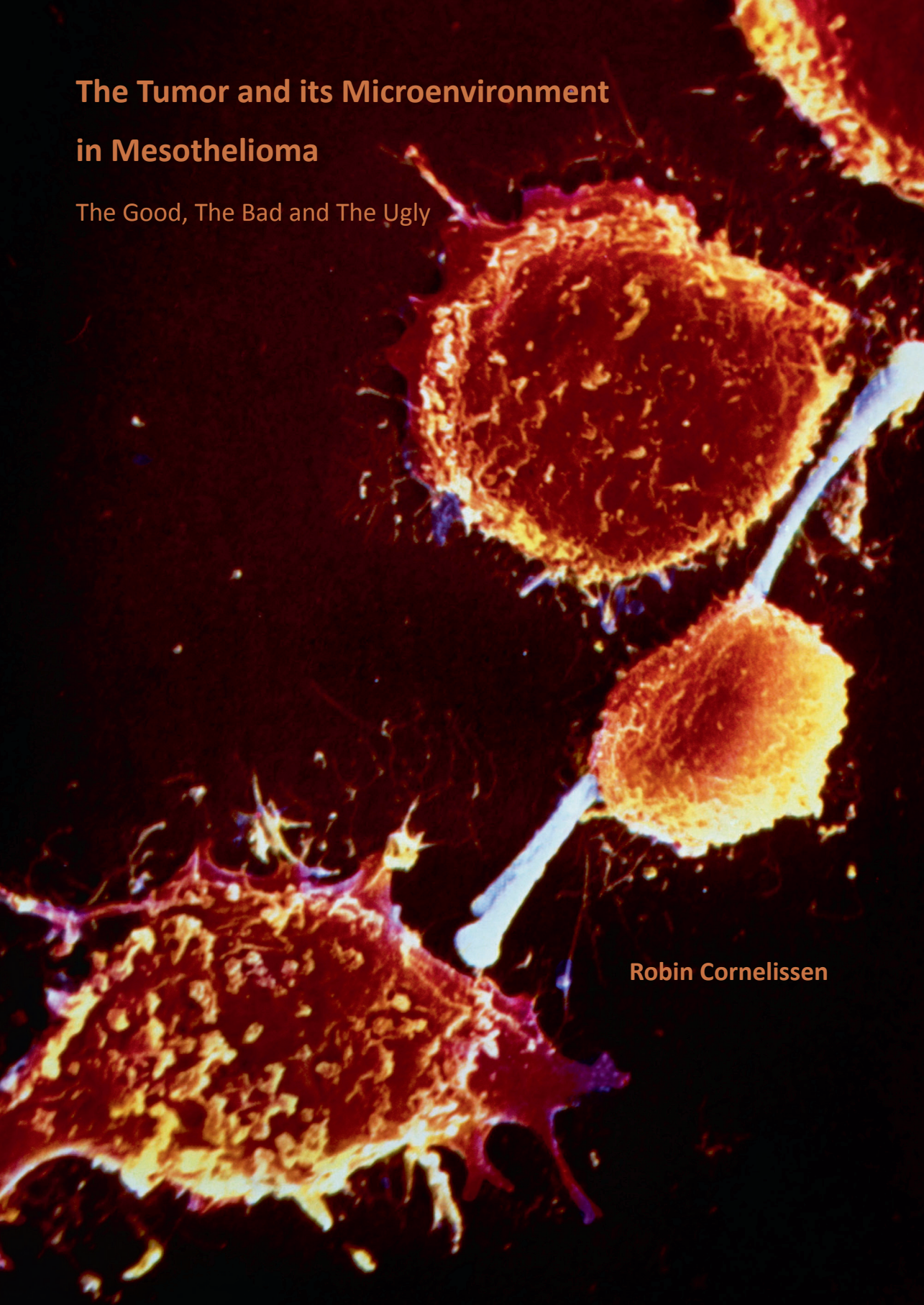
# The Tumor and its Microenvironment in Mesothelioma

## The Good, The Bad and The Ugly



Robin Cornelissen

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Photo on the cover: False-color scanning electron micrograph of macrophages from a lavage of a lung of a person suffering from asbestosis, a lung disease caused by inhalation of asbestos fibers; One macrophage is seen that has an asbestos needle through the whole cell. Normally, foreign particles in the lungs are phagocytized by the macrophages. Asbestos needles, however, are virtually indestructible and are too long to be phagocytized.

Magnification: x980 at 35mm size.



ISBN 978-94-6108-941-0



The studies presented in this thesis were conducted at the Department of Pulmonary Medicine at the Erasmus MC, Rotterdam, The Netherlands, with financial support from Stichting Asbestkanker and the Meso Foundation

Printing of this thesis was financially supported by Amgen B.V., Boehringer Ingelheim B.V., Chiesi Pharmaceuticals B.V., Hitachi Medical Systems, Olympus Nederland B.V., Pfizer Oncology, ProStrakan Pharma B.V., and Verastem, Inc.

Printing and Layout: Gildeprint - www.gildeprint.nl

**The Tumor and its Microenvironment in Mesothelioma**
The Good, The Bad and The Ugly

**Het mesothelioom en zijn micro-omgeving**
De Goede, de slechte en de lelijke

**Proefschrift**

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof. dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 8 april 2015 om 11:30 uur

door

**Robin Cornelissen**
geboren te Rotterdam

Erasmus University Rotterdam

**Promotiecommissie:**

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# Chapter I

**From fiber to disease**

introduction

# INTRODUCTION

**Asbestos**

Asbestos is a natural mineral that was, and unfortunately still is, used a lot by mankind because of the outstanding properties of the substance. It is durable, strong, electric- and heat-resistant and, last but not least, cheap. The discovery of these properties was made millennia ago in ancient Greek, and thus the mining and use of asbestos was initiated over 2000 years ago. During the middle ages, the use of asbestos declined, but in the documents remaining, several descriptions of asbestos use were described. For example, Charlemagne (2 April 747 or 748 – 28 January 814) convinced his guests that he had supernatural powers by throwing his asbestos tablecloth into a fire, and then pulling it out without any singe mark of burning[1]. Marco Polo wore fire-resistant clothing made from fibrous material on his travels in the Ural Mountains in the 13th century[1]. The popularity of the material clearly increased in the 19th century, when asbestos was massively used during the industrial revolution. It further increased in the 20th century; a total of 174 million tons of asbestos was mined during these 100 years[2]. Despite asbestos bans in a growing number of countries worldwide, in the year 2013, a total of 1.94 million tons of asbestos was mined[3].

In the Netherlands, the population mainly exposed to asbestos were employees in ship construction and maintenance, general construction and isolation activities[4]. The main locations of asbestos exposure for workers in the Netherlands were Den Helder, Rotterdam and Vlissingen due to the ship building industries. In addition, the Eternit factory in Goor and the Asbestona factory in Harderwijk manufactured asbestos-based products which resulted in significant local asbestos exposure[5–7]. Also, a considerable number of people were exposed indirectly to asbestos e.g. due to the cleaning of the working clothes by the housemates.

**The asbestos fiber**

Asbestos is the commercial name for a group of hydrated silicate fibrous minerals and is composed of fibers. To inhale these asbestos fibers, these must first get airborne. High dose exposure to asbestos is found when it is processed; hence the fact that most asbestos-related diseases are found in patients working in industrial

and construction setting. Because asbestos fibers appear neither to be metabolized nor interact with deoxyribonucleic acid (DNA), they are unlike most chemical carcinogens. Fiber analysis have led to an toxicology structure paradigm involving length, diameter and biopersistence[8]; The fiber must have a length that is longer than macrophages are able to phagocytose, the diameter of the fiber must be thin enough to enable deposition beyond ciliated airways and the fiber must retain its shape during its residence in the lungs to potentially induce a malignancy.

This fiber paradigm identifies geometry of fibers as their most important toxicological characteristic. The needle-like fibers are classified in the Amphibole class: actinolite, amosite, anthophyllite, crocidolite, and tremolite while the more flexible fibers can be found in the Serpentine class fibers with chrysotile being the only member of this class. Crocidolite, also known as blue asbestos, is known to be the most carcinogenic, and consists of long, thin, biopersistant fibers (figure 1 and 2).

