and infiltrating immune cells qualifies as an attractive potential biomarker for PD-1/PD-L1 blocking antibodies.

The immune contexture of tumors is known to be profoundly heterogeneous and dynamic. The identification of potent biomarkers of immunotherapies is therefore challenging. For example, how well PD-1/PD-L1 blocking antibodies will perform in inducing effective anti-tumor immune responses is dictated by the portion of the anti-tumor immune response which is suppressed by PD-1/PDL-1/2 interaction [16, 56, 57]. Two fundamental mechanisms play a role in the expression of PD-L1 in tumors: innate and adaptive immune resistance. Tumor cells are capable of constitutively expressing PD-L1 regardless of the presence of an active immune response in the tumor (innate immune resistance) [15]. In addition, tumor cells, immune cells and stromal cells will upregulate PD-L1 in a negative-feedback response to intra-tumoral infiltration of activated T cells (adaptive immune resistance) [58, 59]. There is certainly validity in the principle that PD-L1 expression within the tumor microenvironment (on tumor cells and/or infiltrating immune cells) functions as a hallmark of local immune activation and potential response to blockade of the PD-1/PD-L1 axis [59-61]. As described above, however, PD-L1 upregulation may also be independent of an anti-tumor-directed immune response. Despite all the inherent biases, the results of the clinical trials do support the concept that higher levels of PD-L1 expression on immunostaining enriches for patient populations that will respond more robustly to PD-1/PD-L1 inhibition. Table 2 provides an overview of the available data regarding response rates according to stratified PD-L1 expression in landmark clinical trials. Since the PD-1/PD-L1 blocking antibodies are very similar in structure, the differences in the outcomes of the various clinical trials are subject of ongoing debate. It is clear that PD-L1 expression is an enriching factor; therefore studies that include more PD-L1 positive patients or apply a higher cut-off include more responders. Furthermore, there is heterogeneity of the different staining antibodies used to determine PD-L1 expression.

As PD-L1 expression is a continuous and dynamic variable which is heterogeneously expressed throughout the tumor, the challenges concerning the implementation of a PD-L1 expression immunohistochemistry score as a robust biomarker are clear [62, 63]. The International Association for the Study of Lung Cancer (IASLC) has therefore launched a project to compare available PD-L1 immunohistochemistry assays (Blueprint Project) [62]. First results were recently published and demonstrate that for 37% of the reviewed cases, a different PD-L1 classification would be made depending on which assay/scoring system is used [64]. This potential misclassification could also explain the finding that in some study cohorts PD-L1 'negative' patients demonstrate good clinical responses. Whether the differences in study outcomes are attributable to the characteristics of the different monoclonal antibodies or to the study characteristics (e.g. inclusion criteria, PD-L1 immunohistochemistry clone) will need to be determined.

Table 2 Objective response rates according to stratified PD-L1 expression after PD-1-PD-L1 blockade in lung cancer and mesothelioma

Compound	Study	Antibody	PD-L1 score (% positivity)	Prevalence (%)	ORR (%)
Nivolumab	CheckMate 057	Dako 28-8	< 1	47	9
			≥1	53	31
			≥5	41	36
			≥10	37	37
Nivolumab	CheckMate 017	Dako 28-8	< 1	40	17
			≥1	47	17
			≥5	31	21
			≥10	27	19
Pembrolizumab	KEYNOTE-010	Dako 22C3	<1	34	excluded
			≥1-49	38	18
			≥50	28	30
Atezolizumab	BIRCH	Ventana SP142	TC or IC <5	unknown	excluded
			TC or IC 5-49	54	10
			TC or IC ≥50	46	26
	POPLAR	Ventana SP142	TC and IC <1	32	8
			TC or IC ≥1 and <5	31	14
			TC ≥ 5 and <50 or IC ≥ 5 and <10	20	8
			TC ≥50 or IC ≥ 10	16	38
	OAK	Ventana SP142	TC and IC <1	45	8
			TC or IC ≥ 1	55	18
			TC ≥ 5 or IC ≥ 5	31	22
			TC ≥50 or IC ≥ 10	16	31
Durvalumab	ATLANTIC	Ventana SP263	<25 (cohort 2)	61	8
			≥25 (cohort 2)	39	16
			≥90 (cohort 3)	100	31
Avelumab	JAVELIN	DAKO 73-10	<1	22	0
			≥1	78	20
	Mesothelioma		<5	64	8
			≥5	36	14

Abbreviations: ORR overall response rate; TC tumor cells; IC immune cells

Towards biomarkers for CTLA4 blocking antibodies

Since response rates to CTLA4 blockade in lung cancer and mesothelioma are low and grade 3-4 AEs are relatively common, there is a pressing need for the identification of predictive biomarkers. As CTLA4 exerts its effect during T cell priming and activation in the lymph nodes, many studies in search of biomarkers for ipilimumab and tremelimumab have focused on the phenotype of peripheral blood leukocytes before and during treatment. An increased absolute lymphocyte count and specifically increased number of peripheral CD8 T cells have been associated with higher response rates to ipilimumab [65]. Several studies, including the phase 2 study of tremelimumab in mesothelioma, described increased expression of the co-stimulatory molecule, inducible T cell co-stimulator (ICOS) on peripheral CD4 T cells following treatment with CTLA4 blockade [55,56,66]. As this potential biomarker is not a baseline characteristic, however, it is not useful as a selection criterion for patients who are likely to benefit from CTLA4 blockade. Unfortunately, none of the found associations have currently led to the identification of a robust biomarker of response to CTLA4 blockade.

Perhaps the best biomarker for response to CTLA4 blockade is not in the peripheral blood, but rather obtained from minimally invasive assessment (i.e via E(B)US-FNA) of CTLA4 expression on T cells in the draining lymph nodes of patients with lung cancer and mesothelioma [67,68].

Combinatorial immunotherapy in lung cancer and mesothelioma

As it is known that CTLA4 modulates T cell activation in the lymph nodes, while PD-1/PD-L1 controls T cell activation locally at the tumor site, the combined blockade of these checkpoints seems an attractive strategy. Following the example set in metastatic melanoma, the combination of CTLA4 inhibitors and PD-1/PD-L1 blockade is currently being evaluated in multiple cancers. In the phase 1 CheckMate 012 study, 148 advanced NSCLC patients were treated with nivolumab plus ipilimumab in 4 dose cohorts and ORRs ranged from 13% to 39% [69]. The follow-up phase 3 CheckMate 227 trial will establish the role of the nivolumab/ipilimumab combination in advanced NSCLC patients in first line (NCT02477826). In a phase 1 study, the combination of durvalumab and tremelimumab demonstrated an ORR of 23% in the optimal tolerable dosage group [70], leading to the phase 3 MYSTIC trial which is currently being analysed for results. In SCLC, the combination of nivolumab and ipilimumab showed a slight increase in response rates compared to nivolumab monotherapy in the CheckMate 032 phase 2 trial [30]. The phase 3 CheckMate 451 study currently investigates the efficacy of nivolumab versus nivolumab in combination with ipilimumab as switch maintenance therapy versus placebo and aims to enroll 810 advanced SCLC patients with at least stable disease after chemotherapy (NCT02538666). Following the disappointing results of tremelimumab monotherapy in mesothelioma, a phase 2 trial investigating the combination of tremelimumab and the PD-L1 blocking antibody durvalumab has been initiated (NIBIT-MESO-1, (NCT02588131)). Furthermore, first line treatment with the combination of nivolumab

and ipilimumab is currently being investigated in comparison to standard chemotherapy in mesothelioma patients in the phase 3 CheckMate 743 study which aims to enroll 600 patients (NCT02899299).
As stated above, the combination of ipilimumab and nivolumab performed better than ipilimumab monotherapy in melanoma [71, 72]. It should be noted, however, that the addition of ipilumumab to nivolumab only resulted in a modest increase of overall survival at the expense of a clear increase of toxicity, and only in these tumors which were PD-L1 negative. Because single agent nivolumab resulted in long-term survivors, a large percentage of the melanoma patients treated with combination therapy therefore could have responded to nivolumab alone, depending on the PD-L1 positivity of the tumor. These patients were exposed to possibly toxicity due to the lack of proper biomarkers to guide appropriate treatment regimens. The fact that monotherapy may be effective in selected patient populations should play a major role in the design of immunotherapy trials in lung cancer and mesothelioma, as toxicities in general are higher in these diseases, as well as for the significant financial consequences associated with combination treatment. Also in the development of the trials the different tumor microenvironment between lung cancer and mesothelioma compared to other malignancies should be taken into account.

Conclusion

Over the last years, checkpoint blockade has become a part of the treatment backbone of NSCLC and in the near future could possibly prove its efficacy in SCLC and mesothelioma. Following the success in melanoma, numerous clinical trials are being conducted investigating a constantly expanding armamentarium of checkpoint inhibitors. The challenge to fully develop checkpoint blockade in lung cancer and mesothelioma necessitates both the identification and implementation of predictive biomarkers. Furthermore, only when these biomarkers are available can rational combinatorial approaches be designed. In difficult to treat diseases such as lung cancer and mesothelioma, clinicians and scientists have an even stronger obligation to collect and share as much clinical and translational data as possible in order to achieve these goals. Given the potential benefit of immunotherapy in these devastating diseases, implementation of current and new knowledge in trial designs and interpretation of results is essential to move forward.

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Chapter 10

The efficacy of tumor vaccines and cellular immunotherapies in non-small cell lung cancer: a systematic review and meta-analysis

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ABSTRACT

Purpose: PD-1 checkpoint-blockers have recently been approved as second-line treatment for advanced non-small cell lung cancer (NSCLC). Unfortunately, only a subgroup of patients responds and shows long term survival to these therapies. Tumor vaccines and cellular immunotherapies could synergize with checkpoint blockade, but which of these treatments is most efficacious is unknown. In this meta-analysis we assessed the efficacy of tumor vaccination and cellular immunotherapy in NSCLC.