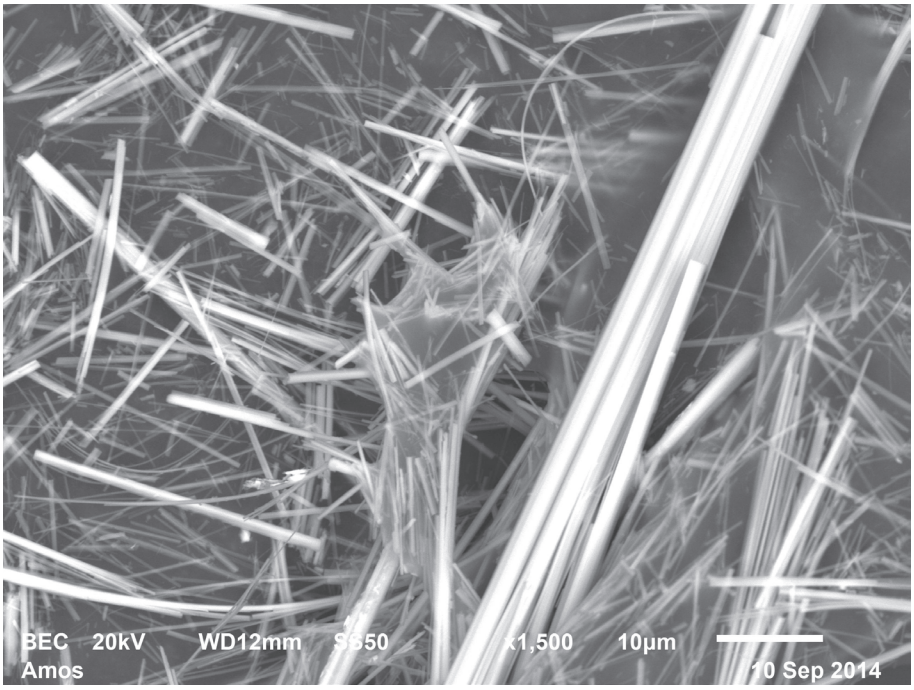


**Figure 1**
Scanning electron microscopic image of amosite, showing the long, thin, biopersistant fibers (Image provided by M.Schaar, Amos Milieutechniek B.V.)

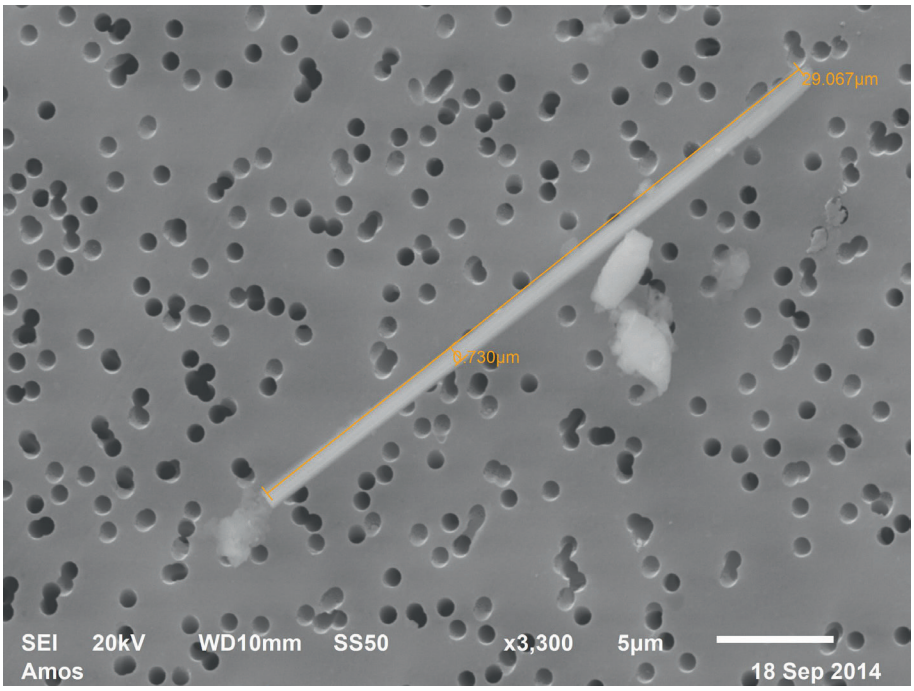


**Figure 2**
Scanning electron microscopic image of amosite on a gold-layered air filter, showing dimensions of a fiber. The length of the fiber has been correlated to the carcinogenicity, strongest associations observed for long fibers (length >10µm) and very thin fibers (diameter <1µm)[10,11] (Image provided by M.Schaar, Amos Milieutechniek B.V.)

The fibers of amosite, brown asbestos, are thicker and even longer than crocodolite, and evenly biopersistant. Exposure to chrysotile, white asbestos, gives 2 to 4 times less chance to develop cancer of the mesothelial cells (mesothelioma)[9]. It is thought that the fact that the fibers are more flexible results in a less toxic profile (figure 3).

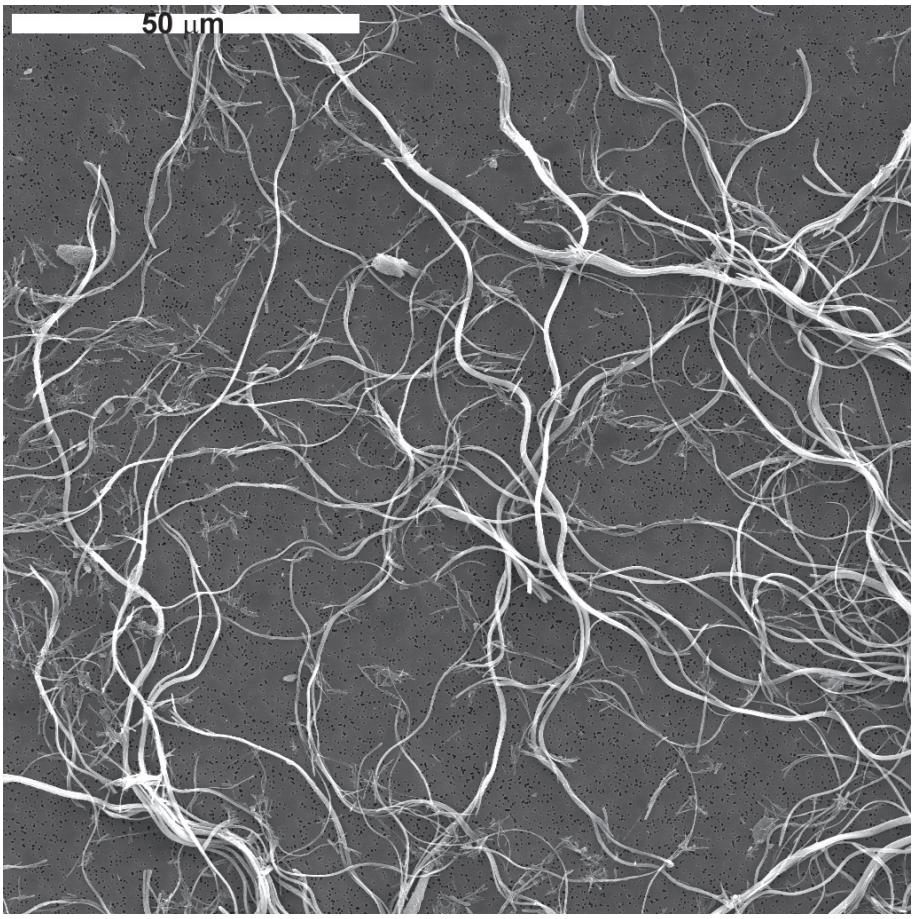


**Figure 3**
Electron microscopic image of chrysotile, showing the long, thin, but more flexible fibers than crocidolite (U.S. Geological Survey Department of the Interior/USGS)