Methods: We searched for randomized controlled trials investigating cellular immunotherapy or vaccines in NSCLC. We used random effects models to analyze overall survival (OS) and progression free survival (PFS) expressed as hazard ratios (HR) and differences in months. The effect of immunotherapy type, disease stage, tumor histology, and concurrent chemotherapy was assessed using subgroup analysis and meta-regression. All procedures were performed according to the PRISMA guidelines.

Results: We identified 18 RCTs that matched our selection criteria, including 6756 patients. Immunotherapy extended NSCLC survival and PFS, expressed as HR (OS: HR=0.81, 95%CI=0.70-0.94, $p=0.01$, PFS: HR=0.83, 95%CI=0.72-0.95, $p=0.006$) and month difference (OS: difference=5.43 months, 95%CI=3.20-7.65, $p<0.005$, PFS: difference=3.24 months, 95%CI=1.61-4.88, $p<0.005$). Cellular therapies outperformed tumor vaccines (OS as HR: $p=0.005$, month difference: $p<0.001$, PFS as HR: $p=0.001$, month difference: $p=0.004$). There was a benefit of immunotherapy in low stage over high stage NSCLC and concurrent administration of chemotherapy only in one of four outcome measures evaluated (PFS in months: $p=0.01$ and PFS as HR: $p=0.031$, respectively) There was no significant effect of tumor histology on survival nor PFS.

Conclusion: Tumor vaccines and cellular immunotherapies enhance overall survival and PFS in NSCLC. Cellular immunotherapy was found to be more effective than tumor vaccination. These findings have implications for future studies investigating combination immunotherapy in NSCLC.

INTRODUCTION

Lung cancer is the leading cause of cancer related mortality worldwide [1]. Non-small cell lung cancer (NSCLC) comprises 85% of all lung cancers and has a 5-year survival rate of 4% in case of metastatic disease [2]. Current treatment options for advanced NSCLC include chemotherapy and radiotherapy, but these treatments only modestly improve survival. Recent advances include the targeting of several driver mutations responsible for tumor progression. Targeting the mutant EGF receptor and the EML4-ALK fusion protein has been found effective; however, resistance to these therapies inevitably ensues [3-5]. Therefore, novel treatment strategies to improve NSCLC survival are warranted.

Immunotherapy aims to establish or enhance an effective immune response toward the tumor. This can be accomplished via different strategies including tumor vaccination, adoptive transfer of immune cells and modification of the immune system to boost an established immune response [6]. The latter category of drugs includes the checkpoint inhibitors anti-programmed death 1 (PD-1) and anti-CTLA-4 that have recently been tested for efficacy in NSCLC [7-9]. The beneficial role for blocking CTLA-4 in NSCLC remains inconclusive [9, 10], but PD-1 blockade has proven to be effective in treating NSCLC [7, 11]. Nevertheless, the majority of patients does not respond to checkpoint inhibition therapy [7, 12, 13].

There are several ways in which an immune response toward a tumor can be induced as has been described in some reviews on this topic [6, 14, 15]. Tumor vaccines elicit an *in vivo* immune response specifically towards a tumor-associated antigen formulated in the vaccine. This form of therapy has proven to be safe and effective in eliciting tumor-specific immune responses in different cancers, including NSCLC [16-18]. It is also possible to circumvent endogenous antigen presentation by directly administering antigen stimulated T cells or dendritic cells (DCs) [19, 20]. Tumor vaccines and cellular therapies are aimed specifically toward tumor antigens and, therefore, have a limited toxicity profile as opposed to established chemotherapy and checkpoint blockade [21-23].

Eliciting potent T-cell response via vaccines or cellular therapies and simultaneously releasing the brakes on these T cells with checkpoint inhibitors may unleash the full potential of immunotherapy and improve the proportion of patients responding to therapy. Synergies between checkpoint inhibitor therapy and tumor or cellular vaccines are currently being investigated in several clinical trials [24]. Which form of immunotherapy; vaccine or cellular therapy, is most promising to combine with checkpoint inhibitors such as PD-1 blockers is currently unknown.

In this meta-analysis we show that vaccination and cellular immunotherapies improved the overall survival (OS) and progression-free survival (PFS) in patients with NSCLC. Cellular therapies outperformed tumor vaccines for all the outcomes assessed. Other factors such as tumor histology or the preconditioning of patients with low-dose cyclophosphamide had no effect on survival or PFS.

METHODS

Database search

On June 17, 2015, we searched for relevant studies in the following databases: Embase, Medline (Ovid SP), Web of Science, Cochrane Central Register for Controlled Trials, Pubmed Publisher and Google Scholar. There were no limitations on the year of publication for all the databases interrogated. Search entries were constructed for each individual database (Appendix, online only). The search initially involved other thoracic malignancies including small-cell lung cancer and mesothelioma. No language restrictions were applied. We also searched manually through conference abstracts and checked references from relevant publications and review articles. All procedures were performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [25].

Eligibility

Articles were included based on title and abstract if they concerned clinical evaluation of a tumor vaccine or cellular immunotherapy in NSCLC. Articles were excluded when they involved less than 5 NSCLC patients, or did not report well-defined clinical endpoints for survival or time to progression of disease. All articles on checkpoint blockade therapy or biological response modifiers (e.g. interferons, interleukins) were excluded. When we obtained all records on vaccine and cellular therapies in NSCLC, we selected the randomized controlled trials to study the efficacy of treatment. Studies that were not randomized or lacked complete outcome data were excluded for this particular research question. If multiple articles covered the same study population, the study with the most recent and complete survival data was used. Remaining studies that investigated the predictive value of immune factors in blood were later used for systematic review. Authors of the individual studies were contacted in case of missing data. Two investigators (F.D. and L.L.) independently screened abstracts and reviewed full texts for eligibility. Data extraction was performed (F.D. and G.V.) according to a predefined data extraction form. Any discrepancies were resolved by consensus with a third reviewer (J.A.).

Data collection and outcomes

Treatment characteristics (type and timing of treatment, dose) patient demographics, tumor histology, disease stage (low stage disease ranging from stage I-II/IIIA and high stage disease being III(B)-IV), and relevant outcome measures were collected according to a predefined data extraction form. The outcome measures OS and PFS were assessed, and when PFS was not available, time to progression and relapse free survival were included to increase the comparability and power of our analysis. These outcome measures were inconsistently reported as either hazard ratios (HRs) or as median months survival or time to disease progression or both, and all were included for further analysis. Tumor response rates were

not evaluated because they were inconsistently reported and have been found to correlate poorly with immunotherapy efficacy [26]. The risk of bias was determined using the Cochrane Collaboration risk of bias assessment tool [27].

Statistical Methods

Random effects models were used to compute summary effect sizes for all the outcome measures investigated, thereby taking heterogeneity across studies into account [28, 29]. When available, HRs from different studies were pooled to calculate the OS benefit of vaccine and cellular immunotherapy in NSCLC. Additionally, median differences were generated and combined when median survival times or median months of PFS were reported. We addressed several possible sources of heterogeneity (expressed as I^2) including type of immunotherapy (tumor vaccine vs. cellular therapy), limited or advanced disease (I-II/IIIA vs. III(B)-IV), histology (percentage of adenocarcinoma), preconditioning therapy with low-dose cyclophosphamide (in case of vaccines), and concurrent administration of chemotherapy, using subgroup analysis and meta-regression (in cases where the percentage of adenocarcinoma was given). Funnel plots were generated to assess the presence of publication bias. In order to define the extent of publication bias, the Duval and Tweedie trim-and-fill test and the classic fail-safe N test were used [30]. The Begg and Mazumdar rank-correlation test and the Egger test were applied in cases of suspected publication bias to quantify the level of bias [31, 32]. All analyses were performed by a biostatistician (L.A.), using a registered copy of Comprehensive Meta-Analysis statistical software (version 2.2.064; Biostat, Englewood, NJ).

RESULTS

Search strategy result and study characteristics of included trials

Our database and manual searches yielded a total of 7832 records of which 5992 records remained following removal of duplicate articles (Figure 1). An additional four records were manually selected from conference abstracts and reference lists. All records were screened on the basis of title and abstract to identify trials investigating the benefit of tumor vaccination or cellular immunotherapy in the context of NSCLC. After screening, a total of 114 potential records remained that were eligible for full-text assessment. Of these, 18 individual randomized controlled trials (RCT) were eligible for subsequent meta-analysis.