### Discovery of hazardous properties of asbestos

Even in ancient times harmful effects of asbestos were noted, mainly in asbestos miners[12]. The first publications of asbestos-related disease in medical literature were dated in the 1920s[13–15]. These publications described patients with anamnestic asbestos exposure that developed lung fibrosis. In addition, asbestos bodies were found in the lung after post-mortem examination. The occurrence of a diffuse pleural neoplasm was described centuries before by Joseph Lieutand in 1767[16]. It is

not known exactly when the term “mesothelioma” was introduced in the medical literature, but one of the earliest reports date back to 1920 by DuBray and Rosson[17]. Klemperer and Rabin further established the term mesothelioma, as opposed to other nomenclature as endothelioma in 1932[18]. Suggestions of a higher incidence of mesothelioma among asbestos exposed persons were also made in these first medical articles in the 1930s. But it was not until 1960 that the link between asbestos fiber inhalation and mesothelioma became incontrovertible with one article published in *The Lancet* entitled “Primary malignant mesothelioma of the pleura” by Eisenstadt and Wilson and another article “Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province” by Wagner, published in the same year in the *British Journal of Industrial Medicine*[19,20]. In Dutch literature, the thesis titled “Asbest in een bedrijfsbevolking, een onderzoek naar het voorkomen van asbestlichaampjes en mesotheliomen op een scheepswerf en machinefabriek” (asbestos in the working population; a study of the prevalence of asbestos bodies and mesothelioma at the harbor and machinery factory) by the industrial medical officer Stumphius was published in 1969[21]. He described 25 patients with mesothelioma, of which 22 patients were directly linked with a working history in the harbor in Vlissingen, where Stumphius was employed. In the Netherlands, this his work is considered a turning point in the view on asbestos use and exposure prevention, despite the evidence in international literature that was already published years before.

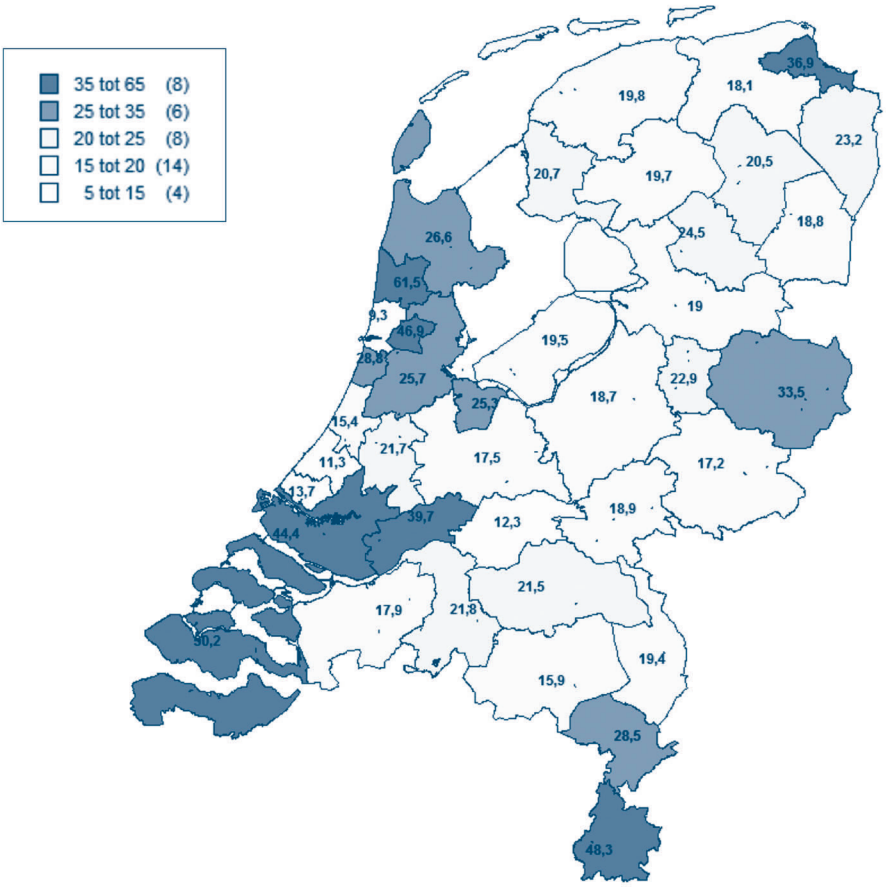


**Figure 4**
Number of cases per 100.000 inhabitants per year that were compensated according to Dutch law for asbestos-related mesothelioma from 2000 until 2012. For this figure, the Netherlands had been divided in large cities with surrounding regions. Regions with high incidence of mesothelioma are in darker blue. It evidently shows the main exposure regions such as the Rotterdam area, Goor and Den Helder. (used with permission from Instituut Asbestslachtoffers and Sociale Verzekeringsbank, L. van Eekelen, march 2013)

### Consequences of asbestos inhalation

Part of the asbestos fibers may remain behind in the alveoli after inhalation. A fraction of these particles eventually reaches the pleural cavity. How this process of relocation exactly takes place is matter of discussion. There is the possibility of

relocation through the lymphatic drainage[22], another is movement of the fibers through pressure related force through the lung parenchyma[23], as shown in figure 5.


**Figure 5**
A field emission scanning electron micrograph of a multi-walled carbon nanotube (similar structure to an asbestos fiber) penetrating the pleura of the lung. (Image courtesy of Robert Mercer, and Diane Schwegler-Berry, NIOSH).

Short fibers and compact particles leave the pleura through the stomatal openings in the parietal pleura. However, long fibers are unable to leave the pleura due to their size and are thus retained in the parietal pleura. This retention of fiber dose at the parietal pleura then serves as the driver that initiates the cascade that eventually can lead to formation of mesothelioma[22]. The association between asbestos exposure and mesothelioma shows a dose–response relationship[24,25].

While asbestos fibers do not interact with the DNA on a molecular level, fibers are able to directly penetrate cells. Therefore, traumatic chromosomal damage is indeed possible. Also, the retained asbestos fibers may adsorb other carcinogens on their surface[26–30]. As a result DNA alterations may occur, such as inactivation of *p16INK4a/p14ARF*, *NF2/Merlin, LATS2*, and the activation of *YAP*[31–33]. In addition,

several receptors are activated by asbestos or its oxidants, leading to direct or indirect activation of several pathways responsible for stimulation of growth-promoting or anti-apoptotic capacities of mesothelial cells. Furthermore, cell-signaling cascades may govern plasticity of mesothelial cells and may impinge on early-response proto-oncogenes and other transcription factors; these encode genes promoting cell proliferation, inflammation, and genetic instability[23].

As mentioned above, the length of the asbestos fibers results in the inability for macrophages, acting as scavengers of the human body, to fully encapsulate these fibers. Macrophages attempt to encapsulate the fibers and deploy mechanisms to break down the nondegradable asbestos particles. This unsuccessful effort is known as the "frustrated macrophage" and leads to chronic inflammation (Figure 6)[23]. This in turn leads to an increased possibility of malignant transformation, due to continued generation of reactive oxygen species and secretion of pro-inflammatory cytokines[34,35].

This results not only in an increase of mesothelioma in asbestos-exposed subjects, but also in an increase in the incidence of lung cancer[36]. The combination of asbestos exposure and smoking, that most asbestos workers did, particularly increases the risk of lung cancer[37].

Asbestos exposure does not only increase the risk of malignancy. Pleural plaques are benign areas of calcification on the lining of the lungs, chest wall, and diaphragm. Their relationship with asbestos exposure is long-known and well established[38,39] The mechanism by which asbestos fibers induce pleural plaque formation is unknown, but several possible theories regarding their pathogenesis exist, although all with their shortcomings[40,41]. It is likely that the complex interactions between resident and inflammatory cells, profibrotic mediators and coagulation, and fibrinolytic pathways are integral to local pleural remodeling and fibrosis[42]. We hypothesize that this type of inflammation could lead to an indolent scar-like tissue, thus protecting the host from the carcinogenic effect of the asbestos fibers or chronic inflammation.

Benign asbestos related pleural effusion can occur after 10-20 years after asbestos exposure. The fluid mostly is exudative, can re-occur and often is self-limiting. However, it can also progress in diffuse pleural thickening, in which the pleural thickening encompasses a much larger surface of the pleura than in the case of pleural plaques. This entity can lead to functional impairment[43].