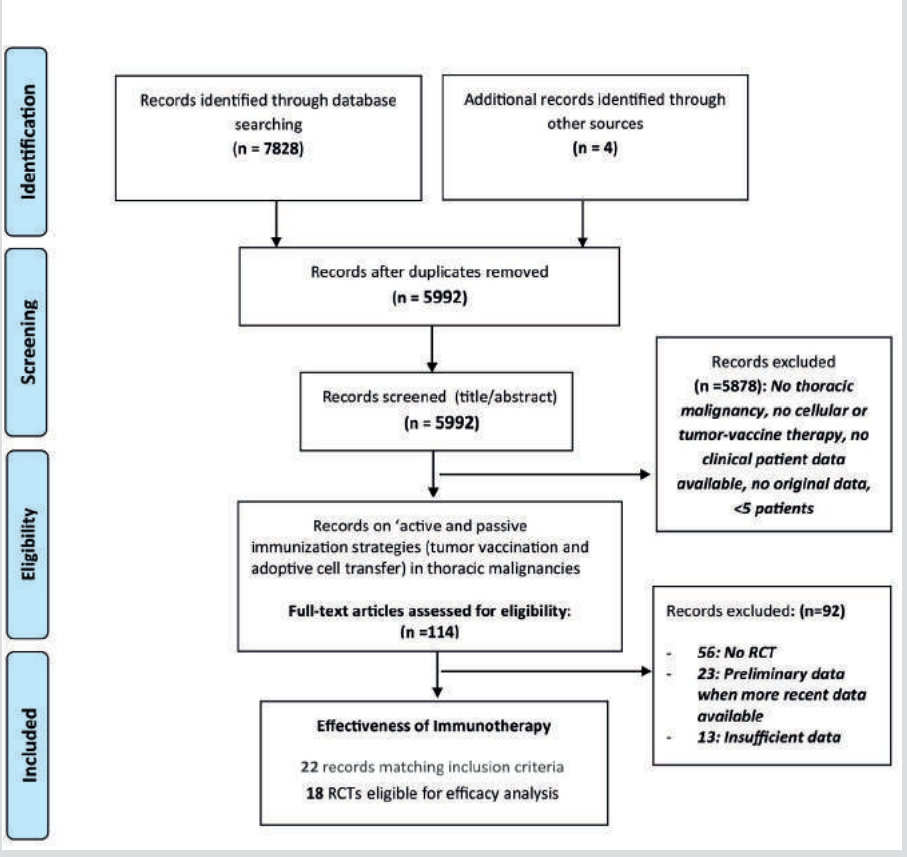

Figure 1 Prisma Flow Chart displaying the search and selection process performed.

Table 1 List of Study Characteristics

Reference	Type Article	Stage	No.of patients	% Adeno-carcinoma	Previous Treatments	Treatment type	Intervention Treatment	Control Treatment
Alfonso et al. 2014[37]	Peer-reviewed	IIIB-IV	176	29%	First line chemotherapy	Tumor Vaccine	Racotumomab (anti-idiotype NeuGcGM3 antibody)	Placebo
Butts et al. 2015[22,61]	Peer-reviewed	IIIA-IIIB	1239	35%	Chemo-radiotherapy	Tumor Vaccine	low-dose cyclophosphamide I.V. (300mg/m2) + Tecemotide (L-BLP25= MUC1 Ag)	Saline + Placebo
Butts et al. 2005[62,63]	Peer-reviewed	IIIB-IV	171	not reported	First line chemotherapy	Tumor Vaccine	low-dose cyclophosphamide I.V. (300mg/m2) + Tecemotide (L-BLP25= MUC1 Ag)	BSC
Giaccone et al.2015[21]	Peer-reviewed	IIIA-IV	532	60%	first line chemotherapy +/- radiotherapy	Tumor Vaccine	Belagenpumatucel-L (4 transforming growth factor (TGF)-ß2 antisense gene-modified, irradiated, allogeneic NSCLC cell lines)	Placebo
Jin et al. 2014[35]	Peer-reviewed	I-IIIA	943	28%	Surgery	Cellular Therapy	CIK-therapy	BSC
Kimura et al. 2015[64]	Peer-reviewed	II–IV	101	76%	Surgery +/- induction chemotherapy (IIIA)	Cellular Therapy	AKT (autologous activated killer T cells) + DC-therapy + Chemotherapy	Chemotherapy
Khranovska et al. 2013[33,65]	Peer-reviewed	IIB-IIIA	120	not reported	Surgery	Cellular Therapy	DC-therapy	BSC
Li et al. Stage IIIB-IV 2012[66]	Peer-reviewed	IIIB-IV	74	62%	No previous treatment	Cellular Therapy	CIK + Chemotherapy (Cisplatin + gemcitabine/paclitaxel/navelbine)	Chemotherapy
Nokihara et al. 2015[67]	Abstract	III	172	67%	Chemo-radiotherapy	Tumor Vaccine	Cyclophosphamide + Tecemotide (L-BLP25= MUC1 Ag)	Saline + Placebo
Quoix et al. 2011[68]	Peer-reviewed	IIIB-IV	148	64%	No previous treatment	Tumor Vaccine	TG4010 (MVA coding for MUC1+IL-2) + Chemotherapy	Chemotherapy

Table 1 List of Study Characteristics

Reference	Type Article	Stage	No.of patients	% Adeno-carcinoma	Previous Treatments	Treatment type	Intervention Treatment	Control Treatment
Quoix et al. 2015[69]	Abstract	IV	222	88%	No previous treatment	Tumor Vaccine	Chemotherapy + TG4010	Chemotherapy + placebo
Shi et al. 2012[41]	Peer-reviewed	IIIB-IV	60	47%	First line chemotherapy	Cellular Therapy	DC-therapy + CIK-therapy	BSC
Shi et al. 2014[70]	Peer-reviewed	IIIB-IV	54	82%	First line chemotherapy	Cellular Therapy	Erlotinib + CIK-therapy + DC-therapy	Erlotinib
Vansteenkiste et al. 2013[40]	Peer-reviewed	IB-II	182	34%	Surgery	Tumor Vaccine	MAGE-A3-vaccine	Placebo
Vansteenkiste et al. 2014[36]	Abstract	IB-IIIa	2272	not reported	Surgery	Tumor Vaccine	MAGE-A3-vaccine	Placebo
Vinageras et al. /Garcia et al. 2008[71,72]	Peer-reviewed	IIIB-IV	74	33%	First line chemotherapy	Tumor Vaccine	Cyclophosphamide + EGF-Vaccine	BSC
Wu et al. 2008[39]	Peer-reviewed	IIIA- IV	59	41%	No previous treatment	Cellular Therapy	Chemotherapy (docetaxel + cisplatin + CIK-therapy	Chemotherapy
Zhao et al. 2014[34]	Peer-reviewed	III	157	56%	Surgery	Cellular Therapy	Chemotherapy (Gemcitabine+Cisplatin) + DC-therapy+ CIK-therapy	Chemotherapy

Abbreviations: NSCLC=Non-Small Cell Lung Cancer, MUC1=Mucin 1, Ag= Antigen, BSC= Best Supportive Care, TGF- ß2= Transforming Growth Factor - ß2, CIK= Cytokine Induced Killer Cells, AKT=Autologous Killer T-cells, DC= Dendritic Cell, MVA= Modified Vaccinia Ankera, IL-2= Interleukin 2, EGF=Epithelial Growth Factor,

The 18 RCTs included in our analysis comprised a total of 6756 patients treated with immunotherapy for NSCLC distributed over different outcome measures. A summary of the main study characteristics is listed in table 1. Most studies focused on late stage disease (15/18 trials stage III(B)-IV). The type of treatment was evenly distributed, with 10 studies investigating tumor vaccines and the other 8 treating patients with either DC-therapy, a form of T-cell therapy (AKT; autologous activated killer T cells or CIK; cytokine induced killer cells) or a combination of the two. The proportion of adenocarcinoma histology varied extensively between studies ranging from 28 to 88% of total cancers. Immunotherapy was administered as monotherapy with or without low-dose cyclophosphamide preconditioning or concurrently with chemotherapy. The control treatment arm was heterogeneously composed of control groups receiving only the placebo or best supportive care (BSC) and others receiving chemotherapy (when immunotherapy was combined with chemotherapy in the experimental arm). There were no studies that investigated the efficacy of tumor vaccines or cellular therapies alone versus chemotherapy treatment.

Immunotherapy significantly prolongs NSCLC survival and delays tumor progression

Studies reported different outcome measures with some reporting hazard ratios of disease progression or survival while others reported only median OS and/or time to disease progression in months. Therefore, we analyzed HRs and the differences in medians for survival and PFS separately. Cancer immunotherapy was found to be effective in extending NSCLC overall survival and PFS, both expressed as HR (cumulative OS: HR=0.81, 95%CI=0.70-0.94, *p=0.01,* PFS: HR=0.83, 95%CI=0.72-0.95, *p=0.006,* Figure 2) and median month difference (cumulative OS: difference=5.43 months, 95%CI=3.20-7.65, *p<0.005,* PFS: difference=3.24 months, 95%CI=1.61-4.88, *p<0.005,* Figure 3*)*.

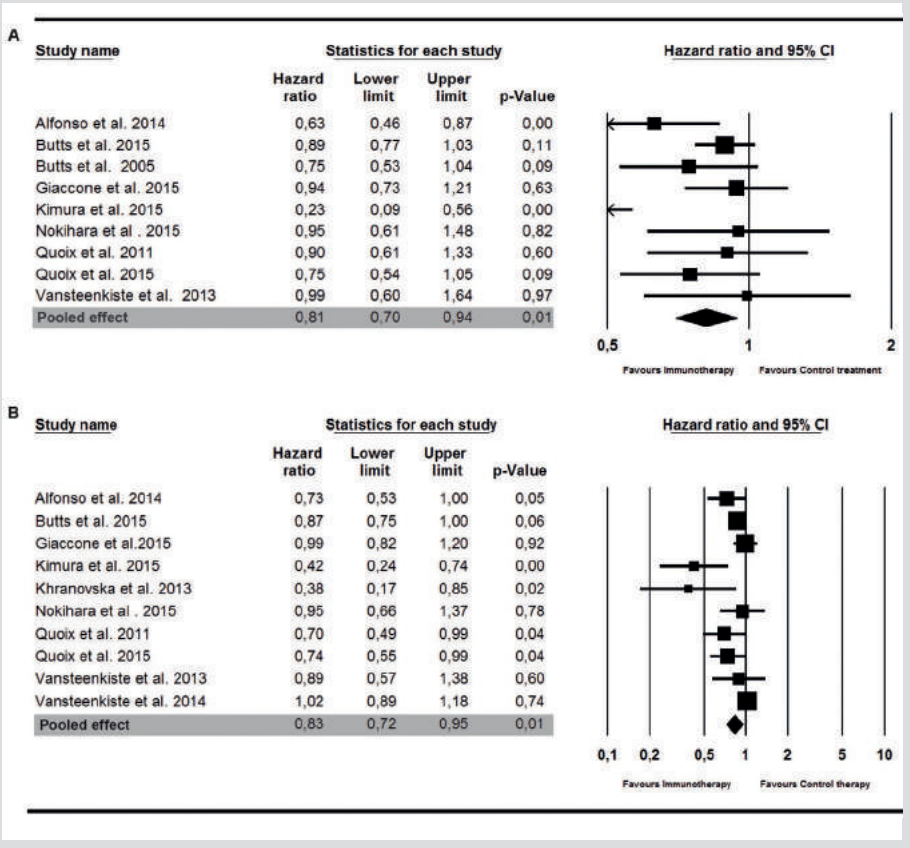

Figure 2 (A) Overall survival and (B) progression free survival when hazard ratios (HR) were reported.

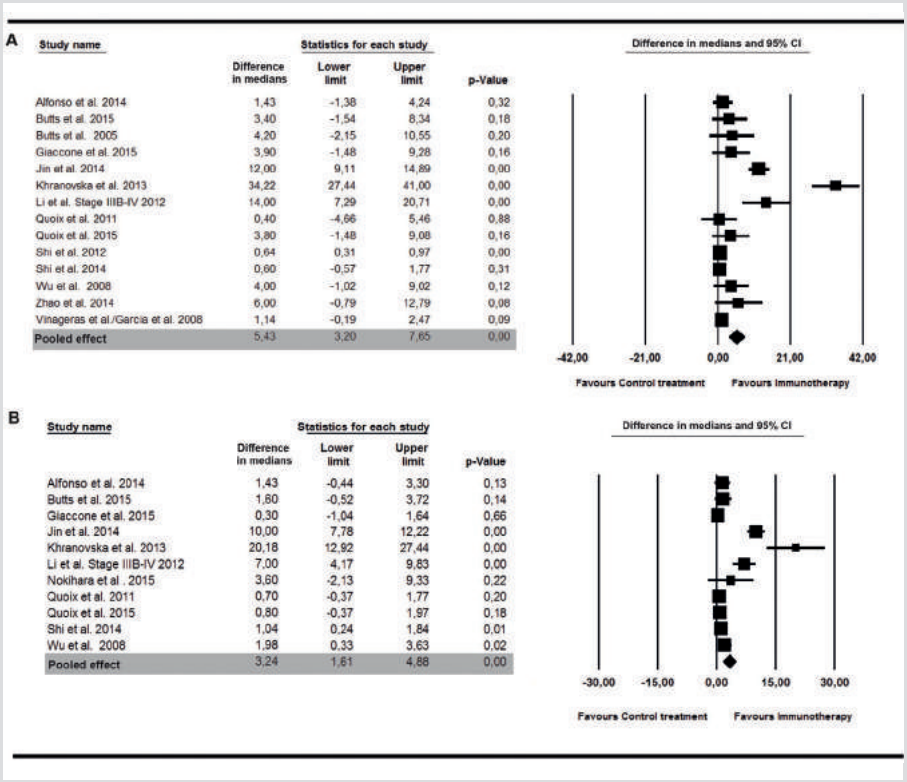

Figure 3 (A) Overall survival and (B) progression free survival expressed as mean differences in months when median overall survival data were reported.

Cellular immunotherapeutic strategies perform significantly better than tumor vaccination therapies

There was a moderate level of heterogeneity between sample estimates for both OS and PFS expressed as HR (OS: I^2=43.9%, PFS: I^2=57.7%) and significant heterogeneity for outcome measures expressed in months (OS: I^2=86.5%, PFS: I^2=85.8%). To test whether this heterogeneity could be attributed to differences in type of immunotherapy (vaccine or cellular therapies), disease stage (low vs. high), concurrent chemotherapy, NSCLC histology (% adenocarcinoma) or preconditioning with low dose cyclophosphamide, subgroup analysis and meta-regression were performed.

Significant heterogeneity existed between studies evaluating tumor vaccination and cellular immunotherapy treatments, with cellular therapies being more effective than tumor vaccines for all outcome parameters evaluated (OS as HR: *p=0.005* and median month difference: *p=0.001*, PFS as HR: *p=0.001* and as median month difference: *p=0.004*, Table 2). There were no significant differences in survival or disease progression between studies investi-

gating immunotherapy in high versus low stage NSCLC disease, except for the difference in median months of PFS, being more favorable for studies involving low-stage NSCLC than those involving high-stage disease (*p=0.010*). Studies that evaluated immunotherapy with concurrent chemotherapy performed better than studies investigating immunotherapy alone, only for time to disease progression as HR (*p=0.030*) There was no correlation between the proportion of patients with adenocarcinoma histology and the standardized mean difference for survival (*p=0.448)* or PFS (*p=0.426*, Appendix figure 1, online only) nor could we detect a benefit of pre-conditioning with cyclophosphamide in case of the tumor vaccines (HR OS: *p=0.577*, HR PFS: *p=0.928*).

Bias assessment

The level of bias varied extensively between studies, with several studies being deficient in thorough methodological reporting, which, in most cases, was because only an abstract was available [33-36]. The studies that properly reported randomization procedures, blinding and all outcome measures were generally low in bias [21, 37, 38], but selection bias and detection bias could be detected in some trials, or were not reported [39-41] (Figure 4). With the exception of three studies with an unclear risk of bias [33, 35, 36], all studies lacked reporting bias, attrition bias and other sources of bias not specifically addressed by the Cochrane Collaboration risk of bias tool.

Funnel plots were constructed to investigate the presence of publication bias. There was no or minimal publication bias for overall survival expressed as HR and median months difference (Appendix figure 2A and 2C, online only). Adjusted values after the Duval and Tweedie trim-and-fill test revealed no significant alteration of the observed point estimate (data not shown). There was no publication bias for the outcome PFS expressed as median month difference (Appendix figure 2D). There was, however, possible publication bias for PFS expressed as HR (Appendix figure 2B), as indicated by a potential loss of significance from the Duval and Tweedie trim-and-fill test (data not shown). Additional tests to quantify publication bias were inconclusive (the Egger test being significant for publication bias but two tailed Begg and Mazumdar's test not). Also, the classic fail-safe N test indicated that 40 additional studies would be required to reach a p-value greater than alpha (p=0.05, data not shown). Therefore, the presence of publication bias for PFS expressed as HR remains uncertain.

Table 2 Heterogeneity explained by factors associated with treatment efficacy

	# studies	OS HR	*p-value*	# studies	OS Median Month Diff.	*p-value*	# studies	Progression HR	*p-value*	# studies	Progression Median Month Diff.	p-value
Cellular Therapies	1	0.229		7	9.071		2	0.410		5	6.967	
Tumor Vaccines	8	0.851	***p=0.005***	7	1.564	***p<0.001***	8	0.890	***p=0.001***	6	0.829	***p=0.004***
High stage NSCLC	7	0.846		12	1.805		6	0.854		9	1.467	
Low stage NSCLC	1	0.990	*p=0.548*	2	22.890	*p=0.058*	3	0.823	*p=0.863*	2	14.479	***p=0.010***
With Chemotx	3	0.631		6	4.119		3	0.654		5	1.761	
Without Chemotx	6	0.853	*p=0.282*	8	6.704	*p=0.313*	7	0.906	***p=0.031***	6	5.304	*p=0.090*
With Cyclophos	3	0.872		3	1.408		2	0.880		2	1.842	
Without Cylophos.	5	0.821	*p=0.577*	5	0.935	*p=0.567*	6	0.872	*p=0.928*	5	0.847	*p=0.343*

Abbreviations: OS= Overall Survival, Diff. = Difference, HR= Hazard Ratio, NSCLC= Non-Small Cell Lung Cancer, Chemotx= Chemotherapy, Cyclophos= Cyclophosphamide