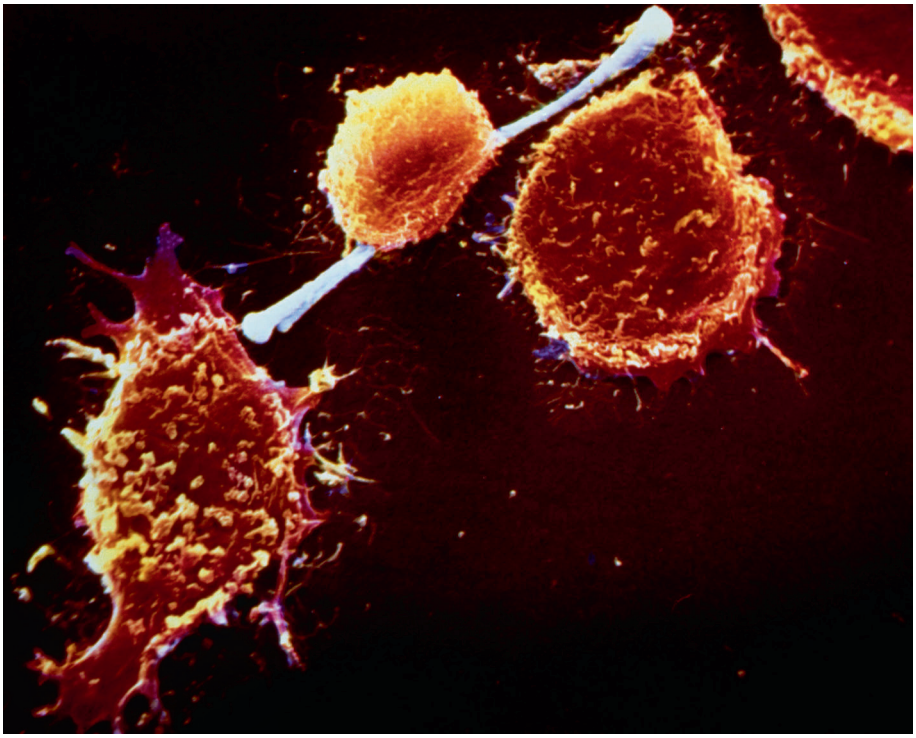

**Figure 6**
The frustrated phagocytosis paradigm as it relates to long and short fibers of asbestos. When confronted by short asbestos fibers the macrophage can enclose them and clear them. However the macrophage cannot extend itself sufficiently to enclose long asbestos fibers, resulting in incomplete or frustrated phagocytosis, which leads to chronic inflammation[22]. (© ANP/Science Photo)

Asbestosis is defined as diffuse fibrotic changes of the lung parenchyma itself as result of asbestos exposure. This leads to a distortion of the lung architecture and a decrease in lung function similar to idiopathic pulmonary fibrosis, but with a slower progression over time[37].

Because mesothelioma is the focus of this thesis, these entities will not be further discussed.

**Mesothelioma**

Mesothelioma is a malignant transformation of mesothelial cells, which are present in the human body in the pleura, the peritoneum, the pericardium and the tunica vaginalis. Around 80 percent of mesothelioma cases present in the pleura, while the peritoneal mesothelioma accounts for almost the rest of the cases. Pericardial mesothelioma and mesothelioma of the tunica vaginalis are extremely rare, with the latter only being reported approximately 100 times in literature[44]. In this thesis, the focus is on malignant pleural mesothelioma (MPM).

MPM is pathologically divided the following subtypes:

- Epithelial mesothelioma accounts for approximately 70% of all diagnosed cases each year. These tumors contain polygonal, oval or cuboidal cells that often mimic reactive mesothelial cells that occur in response to various types of injury[45].
- Sarcomatoid mesothelioma is a less common subtype of MPM, accounting for approximately 15% to 20% of mesothelioma cases each year. Under a microscope, sarcomatoid mesothelioma consist of spindle cells that may mimic malignant mesenchymal tumors such as malignant fibrous histiocytoma, leiomyosarcoma or synovial sarcoma[45].
- Biphasic mesotheliomas are a mix of epithelial and sarcomatoid cell types and account for the remaining percentage of mesothelioma cases.

The latency between asbestos exposure and the first clinical signs of disease in humans is in general long. Mesothelioma can present itself from 15 years post-exposure, with a peak between 30 and 40 years[46]. Exposure to asbestos confers a long-term risk of developing pleural and peritoneal mesothelioma, despite some fiber clearance. While the risk of developing mesothelioma appears to level out for pleural mesothelioma after 40–50 years after exposure to asbestos fibers no one survives long enough for the risk to disappear[47]. However, only a minority of the asbestos-exposed individuals develops mesothelioma[47]. This may be explained by genetic susceptibility, but it may also be explained due to the variety of the immune reactions to the presence of the asbestos fibers.

Currently it is common knowledge that asbestos exposure is the predominant cause of pleural mesothelioma, with a frequently cited attributable fraction in the medical literature of 80%[48]. In the remaining 20%, the patients were not aware of any extensive asbestos exposure. However, the etiology of the malignancy in many of these cases is clear; a fiber count in dry lung of mesothelioma patients in Australia without known asbestos exposure revealed a significant fiber count in nearly all cases[49].

Asbestos alone may not be the only cause of mesothelioma, the following potential alternative causes of mesothelioma are raised in scientific studies; Erionite, a naturally occurring fibrous mineral that belongs to a group of minerals called zeolites, has emerged as an example of nonasbestos-mediated cause of mesothelioma. Exposure to this type of fiber is highly prevalent in regions such as the Central Anatolia Region in Turkey[50–52]. Multi-walled carbon nanotubes (Figure 5) are man-made fibers that have similar dimensions and properties of asbestos with similar carcinogenic effect in animal models[53]. Especially the long fibers seem to reach the parietal pleura[54], thus prevention strategies to minimize exposure are advised[55]. Previous radiotherapeutic treatment, thoracic as well as extra thoracic, results in a small but detectable risk factor for mesothelioma[56–58]. DNA mutation studies show that germline breast cancer 1, early onset (BRCA1)-associated protein 1 (BAP1) mutations are associated with a cancer syndrome characterized by malignant mesothelioma, uveal melanoma, cutaneous melanoma and melanocytic BAP1-mutated atypical intradermal tumors, and possibly by other cancers[59,60]. This assumes that there may be a genetic susceptibility for mesothelioma. It is expected that with the growing amount of knowledge in this field in the following years more data will be collected. The simian virus 40 has been suggested for decades to be a co-carcinogen of asbestos, but a definitive role for the virus in human mesothelioma has not been unequivocally demonstrated and is rigorously debated[61–68].

**Symptoms**

Mesothelioma is difficult to diagnose because the early signs and symptoms of the disease can be subtle or misinterpreted. Therefore, diagnosis of MPM is frequently delayed by months. In pleural mesothelioma, patients frequently experience lower

back pain or side chest pain. In addition, shortness of breath is frequently the presenting symptom if pleural fluid is present. A minority of patients may experience difficulty swallowing, persistent cough, fever, weight loss, or fatigue.

**Establishing the diagnosis**

A long search for an adequate biomarker, with mesothelin and osteopontin being the most studied, has not been successful yet due to poor sentitivity[69–71]. Fibulin-3 (a member of the extracellular glycoprotein fibulin family) in contrast showed very promising results with a sensitivity of 96.7% and a specificity of 95.5% that was published in *The New England Journal of Medicine* in 2012[72]. However, the results in the blinded validation cohort dampened the initial expectations[73]. In addition, a recent study even showed that mesothelin still is a superior diagnostic biomarker for MPM compared with fibulin-3[74].

In most patients, radiological imaging is the first diagnostic tool to detect MPM, with computed tomography(CT)-scan evidently being superior to chest X-ray due to the distinction of pleural thickening and pleural fluid. Positron emission tomography (PET) and PET/CT shows superior results to chest X-ray and CT when pleural thickening is present, but has a limited diagnostic value in the setting of only pleural effusion[75]. It can aid in localizing the fludeoxyglucose (FDG) active parts of the pleura, which can be very useful for the guiding of thoracosopic biopsies or radiographically assisted biopsies. In addition, PET may aid in exploring novel therapeutic approaches[76–80].