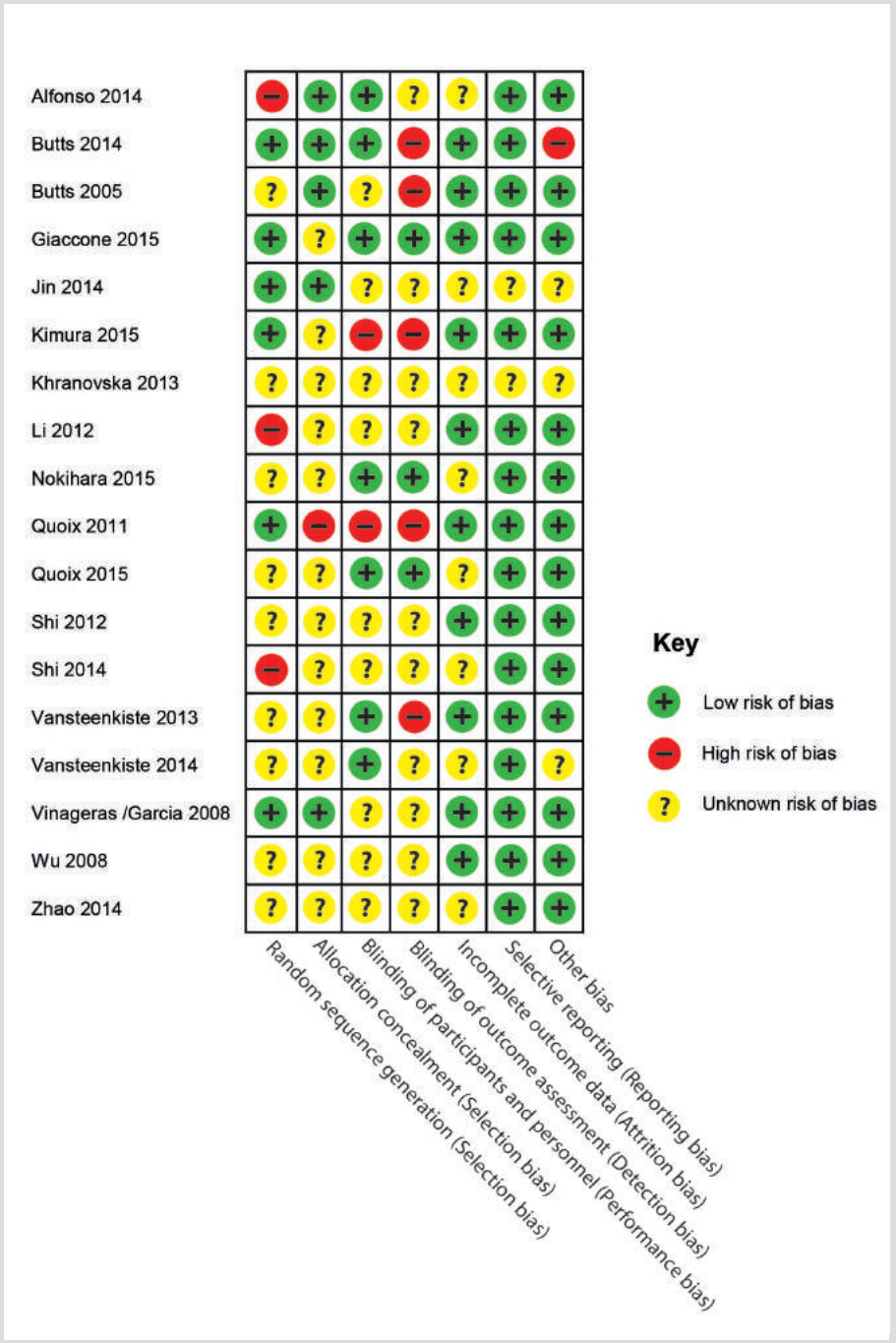

Figure 4 The assessment of bias of included studies using the Cochrane Collaboration Risk of Bias Assessment Tool.

DISCUSSION

Targeting the immune system to combat cancer is an effective way to enhance survival and prolong time until progression in NSCLC. In contrast to disease-modifying drugs such as cytokines (interleukin-2, interferons) and the recently investigated checkpoint inhibitors, tumor vaccines and cellular therapies induce formation of specific tumor-directed cytotoxic T cells capable of destroying cancer cells. Because of their high specificity, based on the selection of only tumor associated antigens, tumor vaccines and cellular immunotherapies are associated with limited side effects, but the efficacy of these therapies, with tumor vaccines in particular, has been controversial [42, 43]. In this meta-analysis, combining the data from all published tumor vaccine trials resulted in a significantly improved survival and prolonged time to disease progression. These results were even more pronounced for the cellular therapies. The distinction between these two immunotherapeutic strategies was the only factor that was consistently different for all outcome measures analyzed. Other factors such as disease stage and combination chemotherapy also significantly differed regarding clinical efficacy albeit not for all outcome measures. Tumor histology was not significantly associated with changes in survival or time to disease progression.

Large phase III studies such as the MAGRIT trial and the belagenpumatucel-L phase III trial were initiated following promising phase II results but they were prematurely terminated due to lack of clinical efficacy [42]. Discordant results of phase II and III studies in NSCLC research have been reported by others in the past and are thought to arise from differences in phase II and III study population characteristics, size and the (intermediate) outcome measures and the target effect size aimed for [44]. Most of the phase III studies included in our analysis concerned tumor vaccination trials, and these studies attributed the majority of patients to our analysis. Given the disappointing results of these trials it is remarkable that in our meta-analysis tumor vaccination therapies significantly enhanced survival and time to disease progression, suggesting insufficient power in previous phase III trials.

Even though there was no or limited publication bias for the majority of parameters investigated, overrepresentation of phase II studies in the cellular therapies included could have attributed to the differences in efficacy compared to tumor vaccination therapy. On the other hand, the differences may be ascribed to the activation of the immune system with cellular therapies such as DC and T-cell therapies. First, cellular therapies partially or totally circumvent potential immune suppression induced by vaccination and, therefore, directly activate anti-tumor T cells capable of targeting the tumor [19, 45]. Second, whereas vaccines target one predominant tumor antigen, DC (and, to a lesser extent, T cells) activated *in vitro* could potentially induce a polyclonal antitumor response [46, 47]. This might prevent immune escape by the tumor and induce immune responses to a multitude of tumor antigens.

Screening patients before vaccination for expression of the targeted tumor antigen and designing personalized tumor vaccines could increase response rates to tumor vaccines [48, 49].

Several studies have correlated a specific immune response to the vaccine with increased survival and tumor responses following therapy [37, 50-57]. Identifying these patients before or after the first vaccination as personalized therapy could specifically benefit those that are most likely to respond and favor the use of combination treatment in nonresponding patients. We found a statistically significant effect of chemotherapy combined with immunotherapy for the HR of disease progression, but not for other outcome measures. Chemotherapy could synergize with immunotherapies by causing immunogenic cell death of cancer cells and by disrupting immune evasion pathways, but timing of combination therapies is crucial [58]. We found no additive effect on tumor vaccine efficacy of low-dose cyclophosphamide in our analysis (Table 2), but this could be due to the limited amount of studies available for analysis. Also, few studies reported antibody responses towards the vaccine, making it difficult to assess the effect of prior immune modulation. Importantly, to further target immune-evasion pathways in patients, enhancing antitumor T-cell functionality with checkpoint inhibitors acting via PD-L1/PD-1 blockade could further improve therapeutic efficacy [59]. The PD-1 blocking antibodies nivolumab and pembrolizumab have been recently approved for NSCLC treatment following positive phase III results [7, 60].

There are several limitations relevant to this meta-analysis. First, there was a limited amount of studies available for some outcome measures investigated with subgroup analysis (e.g. OS HR for type of therapy and disease stage). Second, variability in control treatments with cellular therapies (BSC) and vaccine studies (mostly placebo) could have biased our results in favor of cellular immunotherapy. Because of incomplete reporting and heterogeneity in methodology we did not further investigate potential immune markers (e.g. antibody responses following vaccination) that could be predictive for therapeutic efficacy. More studies will have to be awaited to properly address this issue. Finally, bias could not be assessed in several of the studies included. Therefore, we did not perform sensitivity analysis after exclusion of studies with a high or unspecified degree of bias. Several larger phase III studies on tumor vaccines and cellular therapies are still awaited and it is inevitable in meta-analysis that studies will be missed. However, to our knowledge, this meta-analysis is the first analysis to give a comprehensive overview of specific immunotherapies in NSCLC, assessing both tumor vaccines and cellular therapies.

In conclusion, specific immunotherapies significantly prolonged NSCLC survival and PFS. Cellular immunotherapies were more effective than tumor vaccines for all outcome measures evaluated. Low-stage disease and the concurrent use of chemotherapy improved efficacy but only for disease progression in months and as HR, respectively. There was no association between treatment efficacy and adenocarcinoma histology or preconditioning low-dose cyclophosphamide. These findings are useful for the design of future studies investigating immunotherapies in NSCLC and possible synergistic combination strategies that could improve patient survival.

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Chapter 11

Summary and Discussion

SUMMARY

At the start of the studies described in this thesis we knew that macrophages are the predominant cell type in the microenvironment of mesothelioma tumors. Evidence regarding their potential role in tumor initiation, outgrowth and dissemination was arising from preclinical studies in other tumor types. In the studies described in this thesis we used multiple translational approaches to investigate the role of TAMs in the mesothelioma environment and their potential as a therapeutic target. We started in **chapter 2 and 3** by evaluating the presence, phenotype and correlation with patient survival and local tumor outgrowth (LTO). We demonstrated that the ratio of M2 macrophages to total macrophages present in tumor biopsies is a potential prognostic marker. Furthermore, determination of this ratio in diagnostic biopsies made it possible to predict the occurance of LTO. In addition to the infiltration of TAMs in the tumor microenvironment, we investigated their phenotype and function in pleural effusion of mesothelioma patients (**chapter 4**). Pleural effusion was shown to be able to induce a profound immunosuppressive macrophage phenotype capable of inhibiting T cell proliferation. In **chapter 5**, we further investigate the properties of pleural effusion and demonstrate that its composition is dynamic, influenced by treatment and doesn't necessarily mirror the immunological properties of the tumor. Together, **chapter 4 and 5** provide novel insights regarding the presence and phenotype of TAMs in pleural effusion, which should be considered when applying (intrapleural) immunotherapy in mesothelioma. In the first part of the thesis we validated the hypothesis that TAMs are an important and detrimental part of the mesothelioma microenvironment. Targeting TAMs as an immunotherapeutic approach has proven to be challenging. Multiple strategies are described in the literature which aim mainly at the re-education or depletion of TAMs. In **chapter 6** we use a CD40 agonistic antibody in a pilot study and demonstrate that it has an additive effect to DC immunotherapy. CD40 agonists have been described to exert their effect through the stimulation of local TAMs, whether this mechanism also underlies our results in a mesothelioma mouse model will be the subject of future studies. The reduction of TAMs using a CSF1R-inhibitor as described in **chapter 7**, did not improve survival but the combination with DC-immunotherapy showed clear synergistic effects in mesothelioma mouse models. As these preclinical studies provide leads for future clinical trials, it is pivotal to critically evaluate the design of these trials. Checkpoint blockade is quickly conquering the field of cancer immunotherapy thanks to the promising results of clinical trials in multiple tumor types. However, in order to benefit most from these novel agents in pulmonary oncology, it is pivotal that predictive biomarkers will be identified. In **chapter 8 and 9**, we discussed this need for biomarkers, provided an overview of the landmark clinical studies and the hurdles to overcome to implement checkpoint blockade in pulmonary oncology. **Chapter 10** demonstrated that cellular immunotherapy is more effective than tumor vaccination in NSCLC which opens new roads for combinatorial immunotherapy in pulmonary oncology.