Obtaining a pathological diagnosis in MPM can be a daunting task. When pleural fluid is present, thoracocentesis can be used to obtain the pleural fluid for analysis. However, this material provides a diagnosis in only 20-50% of patients. In addition, the cytological sample that is obtained gives way to a large diagnostic error and is therefore not recommended for establishing the diagnosis of mesothelioma[81]. However, in a setting of cytological recognition of an atypical mesothelial proliferation in pleural effusion from patients with the clinical background and imaging studies compatible with MPM, and when biopsy is considered inadvisable or unnecessary, cytology may be sufficient for diagnosis in most patients[45]. Of note, analysis of pleural fluid in patients with mesothelioma reveals a plethora of immune cells, cytokines and chemokines. This may prove to be a very convenient method of gaining insight

into the immunological status of the tumor, which is further discussed in this thesis. For establishing the diagnosis of mesothelioma, Abrams or Castelain needle biopsies have an accuracy of only 30% of cases and are not recommended[81].

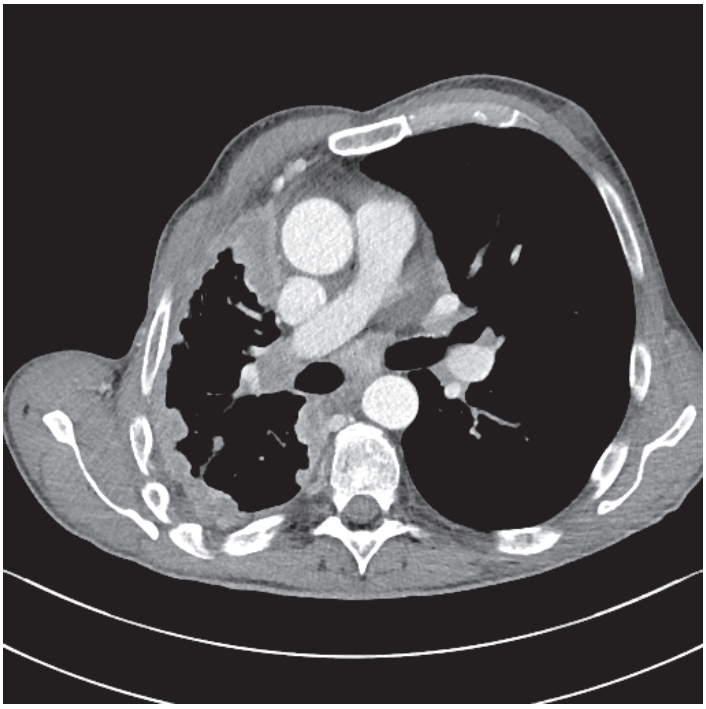

**Figure 7**
CT scan of a patient with mesothelioma. The nodular pleural thickening is seen in the right hemithorax, covering nearly the complete pleural surface. In addition, an evident shrinking of the right hemithorax can be seen.

Thoracoscopic biopsies, in The Netherlands performed by either a thoracic surgeon or a pulmonologist, are the preferable means to establish the pathological diagnosis of mesothelioma. In most cases, a relatively large histologic sample is obtained of an abnormal site of the parietal pleura by the performing physician. The diagnostic yield is over 90%[81]. When a sufficiently pleural thickening is present, ultrasound guided biopsies are also possible and have a diagnostic accuracy of 80%[82]. Endosonographic techniques, endoscopic ultrasound (EUS) and endobronchial ultrasound (EBUS), with fine needle aspiration have widely become available to the pulmonologist in the last

decade and are largely being used to stage lung cancer. In the author's experience and others, these techniques in some cases can provide the diagnosis of mesothelioma by obtaining material either from mediastinal/hilar lymph nodes or from the pleural thickening itself[83,84]. Until recently only cytology samples were obtained, resulting in inferior samples for the pathologist. But recently, EUS and EBUS needles that are able to obtain histology were introduced[85], their value in establishing the diagnosis of mesothelioma is yet to be studied.

**Prognosis**

The prognosis of MPM is dismal with a median survival of 9-12 months after the first signs of illness. However, with great variability ranging from a few weeks to over 10 years. Prognostic markers, such as epithelioid histology, good performance status and low volume of disease may aid clinicians and patients in choosing the optimal therapeutic strategy[86,87]. In addition, a recent large data set confirmed that although MPM is less common in women, survival is far better in women compared with men, independent of confounders such as age, stage, and treatment[88]. Differences in tumor biology and the impact of circulating hormones on host response are possible explanations for this sex difference, but this is subject for future research. Prognostic scores have been published and validated[89–91], but these tools make use of patient reported data such as performance scores and serum parameters that can vary just over several days. A more objective tool to predict the prognosis or predict the effect of treatment would be a welcome addition to the tools currently available. Gene-signatures and tumor infiltrating T-cells are among the tested methods[92–98], but most of the patients studied are surgical patients, therefore a selected minority of the total mesothelioma patient population. In a recent retrospective study, circulating fibrinogen was found to be an independent prognostic biomarker in MPM. In addition, fibrinogen predicted treatment benefit achieved by surgery within multimodality therapy[99]. Whether fibrinogen itself has an active role during cancer progression by promoting cell adhesion, proliferation, angiogenesis and cell migration or as a bystander has not been elucidated yet[100]. Given the highly variable nature of this disease, the need for a simple, but well validated prognosis and predictive tool is apparent.

**Treatment**

The main classical treatment options in cancer are chemotherapy, radiotherapy, and surgery. All these modalities are used in MPM; however, treatment tends to differ between regions. Current guidelines evidently reflect this apparent gap in opinion of optimal treatment[81,101]. Conventional treatment options and targeted treatment options in research are discussed below.

*Surgery*

To achieve macroscopic complete resection is a key prognostic factor after surgery[102–104]. There are two main surgical approaches in mesothelioma:

- Extrapleural pneumonectomy (EPP) is en bloc resection of the tumor, e.g. resection of the lung, the parietal and visceral pleurae, and portions of the ipsilateral pericardium and diaphragm.
- Pleurectomy/decortication (P/D) involves resection of the macroscopic visible tumor, including the parietal and visceral pleurae, pericardium, and diaphragm when necessary, but spares the lung.

Only a minority of patients, 10 to 15%, is deemed eligible for surgical treatment. EPP gained wide acceptance after the publications of studies by Sugarbaker et al. and Rusch et al. showing a favorable outcome in patients with epithelioid histology[105,106]. Institutional experience in patients with best prognostic factors reported an outcome of median survival of over 59 months and a 5-year survival rate of more than 53%[107]. However, surgery alone leads to curration; locoregional recurrence is the most common cause of treatment failure after successful EPP with an incidence ranging between 51% and 68% between studies[108–110]. Furthermore, in most surgical series of EPP in MPM, median survival is only less than 2 years[106,108,111–114]. Therefore, whether surgical treatment of mesothelioma prolongs survival remains a matter of debate due to the absence of large randomized controlled trials[115–118]. In addition, surgery is almost exclusively performed in a multimodality setting with chemotherapy and radiotherapy, making it impossible to address the question if the surgery solely is beneficial. Numerous surgical case-series have been published, however, this series are troubled by several confounding factors including lead-time shift, case selection

and the absence of an intention-to-treat analysis[116]. The only, small, randomized controlled trial "Mesothelioma and Radical Surgery" (MARS) did not show an improvement in outcome when EPP was performed versus no EPP[117]. Data suggest a detrimental effect of EPP, however, the study was a feasibility study and was not powered for this outcome[119,120].

Pleurectomy/decortication has gained in popularity over the last decade due to the surgical procedure resulting in less mortality and morbidity. This is in spite of absence of randomized controlled trials comparing P/D against EPP or even placebo. There have been nonrandomized trials and retrospective series described[121–123], but these are subject to evident selection bias; In most cases where P/D is deemed inadequate to archive macroscopic complete resection, EPP was chosen as surgical option. This way, the larger and more invasive tumors received EPP and the smaller, less invasive P/D. An additional factor that adds to the difficulty of the evaluation of the effectiveness of surgery is that there is a significant variation regarding surgical technique and nomenclature for procedures performed in patients with MPM. Currently, several initiatives are ongoing to achieve consensus in the surgical treatments in MPM[73–78].

Most intriguingly, long-term survivors after surgery do exist and have been well-described[114,125,126], acting as the fuel for surgeons to continue pursuing a macroscopic complete resection in mesothelioma.

Surgery also is performed in the palliative setting to release a trapped lung. In such a setting, a thoracoscopic debulking of the parietal pleura and visceral pleurectomy with decortication can be performed (video-assisted thoracoscopic partial pleurectomy (VAT-PP)). Non-randomized studies suggested that VAT-PP improved symptom control compared with EPP and possibly increased survival compared with biopsy alone[127,128]. Recently, an open-label, parallel-group, randomized, controlled trial addressed this subject in which patients were randomly assigned to either VAT-PP or talc pleurodesis. It was shown that VAT-PP did not improve overall survival and talc pleurodesis might be preferable considering the fewer complications and shorter hospital stay associated with this treatment[129].

*Radiotherapy*

Radiotherapy is commonly used in multimodality setting following surgery, despite of the absence of randomized trials that show its benefit. The use of traditional external beam radiotherapy was however limited due the large treatment volumes required and the radiation limitations because of damage to the surrounding organs. Intensity-modulated radiation therapy (IMRT), a novel, more accurate radiotherapy technique gives radiotherapists a superior tool to deliver a more effective radiation dosage on the total required treatment field. Especially in the adjuvant setting after P/D, outcomes of IMRT are promising and is associated with low rates of locoregional recurrence[130–132]. However, no prospective randomized controlled trial (RCT) is performed yet. Of note, it's safety profile varies between the studies, with fatal pneumonitis being the most feared complication[133–135]. Novel forms of radiation therapy are currently under investigation for treatment of MPM, with early experiences with proton beam radiotherapy showing its feasibility with lung-intact MPM patients very recently[136].

In the palliative setting, treatment regimens are used to relieve symptoms arising from tumor growth, such as obstruction of a major blood vessel or pain due to destruction of bone structures, although a recent systemic literature review showed that no high quality evidence currently exists to support radiotherapy in treating pain in MPM[137].

Furthermore, radiation therapy may play a role by preventing local tumor outgrowth at intervention sites. Mesothelioma frequently grows along the tracts of biopsies, chest tubes, thoracoscopy trocars, and surgical incisions, producing uncomfortable subcutaneous nodules. Although the initial RCT did show a beneficial effect of this prophylactic radiotherapy to intervention sites (PIT)[138], two later RCTs did not reproduce that effect[139,140]. The negative studies, however, were hampered by sub-optimal radiation techniques and/or low incidence of local tumor outgrowth[141]. The question whether PIT is an effective intervention remains.

*Chemotherapy*

Currently, chemotherapy is the only treatment that improved survival in randomized controlled trials in mesothelioma patients. The most pivotal RCT being the study

published by Vogelzang and colleagues in 2003 in which they compared cisplatin alone to a combination of cisplatin and pemetrexed that resulted in a survival advantage of 3.3 months of the combination therapy arm[142]. Important to note is that there was no placebo group included in this trial, thus cisplatin group was used as the control arm, in spite of the fact that this was not evidence-based therapy. Nevertheless, this study led to the approval of the combination of cisplatin and pemetrexed as 'standard of care' for the treatment of patients with "unresectable" mesothelioma. Pemetrexed proved not to be the only effective antifolate treatment in MPM, since similar outcomes were reached with cisplatin and raltitrexed compared to cisplatin alone[143]. Whether the antifolate/cisplatin combination is the most effective chemotherapeutic option remains uncertain, since no head-to-head chemotherapeutic comparison has been performed in mesothelioma with other chemotherapeutic combinations[33]. In this setting, especially the combinations of cisplatin with either gemcitabine or vinorelbine seem to be the most relevant[144]. Recently, a multi-center trial was commenced in the Netherlands to investigate whether gemcitabine has an additional effect on survival if it is used as switch maintenance therapy after the completion of pemetrexed/cisplatin (NVALT 19 trial, Dutch trial registry number NTR4132). There is no agent that has proven its efficacy in second line setting in MPM, thus it is advised to include patients in clinical trials[81]. If patients cannot be included in clinical trials, numerous phase II trials can give an insight in possible alternative treatment options. Re-treatment with cisplatin/pemetrexed appears to be a feasible and effective option if progression-free survival exceeds 12 months[145,146]. Vinorelbine and gemcitabine have been the most studied option in patients that progress after first line chemotherapy, each as single agent, in combination with a platinum derivate, or combined with each other. In most studies, the activity of these agents was modest, at most[144,147–151]. A recent retrospective study of 60 patients concluded that the response rate of vinorelbine and gemcitabine was low enough (2%) to justify the choice of a placebo arm in randomized trials of novel agents in previously treated patients[152]. Importantly, in a limited number of patients these two chemotherapeutic agents have been described to be very effective[153,154]. The way to unleash this effect could prove to be selection of the right chemotherapeutic drug for each patient. In the Netherlands Cancer Institute in

Amsterdam, a trial is currently conducted in which tumor cells are collected from the pleural fluid of patients. These cells are cultured and tested for sensitivity to several chemotherapeutic drugs. Using this method, the most effective chemotherapeutic agent in vitro is selected for treatment of each patient (PROOF trial, Dutch trial registry number NTR4775). Preliminary results are promising[155].

*Targeted therapy*

Several targeted agents have been extensively studied in mesothelioma. Epidermal growth factor receptor (EGFR) offers the malignant cell a way to enhance growth signals to the cell nucleus. It is a thoroughly studied therapeutic target due to the availability of proven tyrosine kinase inhibitors (TKi) in for example non-small cell lung cancer[156]. In MPM development, upregulation of EGFR also plays an important role. Unfortunately, EGFR-TKi failed to prove to be a therapeutic option[157,158]. This is due to the fact that the intracellular tyrosine kinase domain, which is responsible for the signal transduction and the target for the EGFR-TKi, does not show a sensitizing mutation in the EGF receptor[159,160]. Recent *in vitro* research, however, shows the EGFR-TKi gefitinib does inhibit MPM cell growth and survival, giving way to further in depth studies for the working mechanism and possible use of EGFR-TKi in the future[161]. An alternative therapeutic option can be achieved by blocking of the extracellular domain of the EGFR receptor with a monoclonal antibody. This theory was recently studied in a phase II study using cetuximab in addition to standard chemotherapeutic treatment in the first line setting in Belgium and the Netherlands (ClinicalTrials.gov Identifier: NCT00996567) and results are expected soon.

Anti-angiogenic agents are another class of targeted therapy, among which thalidomide is the most extensively studied drug. The pivotal phase III NVALT 5/MATES (Maintenance Thalidomide in Mesothelioma Patients) trial could unfortunately not prove a survival advantage when given as maintenance treatment[33,162]. Bevacizumab, a humanized anti-VEGF antibody, is currently being studied for its use in mesothelioma in addition to chemotherapy in France and Belgium in a phase III trial (ClinicalTrials.gov Identifier: NCT00651456) following several phase II trials with variable results[33,163–165].

A multicenter, randomized, placebo-controlled phase III study of the histone deacetylase inhibitor vorinostat in patients with advanced mesothelioma which did not improve survival compared with placebo as second-line therapy for mesothelioma[166]. However, the poor solubility, fast metabolism, and high toxicity of vorinostat are responsible for its limited dosing in the clinic. This could lead to a sub therapeutic dosing of this drug. A new type of nontoxic pH-responsive delivery system recently showed improved *in vivo* vorinostat delivery in solid tumors[167]. This strategy should limit the clearance and metabolism of vorinostat and yield better clinical results.

Recently, several new targets have been discovered that are currently being studied. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B(AKT)/mechanistic target of rapamycin(mTOR) pathway is involved in a number of cellular processes that regulate proliferation, survival, and motility. In MPM this pathway is frequently dysregulated which makes it an interesting target for therapy[168,169]. This is currently being tested in mesothelioma with promising results[162,163]. Furthermore, the Focal Adhesion Kinase(FAK) kinase pathway, especially targetable in cells with a loss of Merlin/NF2[172,173] has led to a large international trial using the FAK inhibitor defactinib that is currently being performed (ClinicalTrials.gov Identifier:NCT01870609). Very recently, arginine deprivation has been used as a novel antimetabolite strategy for the treatment of arginine-dependent MPM. Pegylated arginine deiminase (ADI-PEG 20) is an arginine-depleting drug that has demonstrated interesting results in a phase II as single agent[174].