DISCUSSION

Macrophages are key players in the mesothelioma environment

TAMs are the predominant immune cells in the mesothelioma environment. Characterization of their phenotype and more importantly local function is challenging. In chapter 2 and 3 of the thesis we attempted to quantify and qualify the infiltrating TAMs in mesothelioma biopsies. The scavenger receptor CD163 is often used to identify alternatively activated or M2 macrophages in human studies whereas CD68 is recognized to be a general macrophage marker [1]. Using immunohistochemistry as a relatively simple method we were able to establish the clinical value of the TAM infiltrate. We demonstrated in chapter 2 that although the total number of TAMs in tumor tissue did not correlate with survival, the CD163/CD68 ratio did in the total patient group (surgery or non-surgery). Therefore we identified this CD163/CD68 ratio as a potential prognostic marker, however it cannot be used as a predictive marker for outcome after surgery. Another group showed that in a large group of mesothelioma patients the combination of a high CD163+ TAM infiltration and a low CD8 T cell infiltration indicated a worse prognosis [2]. Furthermore, in chapter 3 we demonstrated that the CD163/CD68 ratio and amount of CD8 T cells within diagnostic tumor biopsies were predictive of local tumor outgrowth after a local intervention. Although only performed in limited patient groups, these results do indicate the regulation of a local immunological profile in mesothelioma which has clinical consequences. Evidently, when applying immunohistochemistry to characterize TAMs, the subtleties of the phenotype cannot be appreciated. Furthermore, whether the taken biopsy is representative of the entire tumor remains questionable. However, the studies presented in this thesis do show a correlation of the CD163/CD68 ratio with survival and local tumor outgrowth, thus providing a potential easy to perform clinical tool and the validation of TAMs as a therapeutic target in mesothelioma.

Expansion of the environment: pleural effusion

Malignant pleural effusion occurs as a consequence of pleuritis carcinomatosa in many cancers. In mesothelioma, it occurs in 70% of patients and the dyspnea it causes often is the first clinical sign [3, 4]. Drainage of pleural effusion is performed to relieve symptoms and for diagnostic purposes. In chapter 4 and 5 we further investigated the immunological properties of pleural effusions derived from mesothelioma patients. Initially we hypothesized that pleural effusion might be a direct reflection of the pleural tumor and could therefore function as an easy-access liquid biopsy. However in the proof-of-concept study we described in chapter 5 we demonstrated that the composition of pleural effusion is dynamic and influenced by treatment. Furthermore we established that the immune cell composition of pleural effusion does not necessarily mirror the composition of the tumor. These data will have to be confirmed in larger patient studies, indicating the importance of patient enrollment in clinical studies in a rare disease as mesothelioma. However our data do indicate that pleural effu-

sion should be considered a separate part of the mesothelioma environment. In chapter 4 we investigated the immunosuppressive properties of pleural effusions from mesothelioma patients prior to any treatment. We show that as in tumor biopsies, macrophages are the most abundant immune cells present although clear patient to patient variety is present. The capability of pleural effusion to induce macrophages with a M2 phenotype which suppress T cell proliferation illustrates the profound immunosuppressive character of this compartment. Therefore, the characteristics of pleural effusion should be taken into account when applying (intrapleural) immunotherapy in mesothelioma. As we identified PGE2 as a potential key regulator of the immunosuppressive environment, an elegant application of personalized immunotherapy would be to consider to administer COX-2 inhibitors to patients with high levels of PGE2 and M2 macrophages in their pleural effusion [5-7]. In contrast, the drainage of pleural effusion followed by pleurodesis to eliminate the immunosuppressive compartment seems an intuitively logical suggestion. However, as the intrapleural cavity is a potentially attractive site to administer (immuno)therapies we suggest to characterize the local profile and add a personalized adjuvant (e.g. COX-2 inhibitor, CD40 agonist) to the proposed therapy in order to be able to take advantage of the macrophages present in pleural effusion.

Infiltrating the environment: TAMs as a therapeutic target

The clinical and *in vitro* studies described in the first part of the thesis identified TAMs as an attractive potential therapeutic target in mesothelioma. There are several therapeutic strategies available, including the inhibition of recruitment of macrophages, the depletion of (M2) macrophages, the inhibition of (M2) macrophage function and the induction of a phenotypic switch from M2 to M1 [8, 9]. This induced re-polarization is the most attractive strategy since it would make use of the unique plasticity of macrophages and therefore allows to skew an immunosuppressive environment to a more pro-inflammatory environment. Stimulation of CD40 using agonistic antibodies has been described to skew macrophages towards a more M1-like phenotype and has shown promising results in other tumor types [10]. Therefore in chapter 6 we performed a pilot study with a CD40 agonistic antibody in a mesothelioma mouse model. In addition to the treatment with the CD40 agonist we also combined the antibody with DC immunotherapy. As DC immunotherapy relies on the activation of T cells followed by their infiltration and local anti-tumor response, DC immunotherapy could benefit from a M1-prone pro-inflammatory local environment [11, 12]. The combination therapy indeed showed additive results, with an increased systemic immune activation and survival in this treatment group. However, whether this additive effect can be attributed to the local re-polarization of TAMs could not be concluded from this pilot study. Given the pleiotropic expression of CD40, multiple cell types could contribute to the observed effect [13]. However, others have shown that in pancreatic cancer, a tumor type with a microenvironment dominated by macrophages as mesothelioma, tumor regression after treatment with a CD40 agonist required macrophages, not T cells [10]. Furthermore, in a mouse model of pancreatic

cancer, extra-tumoral macrophages residing in the lymph nodes and secondary lymphoid organs have been demonstrated to regulate the intra-tumoral infiltration of T cells following CD40 agonistic therapy, a finding which potentially expands the influence of macrophages beyond the tumor microenvironment [14]. Interestingly, in a recent study this group demonstrates that CD40 activation induces macrophages which originate from peripheral blood monocytes to induce fibrosis via the production of specific MMPs [15]. This effect depended on the systemic release of IFN-γ and CCL2 following CD40 activation. Degradation of the local fibrosis increased sensitivity to chemotherapy. These findings elegantly demonstrate the potential of the targeting of TAMs in tumors with a robust TAM infiltrate. As scavenging of local fibrotic material would originally be classified as a typical M2 trait, these findings also illustrate the evolving insights regarding macrophage plasticity. The continuum of macrophage phenotypes allows for the induction of specific traits, e.g. phagocytosis, antigen presentation or fibrosis degradation, depending on the required local effect.

In addition to the skewing of TAMs we investigated the potential of TAM depletion in chapter 7. Using the CSF1R-inhibitor in mesothelioma mouse models we were able to reduce TAMs without improving survival. However in combination with DC immunotherapy there was a synergistic effect improving survival, decreasing TAMs and enhancing CD8 T cell frequency and functionality. These findings illustrate that targeting of the tumor microenvironment as a single strateqy will unlikely be sufficient to unleash a potent anti-tumor immune response in a tumor with a profound immunosuppressive environment as mesothelioma. However, these preclinical data do illustrate the great potential of combinatorial approaches. As this thesis focuses on immunotherapy, (combinations with) conventional therapies were not investigated. Evidently, it is essential to evaluate immunotherapy in the context of treatment modalities which have proven their benefit in the past.

Towards combinatorial immunotherapy

The final part of the thesis focuses on how checkpoint blockade should be implemented in pulmonary oncology. Since this class of immunotherapy is evolving quickly and multiple agents are now clinically available, a rational design of clinical studies investigating these compounds is key. Although initial results are promising and have lead to the approval of PD-1 blockade in NSCLC patients, the vast majority of lung cancer and mesothelioma patients do not respond to the currently available checkpoint blocking antibodies [16-19]. As discussed in chapter 8 and 9, the identification of robust biomarkers is crucial for the successful clinical implementation of checkpoint blockade. Given the costs and potentially serious side effects research should be focused on identifying those patients who are most likely to benefit from checkpoint blockade. The local and systemic immunosuppressive environment in lung cancer and mesothelioma hampers the efficacy of checkpoint blockade. We propose a combinatorial immunotherapeutic approach based on the targeting of both systemic and local immunosuppression in combination with cellular immunotherapy. In our view, this approach should

be personalized and adapted to the suppressive mechanisms in action in each particular patient and in every compartment (peripheral blood, pleural effusion and tumor). In our model we propose the use of cellular immunotherapy as the strategy to induce a specific anti-tumor T cell response. This is based on the findings in our meta-analysis in chapter 10 which demonstrates that cellular immunotherapy is more effective in NSCLC compared to tumor vaccination. Given the up-regulation of the PD-1/PD-L1 axis following a potent local T cell response, combination of cellular (DC) immunotherapy with PD-1/PD-L1 blockade is reasonable. Furthermore, traditional treatment modalities as chemotherapy and in selected mesothelioma patients, surgery, could be very useful neoadjuvant sensitizing approaches. Various chemotherapies have been demonstrated to induce immunogenic cell death which can lead to the release of tumor associated antigens, enhancing the potential of immunotherapeutic approaches [20].