In summary, the current outcomes of surgery, radiotherapy, chemotherapy, and targeted therapy show a modest, if any, improvement in outcome. This gives way to novel forms of therapy that requires a small introduction.

**Inflammation and cancer**

In 2011, cancer–related inflammation was finally acknowledged as one of the hallmarks of cancer in the article “Hallmarks of Cancer: The Next Generation” which is an updated version of the pivotal “Hallmarks of Cancer” review by Hanahan and Weinberg, published in 2000[175,176]. Preceding this article, Mantovani and others in

the fields of the interplay between immunology and cancer surmised that chronic inflammation should be included as one of the hallmarks of cancer[177]. However, the observation that there is a link between inflammation and cancer dates almost 150 years earlier by observations of Rudolf Virchow[178]. Indeed, malignancies are surrounded by a vast amount of immunological cells, but not all of these cells have the same function. Partly, these cells are under influence of the host and have anti-tumoral capacities. On the other hand, there are immunological cells that, under influence of substances secreted by the tumor or other cells that are already under influence of the tumor, aid the tumor in growth, angiogenesis and resistance to the anti-tumor response.

**Immunotherapy**

Cancer immunotherapy makes use of the host immune system to induce or enhance an effective immune response against the cancer cells. This can be established in a variety of ways, as discussed in **chapter 2**. In recent years, immunotherapy has finally made its steps into clinical practice in the form of ipilimumab in melanoma and sipuleucel-T-cell treatment in prostate cancer[179,180]. In recent clinical trials, preliminary results of new forms of immunotherapy in cancer yield promising and unprecedented results[181,182]. The editors of Science have chosen this upcoming strategy to treat cancer as the "Breakthrough of the Year" for 2013[183]. In mesothelioma, progress in immunotherapy has been slow, but gradual. Entry of this form of therapy into the clinical setting is however expected in this decade.

Passive antibody therapy, directly targeted at the tumor, in the form of CAT-5001 and amatuximab have reached clinical trials with modest response[184–189]. Recently, a phase I study of SS1(dsFv)PE38 (SS1P), a recombinant anti-mesothelin immunotoxin, thus the combination of an antibody-based targeting domain fused to a bacterial toxin for cell killing, showed promising results when combined with first line chemotherapy[190]. However, the rapid development of antibodies likely hampered the efficacy of SS1P.

Active immunotherapy, using the stimulation or infusion of immune cells, has made progress over the last decade as well. Lentiviral or retroviral vectors can be used to transduce T cells with modified T-cell receptors engineered to attack specific

tumor antigens[191]. Preclinical results of this method are promising and a phase I study showed partial response in 1 and stable disease in 4 out of 9 patients[192]. Adoptive transfer of tumor-reactive T cells expressing chimeric antigen receptors (CAR) with tumoricidal properties were also found to mediate regression of the tumor in a preclinical mesothelioma model[193]. When given intrapleurally, mesothelin-targeted CAR T cell therapy generated long-lasting CD4-dependent tumor immunity in a murine model and this model has progressed to a clinical phase I trial in Memorial Sloan Kettering Cancer Center, New York[194]. However, CAR T cell therapy has the risk of a cytokine release syndrome, which can be fatal[195].

Listeria monocytogenes can be engineered to express human mesothelin (CRS-207). Subsequent vaccination leads to uptake and multiplication by phagocytic cells and mesothelin is expressed and released into the cytosolic compartment. Mesothelin is then processed through the endogenous MHC class I presentation pathway, resulting in activation of anti-mesothelin cell-mediated immunity. Results of a phase I trial combining CRS-207 with chemotherapy as front-line treatment in mesothelioma patients were presented recently and showed promising results[196].

Of importance, mesothelin-targeted CAR T cells and CRS-207 can only target this specific antigen and can result in escape of mesothelin-negative tumor cells. This can be illustrated by a promising publication in *The New England Journal of Medicine* in April 2013 in which two children were described with relapsed and refractory pre-B-cell acute lymphocytic leukemia. They received infusions of T cells transduced with CD19-reactive CAR, the target for B-cell acute lymphocytic leukemia. Rapid complete remission was observed in both patients, showing that CAR-modified T cells are capable of killing even aggressive, treatment-refractory acute leukemia cells *in vivo*. However, approximately 2 months after treatment one patient had a relapse, with blast cells that no longer expressed CD19[197]. This example exposes the Achilles heel of single antigen therapy.

The stimulation of antigen presenting cells is another method of boosting the immune response. Particularly, it has become clear that dendritic cells (DCs) are at the center of the immune system owing to their ability to control both immune tolerance and immunity[198]. Therefore, DCs are an essential target in efforts to generate therapeutic immunity against cancer. DC's are not only capable of inducing

a cytotoxic T cell response, but they can also interact with B cells and natural killer cells[198,199].

DC based therapy has been studied by our group for years; first in a murine model, followed by a phase I clinical trial using autologous tumor loaded DCs[181,200]. The result of this phase I trial was promising, with a partial response seen in 3 out of 10 patients. Furthermore, DC immunotherapy proved to be feasible and safe. To improve the outcome of DC-based immunotherapy, a murine model was tested for the combination of cyclophosphamide (CTX) and vaccinations with loaded DC. The rationale behind CTX was that it could deplete the regulatory T cells, which in turn downregulate the induced cytotoxic immune response. Thus administration of CTX could lead to an unhampered immune response, a theory that was eventually proven[201]. Following this pre-clinical model, another phase I trial was commenced, which is discussed in **chapter 7**.

## CONCLUSION

Malignant pleural mesothelioma is a lethal disease caused by the inhalation of asbestos fibers. Chemotherapy is the only scientifically proven treatment increasing survival. In spite of decades of research on this disease, many questions remain; for example, how to predict prognosis of patients given the variability of survival, how to predict the occurrence of local tumor outgrowth or, the unknown impact of the immunological cells present in the pleural effusion. The focus of the first part of this thesis lies on the interaction of the immune cells in the tumor microenvironment with the tumor cells.

Currently, a vast number of new therapeutic options are under investigation. One of those treatment options is immunotherapy; a therapy that tries to induce or enhance an anti-tumor immunoresponses. Dendritic cells are recognized as the most potent antigen presenting cells able to induce an immunoresponse. Dendritic cell-based immunotherapy is of our most interest and is studied by our research group. One completed clinical trial and the current clinical trial are the focus of the second part of this thesis.

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# Chapter II

## New roads open up for implementing immunotherapy in mesothelioma

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Clinical and Developmental Immunology, 2012:927240

## ABSTRACT

Treatment options for malignant mesothelioma are limited and the results with conventional therapies have been rather disappointing to this date. Chemotherapy is the only evidence-based treatment for mesothelioma patients in good clinical condition, with an increase in median survival of only 2 months. Therefore, there is urgent need for a different approach to battle this malignancy.

As chronic inflammation precedes mesothelioma, the immune system plays a key role in the initiation of this type of tumour. Also, many immunological cell types can be found within the tumour at different stages of the disease. However, mesothelioma cells can evade the surveillance capacity of the immune system. They build a protective tumour microenvironment to harness themselves against the immune system's attacks, in which they even abuse immune cells to act against the anti-tumour immune response.

In our opinion, modulating the immune system simultaneously with the targeting of mesothelioma tumour cells might prove to be a superior treatment. However, this strategy is challenging since the tumour microenvironment possesses numerous forms of defence strategies. In this review, we will discuss the interplay between immunological cells that can either inhibit or stimulate tumour growth and the challenges associated with immunotherapy. We will provide possible strategies and discuss opportunities to overcome these problems.