Concluding remarks and future directions

The main goal of this thesis was to investigate the role of tumor-associated macrophages in the mesothelioma environment and their potential as a therapeutic target. We established that macrophages are key immunosuppressive players in mesothelioma (both locally and in pleural effusion) and their presence and phenotype has major clinical implications. The preclinical studies in this thesis demonstrate the great therapeutic potential of targeting macrophages in mesothelioma, especially in combination with DC immunotherapy. Although questions have been answered, new questions arose which will lead the way of future studies. One of the major insights from this thesis is the pressing need for patient-tailored immunotherapy in mesothelioma and probably multiple other tumor types. Since mesothelioma is a rare disease, patient numbers in our clinical studies were relatively low. On one hand this evidently is a drawback, on the other this allowed us to recognize the profound patient-to-patient variability regarding the characteristics of the tumor environment in mesothelioma. Future studies should be directed at the standardized characterization of the immunosuppressive mechanisms at play. Ideally, in concordance with the molecular profiling of a tumor an additional immunological profiling should take place which includes both the local and systemic dominant cell types and phenotype. For mesothelioma, local tumor-associated macrophages and systemic myeloid cells should be among the main cells of interest. As immunological reactions and tumor characteristics are dynamic by nature, a single diagnostic biopsy is not sufficient to investigate potential biomarkers and treatment effects. Although stressful for the patients, multiple biopsies during a treatment regimen will allow for the identification of working mechanisms and therefore further improvement of immunotherapy. Future preclinical studies are necessary to design rational treatment combinations depending on the immune profile identified. Clinical studies investigating immunotherapy should focus on the identification of potential biomarkers based on hypotheses derived from preclinical work. Therefore, a close collaboration between basic scientists and clinical researchers is essential to fully develop cancer immunotherapy.

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Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Kanker en het immuunsysteem

Aan het einde van de 19e eeuw poneerde Stephan Paget (Engelse chirurg, 1855-1926) de hypothese dat niet alleen de intrinsieke eigenschappen van de tumorcel belangrijk zijn, maar dat ook de omgeving waar een tumorcel in terecht komt bepalend is voor de manier waarop een tumor zich verspreidt door het lichaam. Paget observeerde namelijk dat bepaalde soorten van kanker, zoals borstkanker, voornamelijk uitzaaien naar specifieke organen zoals de lever. Het uitzaaiingspatroon wordt niet bepaald door toeval. Sommige organen vormen namelijk een betere voedingsbodem voor bepaalde tumorcellen dan andere. Inmiddels weten we dat normale lichaamscellen niet alleen invloed hebben op de plek van uitzaaiing van een tumor, maar ook bepalend kunnen zijn voor de ontwikkeling van de tumor zelf.

Gedurende de ontwikkeling van een tumor ontstaat er een ingewikkeld netwerk van niet alleen tumorcellen, maar ook van bloedvaten, bindweefsel en cellen van het afweersysteem; de immuuncellen. Dit netwerk wordt het micromilieu van de tumor genoemd.

De primaire taak van het immuunsysteem is de bescherming van ons lichaam tegen infecties door onder andere bacteriën en virussen. Om deze bescherming mogelijk te maken is ons immuunsysteem uitgerust met verschillende type immuuncellen die in staat zijn om lichaamsvreemde indringers te herkennen en vervolgens onschadelijk te maken. Macrofagen spelen een grote rol bij de eerste verdediging tegen indringers en zijn in staat om lichaamsvreemd materiaal op te nemen en te vernietigen.

Dendritische cellen zijn gespecialiseerd in het herkennen van lichaamsvreemd materiaal en presenteren dit vervolgens aan T cellen die een belangrijk deel uitmaken van de specifieke afweer.

Als een tumorcel ontstaat wordt deze normaal gesproken ook herkend door het immuunsysteem en opgeruimd waardoor de uitgroei van een tumor voorkomen wordt. Echter, soms ontsnapt een kwaadaardige cel aan het immuunsysteem waardoor de cel zich kan gaan vermenigvuldigen en een tumor de kans krijgt zich te ontwikkelen. Een tumor kan daarnaast in staat zijn om immuuncellen te onderdrukken of zo te beïnvloeden dat ze de groei van de tumor ondersteunen.

Het doel van immuuntherapie is om het immuunsysteem zo te stimuleren dat het de tumor herkent als lichaamsvreemd en vervolgens aanvalt.

Macrofagen en kanker

De macrofaag speelt een belangrijke rol in de eerste verdedigingslinie van het immuunsysteem. Een bijzondere eigenschap van macrofagen is dat deze cellen veel verschillende soorten functies kunnen uitoefenen, afhankelijk van de omgeving waarin de macrofaag zich bevindt. Als weefsels beschadigd raken door trauma of infectie worden de voorlopers van

macrofagen, monocyten, vanuit de bloedbaan naar de plek van schade gerekruteerd. Het hangt af van de omgeving waarin de monocyt terecht komt tot wat voor type macrofaag hij zich ontwikkelt.
De macrofaag fenotypes zijn in te delen in een breed spectrum met aan de uiteindes de M1 en de M2 macrofaag. De M1 macrofaag is gespecialiseerd in het aanvallen en opruimen van indringers en lichaamsvreemde cellen zoals bacteriën en tumorcellen. De M2 macrofaag speelt een belangrijke rol bij verschillende aspecten van de wondgenezing zoals het vormen van bindweefsel en nieuwe bloedvaten. De M1 macrofaag heeft een belangrijke pro-inflammatoire functie, de M2 macrofaag onderdrukt de immuunrespons juist. Een M1 macrofaag wordt dan ook anti-tumor genoemd, terwijl een M2 macrofaag als pro-tumor bekend staat. Een belangrijke eigenschap van de macrofaag is de plasticiteit van het fenotype; een macrofaag is in staat om het fenotype te veranderen afhankelijk van de signalen die hij ontvangt uit zijn omgeving. Tumoren kunnen voor een groot deel uit macrofagen bestaan en het fenotype van deze macrofagen (M1 of M2) heeft klinische betekenis.

Zo heeft onderzoek in verschillende vormen van kanker aangetoond dat patiënten met tumoren waar veel macrofagen met het M1 fenotype in zitten een betere overleving hebben dan patiënten met veel M2 macrofagen in de tumor.

Mesothelioom en Longkanker

Als iemand asbestvezels inhaleert kunnen deze terecht komen op het borstvlies (pleura) of buikvlies (peritoneum). Asbest is niet afbreekbaar lichaamsvreemd materiaal wat lokale macrofagen op proberen te ruimen, maar gezien het formaat van de asbestvezels lukt dit niet. Vaak vele jaren na blootstelling aan asbest kan er kanker ontstaan van het long- en/of buikvlies; het mesothelioom. In Nederland worden jaarlijks ongeveer 500 mensen gediagnosticeerd met mesothelioom en de verwachting is dat dit aantal de komende jaren verder zal stijgen. Mesothelioom kan (nog) niet genezen worden en de gemiddelde overleving na diagnose is ongeveer 12 maanden, ondanks behandeling met chemotherapie.
Longkanker wordt meestal veroorzaakt door roken en wordt jaarlijks in Nederland bij ruim 10.000 patiënten vastgesteld. Voor de prognose van de levensverwachting is het stadium waarin longkanker wordt vastgesteld van groot belang. De 5-jaars overleving is slechts 17%, met name doordat longkanker vaak pas in een vergevorderd stadium gediagnosticeerd wordt waardoor de behandelopties beperkt zijn.

Het is duidelijk dat er voor zowel mesothelioom als longkanker behoefte is aan nieuwe manieren om deze ziekten te behandelen. Immuuntherapie is daarvan een veelbelovende optie.

Doel en resultaten van het onderzoek

Het doel van het onderzoek beschreven in dit proefschrift is om de bijdrage van macrofagen aan de progressie van het mesothelioom (deel 1) en hun potentie als therapeutisch aangrijpingspunt (deel 2) te onderzoeken. Daarnaast wordt ook de potentie van immuuntherapie bij longkanker en mesothelioom in beschouwende zin onderzocht (deel 3).

Hoofdstuk 1 van dit proefschrift geeft een uitgebreide introductie van de achtergronden waar dit onderzoek op gebaseerd is. Het principe en de verschillende vormen van immuuntherapie wordt besproken. Verder wordt een algemene introductie gegeven over het ontstaan, de klinische kenmerken en de mogelijke behandelingen van mesothelioom en longkanker. Daarnaast beschrijft de introductie de huidige kennis over de eigenschappen van macrofagen en hun rol bij het ontstaan en de progressie van kanker. In **hoofdstuk 2 en 3** wordt allereerst de aanwezigheid en het fenotype van macrofagen in mesothelioom onderzocht. Het grootste gedeelte van de macrofagen bleek van het M2 (pro-tumor) fenotype te zijn. Er wordt aangetoond dat hoe meer van de aanwezige macrofagen in mesothelioom een M2 fenotype hebben, hoe slechter de prognose van patiënten is. Daarnaast wordt aangetoond dat dit M2 fenotype ook de kans op een lokale uitgroei van het mesothelioom vergroot.

Bij patiënten met een pleuraal mesothelioom is er vaak sprake van vocht tussen het borst- en longvlies; dit wordt pleuravocht genoemd. Aangezien pleuravocht zorgt voor kortademigheid wordt het regelmatig weggehaald door middel van drainage. Dit biedt de mogelijkheid om eigenschappen van dit pleuravocht nader te onderzoeken. In **hoofdstuk 4** wordt het fenotype en de functie van macrofagen in pleuravocht onderzocht en wordt aangetoond dat macrofagen onder invloed van factoren in het pleuravocht van mesothelioompatiënten in staat zijn om de anti-tumor immuunrespons te onderdrukken. **Hoofdstuk 5** toont verder aan dat de eigenschappen van pleuravocht dynamisch zijn en worden beïnvloed door therapie. Daarbij bleek pleuravocht geen afspiegeling te zijn van kenmerken van de tumor maar een op zichzelf staand immunologisch compartiment. De in hoofdstuk 4 en 5 aangetoonde eigenschappen van pleuravocht zijn belangrijke factoren om rekening mee te houden bij de toepassing van, in het bijzonder intrapleurale, immuuntherapie bij mesothelioom. Daarnaast resulteert het eerste gedeelte van het proefschrift in de hypothese dat macrofagen een interessant potentieel therapeutisch aangrijpingspunt zijn bij mesothelioom. In de literatuur worden verschillende manieren beschreven om macrofagen aan te vallen. In de eerste plaats kan de therapie zich richten op het verwijderen van alle aanwezige macrofagen. Deze opzet heeft als nadeel dat ook de aanwezige anti-tumor M1 macrofagen verwijderd worden. Een andere optie is dan ook om te trachten de aanwezige pro-tumor M2 macrofagen te veranderen in anti-tumor M1 macrofagen. In het tweede deel van het proefschrift, **hoofdstuk 6 en 7**, zijn met behulp van preklinische mesothelioom muismodellen verschillende opties om macrofagen te beïnvloeden onderzocht. Het aangrijpen van macrofagen is steeds gecombineerd met een andere vorm van immuuntherapie; het toedienen van dendritische cellen. Door dendritische cellen die specifieke eigenschappen van de tumor herkennen toe te dienen wordt

de anti-tumor immuunrespons geïnitieerd. Aangezien deze immuunrespons lokaal ook weer teniet gedaan kan worden door pro-tumor macrofagen zou de effectiviteit van dendritische cel immuuntherapie vergroot kunnen worden door deze te combineren met macrofaag-gerichte therapie. In hoofdstuk 6 is gebruik gemaakt van een CD40-agonistisch antilichaam en wordt aangetoond dat dit een additief effect heeft op dendritische cel immuuntherapie in een mesothelioom muismodel. Van deze CD40-stimulerende antilichamen is beschreven dat het werkingsmechanisme berust op het effect op het fenotype van macrofagen. Of dit mechanisme ook ten grondslag ligt aan de gevonden effecten in mesothelioom is het onderwerp van toekomstige studies. In hoofdstuk 7 is gebruik gemaakt van een techniek om het aantal macrofagen in een mesothelioom te verminderen. Zoals eerder beschreven worden initieel de voorlopers van macrofagen, monocyten, vanuit de bloedbaan naar de tumor toegetrokken waarna deze monocyten zich lokaal differentiëren tot macrofagen. De aantrekking en overleving van macrofagen kan verstoord worden door gebruik te maken van een CSF1R inhibitor. In hoofdstuk 7 wordt gedemonstreerd dat een CSF1R inhibitor alleen geen effect heeft, maar in combinatie met dendritische cel immuuntherapie een sterke toename van de overleving laat zien in een mesothelioom muismodel. Deze preklinische studies tonen de potentie van macrofagen als therapeutisch aangrijpingspunt bij mesothelioom, zeker in combinatie met dendritische cel immuuntherapie.

Het derde deel van het proefschrift is gericht op het beschouwen van nieuwe vormen van immuuntherapie bij mesothelioom en longkanker. In **hoofdstuk 8 en 9** worden de beschikbare klinische studies die de effectiviteit van checkpoint inhibitors onderzoeken in mesothelioom en longkanker geëvalueerd. Checkpoint inhibitors zijn antilichamen gericht tegen immuuncel gebonden signalen die de immuunrespons remmen. Door deze negatieve signalen te blokkeren wordt de rem op het immuunsysteem opgeheven. Hoewel deze antilichamen veelbelovend zijn, heeft maar een beperkt gedeelte (ongeveer 20%) van de longkanker en mesothelioom patiënten profijt van deze behandeling. Het is daarom essentieel dat er onderzoek verricht wordt naar het identificeren van zogenaamde biomarkers; eigenschappen van een patiënt of tumor die de respons op behandeling kunnen voorspellen zodat er geen patiënten onnodig behandeld worden.

In **hoofdstuk 10** wordt tenslotte aangetoond dat bij longkankerpatiënten cellulaire immuuntherapie een effectievere vorm van immuuntherapie is dan therapie op basis van vaccinatie. Dit is een belangrijke bevinding met het oog op de combinaties van immuuntherapieën die steeds vaker uitgevoerd worden. De eerdergenoemde checkpoint inhibitors zouden derhalve goed gecombineerd kunnen worden met cellulaire immuuntherapie (zoals dendritische cellen) in mesothelioom en longkanker.

Conclusie

Macrofagen spelen een essentiële rol in het micromilieu van het mesothelioom door hun vermogen om de immuunrespons te onderdrukken, zowel lokaal als in pleuravocht, en hun aanwezigheid en fenotype hebben klinische consequenties. De preklinische studies in het mesothelioom muismodel demonstreren de potentie van macrofagen als therapeutisch aangrijpingspunt in mesothelioom, in het bijzonder in combinatie met dendritische cel immuuntherapie. Aangezien de studies beschreven in dit proefschrift laten zien dat de samenstelling van het micromileu van de tumor sterk kan verschillen van patiënt tot patiënt, ondersteunen deze resultaten de ontwikkeling van persoonlijke, op maat gemaakte therapie. Preklinische studies zullen nodig blijven om rationele combinaties van immuuntherapieën te ontwerpen en te testen. Een nauwe samenwerking tussen basale wetenschappers en klinische onderzoekers is dan ook essentieel voor de toekomst van immuuntherapie in de (pulmonale) oncologie.

Dankwoord

DANKWOORD

Zoals een belangrijke hypothese die ten grondslag ligt aan dit proefschrift voorschrijft, zijn er twee dingen belangrijk voor groei; 'seed and soil' oftewel 'nature and nurture'. Hetzelfde geldt voor de totstandkoming van een proefschrift en er zijn een aantal mensen die de afgelopen jaren op verschillende manieren een bijdrage hebben geleverd aan de omgeving waarbinnen ik mijn onderzoek heb mogen doen.

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Sanne
Breda, januari 2017

About the author

ABOUT THE AUTHOR

Sanne Lievense was born in Breda on February 26th in 1985. She attended the Stedelijk Gymnasium in Breda and graduated cum laude in 2003 after which she started her studies in Molecular Life Sciences at Maastricht University. After obtaining her Bachelor of Science degree Sanne continued with a Master of Science in Clinical Molecular Science at Maastricht University and Hasselt University in Belgium and obtained her degree in 2007. Although Sanne enjoyed studying basic science she decided to broaden her horizon and applied for the MSc program MD-Clinical Investigator at Maastricht University. In the final year of this four year program, Sanne investigated pulmonary outcomes of prematurity under the supervision of prof. dr. Boris Kramer and dr. Jasper Been. In 2011 Sanne started to work as a resident at the Intensive Care Unit of the Amphia hospital in Breda where she came in contact with prof. dr. Joachim Aerts. She started her PhD project which resulted in this thesis in 2012 at the research laboratory of the department of Pulmonary Medicine at the Erasmus Medical Centre in Rotterdam under the supervision of dr. Joost Hegmans, prof. dr. Rudi Hendriks and prof. dr. Joachim Aerts. In April of 2016 Sanne started her clinical training in pulmonary medicine (supervisor dr. Marco Grootenboers) with two years of internal medicine (supervisor dr. Joost van Esser) at the Amphia hospital. After completing her PhD, Sanne aims to combine a clinical career in pulmonary medicine with performing research. Sanne lives in Breda with Jeroen and their son Lukas.

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PhD Portfolio

Lysanne Annelie Lievense

Project title	Macrophages in Mesothelioma – Improving immunotherapy in pulmonary oncology
Supervisors	Prof.dr. J.G.J.V. Aerts Prof.dr. R.W. Hendriks Dr. J.P.J. Hegmans
Department	Pulmonary Medicine, Erasmus MC Rotterdam The Netherlands
Research School	Molecular Medicine Postgraduate School Erasmus MC Rotterdam, The Netherlands
PhD period	2012-2016

Courses

2012	Basic Flow Cytometry
2012	Good Clinical Practice
2012	Laboratory Animal Experimentation (art. 9)
2013	Microscopic Image Analysis
2014	Molecular Immunology
2014	Dutch Lung Cancer Course (New York City, USA)
2015	Good Clinical Practice Update

Scientific Presentations and Meetings

2012	American Assocation of Cancer Research (AACR) *Chicago, USA*
2012	International Mesothelioma Interest Group (iMig) *Boston, USA (2 poster presentations*)

2012	Cancer Immunotherapy (CIMT) *Mainz, Germany (2 poster presentations)*
2013	Netherlands Respiratory Society (NRS) Longdagen *Utrecht, The Netherlands (2 poster presentations)*
2013	17th Molecular Medicine Day *Erasmus MC Rotterdam, The Netherlands (poster presentation)*
2013	Cancer Immunotherapy (CIMT) *Mainz, Germany (2 poster presentations)*
2014	GREAT meeting (Ghent – Rotterdam collaboration) *Ghent, Belgium (oral presentation)*
2014	18th Molecular Medicine Day *Erasmus MC Rotterdam, The Netherlands (poster presentation)*
2014	Society of asbestos victims annual meeting Rotterdam, The Netherlands (oral presentation)
2014	Keystone Symposium Immune Evolution in Cancer *Whistler, Canada (2 poster presentations)*
2014	Netherlands Respiratory Society (NRS) Longdagen *Utrecht, The Netherlands (oral presentation)*
2014	Cancer Immunotherapy (CIMT) *Mainz, Germany (poster presentation)*
2015	Society of asbestos victims annual meeting *Rotterdam, The Netherlands (oral presentation)*
2012-2016	Periodic presentations at department of Pulmonary Medicine *Erasmus MC Rotterdam, The Netherlands*
2014-2016	Chairmanship Tumor Immunology Platform – Taskforce Immunomonitoring *Erasmus MC Rotterdam, The Netherlands*

Teaching

2013-2014	Journal Club supervision first-year medical students *Erasmus MC Rotterdam, The Netherlands*
2013	Supervision graduation project BSc student
2014	Supervision graduation project MSc student
2015	Supervision graduation project MSc student
2014-2015	Lecture Winter Course Infection & Immunity Master

Grants and Awards

2013	Mesothelioma Applied Research Foundation – Larry Davis Memorial Grant ($100.000,- for research necessities)
2014	NRS travel grant – Keystone Symposium
2014	Best oral presentation, NRS Longdagen