## INTRODUCTION

Links between cancer and inflammation were first noted by Rudolf Virchow in 1863, on observations that tumors often arose at sites of chronic inflammation and that inflammatory cells were present in biopsy samples from tumors[1]. In a severe combined immunodeficiency (SCID) mouse xenograft model, it has recently been shown that inflammation precedes the development of human malignant mesotheliomas[2]. Also, epidemiological studies have revealed that chronic inflammation caused by chemical and physical agents, autoimmune and by inflammatory reactions of uncertain etiology, predisposes for certain forms of cancer[3,4]. Recently our group demonstrated a significantly shorter survival in patients with lung cancer in subjects with a history of pulmonary tuberculosis than patients without tuberculosis[5], revealing even a more complex interplay between inflammation and cancer. Increasing evidence indicates that the "inflammation-cancer" connection is not only restricted to the initiation of the cancer process, since all types of clinically manifested cancers appear to have an active inflammatory component in their microenvironment. These experimental findings and clinical observations have led to cancer–related inflammation being acknowledged as an important hallmark of cancer[6].

## IMMUNO-ONCOLOGY

*Tumor-immune surveillance*

Lloyd J. Old, George Klein and others investigated murine tumor transplantation models and showed that the immune system of healthy recipient mice was able to distinguish transformed malignant cells from normal cells[7,8]. Even preceding these publications, Frank MacFarlane Burnet and Lewis Thomas formulated their cancer immunosurveillance hypothesis: "It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumor and no clinical hint of its existence"[9]. At that time this hypothesis was controversial, however, with the current knowledge and ongoing research,

it is apparent their premise seems to be correct because there is strong evidence from animal studies that cells of the immune system carry out surveillance and can eliminate nascent tumors[10].

Several immunological cell types are involved in the recognition and destruction of tumors. These include cells and factors of the innate immune system, including macrophages, neutrophils, complement components, γδ T cells, natural killer (NK) cells, NKT cells and certain cytokines (IL-12, IFN-γ) and cells of the adaptive immune system, including B lymphocytes, helper T cells (Th cells) and cytotoxic T lymphocytes (CTL).

In order for the adaptive immune system to function, tumor-associated antigens (TAA) need to be presented to the cells of the adaptive immune system. TAA are antigens acquired by tumor cells in the process of neoplastic transformation that can elicit a specific immune response by the host. Mutations leading to synthesis and overexpression of these abnormal proteins lead to expression of these antigens. In this manner, the immune system can discriminate between normal cells and their malignant counterparts through recognition of these TAA.

Dendritic cells (DCs) are widely acknowledged for their potent antigen presenting capacity and play a key role in the initiation of this adaptive immune response by activation and modulation of lymphocyte subsets[11]. DCs originate from bone marrow precursor cells and are found at low frequencies in peripheral tissues where they maintain an immature phenotype and search their surroundings for foreign substances. Immunogenic TAA are secreted or shed by tumor cells or released when tumor cells die and can be taken up by DCs or other antigen presenting cells (APCs). Upon encountering an antigen, DCs mature and migrate to regional draining lymphoid organs. The captured antigen is processed and presented by major histocompatibility complex (MHC) class I and class II molecules on their cell membrane leading to the activation of antigen-specific lymphocytes. This results in antibody production by B lymphocytes and tumor-specific CTLs to assist the innate immune responses in the killing of tumor cells.

*Tumor immune escape*

There is increasing evidence that shows when tumor progresses in time, tumor cells differentiate to escape the – from the tumor's view – harmful effects of the immune system. This process encompasses three phases: Elimination, Equilibrium, and Escape. During the first phase, immune surveillance takes place that protects the host from malignancy. Thus in order for the tumor cells to survive, they have to escape the immune surveillance. Then these surviving tumor cells can enter the equilibrium state, in which there is equilibrium between tumor growth and tumor killing by cells of the immune system. In this stage, tumors can persist for years without progressing to more advanced tumor stages. However, during this period, tumor cells may undergo mutations caused by their genetic instability; potentially generating variants that can escape the immune system, by either evading the induction of an immune response or by inhibiting anti-tumor responses via a variety of immune suppressive mechanisms. This ultimately leads to the possibility for the cancer cells to grow and become a clinical entity.

*Immune suppressive mechanisms*

The tumor immune escape mechanism can be greatly enhanced by the induction of an immune suppressive tumor microenvironment. In this microenvironment, inflammatory cells and molecules have a major influence on cancer progress. Effective adaptive immune responses are suppressed through the activation of several pathways. For example, the differentiation and activation of dendritic cells, which are the key initiators of adaptive immune responses, are inhibited by signals (such as IL-10 and VEGF) present in the tumor microenvironment. In addition, tumors, peripheral blood and lymph nodes contain increased amounts of regulatory T cells (Tregs), which suppress both the adaptive and innate immune responses[12]. Also, a heterogeneous population of myeloid-derived suppressor cells (MDSCs) is induced in tumor-bearing hosts; these cells, as well as tumor-associated macrophages (TAMs) that are skewed into M2 phenotype, are potent suppressors of antitumor immunity. Not only do MDSCs and M2 TAMs suppress the antitumor response, they also assist the malignant behavior of tumor cells by secreting cytokines, growth factors, matrix-degrading enzymes and proteases, which promote tumor progression or enhance metastasis.

In conclusion, immune cells can either protect the host against cancer development or promote the emergence of tumors with reduced immunogenicity leading to a complex interplay of tumor growth and tumor regression mechanisms (Figure 1)[13].

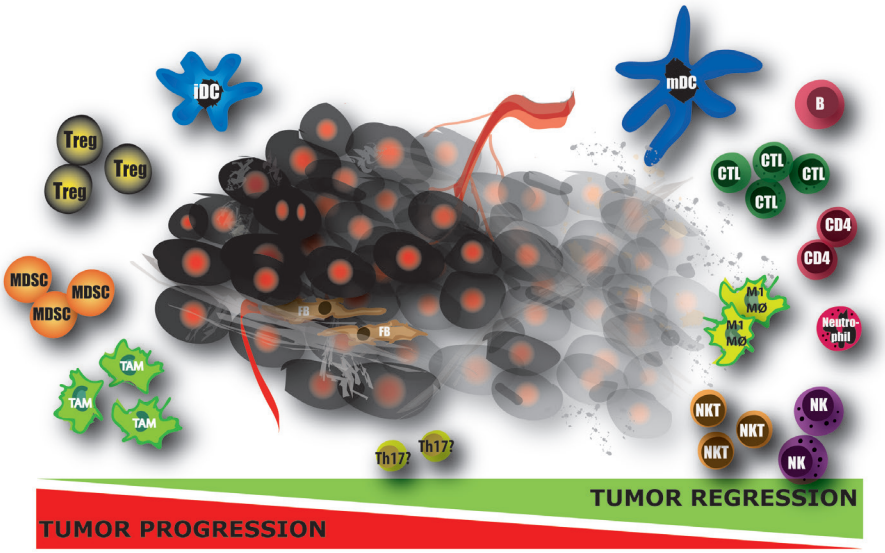


**Figure 1.** Interplay between immunological cells that inhibit tumor growth on the right of the tumor and cells that aid in tumor progression on the left. (Tumor is depicted as black cells with a red nucleus in the middle). iDC = immature dendritic cell, Treg = regulatory T cell, MDSC = myeloid derived suppressor cell, TAM = tumor-associated macrophage of M2 phenotype, mDC = mature dendritic cell, B = B cell lymphocyte, CTL = cytotoxic T lymphocyte, M1 MØ= M1 macrophage, NK(T) natural killer (T) cell, Th17 = helper T lymphocyte 17, FB = fibroblast.

## IMMUNOTHERAPY

Cancer immunotherapy attempts to activate or enhance the anti-tumor effects of the immune system of the patient, or it may assist in the capabilities of the immune system to fight cancer. Multiple approaches for immunotherapy have been developed over the years and many are in various stages of (pre-)clinical research. Immunotherapy can be divided into two main categories: passive and active immunotherapy[14].

*Passive immunotherapy*

Passive immunotherapy makes use of *in vitro* produced immunologic effectors that are capable of influencing tumor cell growth. The most common form of passive immunotherapy is called monoclonal antibody therapy. It consists of humanized monoclonal antibodies that are investigated in several human malignancies. Monoclonal antibodies can target cells directly[15] or indirectly. Monoclonal antibodies are also used as immune modulators to inhibit immune suppressive molecules/cells or activate immune stimulatory molecules. Efficacy of this approach can sometimes be enhanced by linking a toxin to these antibodies (e.g. radionucleotides or anticancer drugs).

In mesothelioma, preclinical studies targeting mesothelin with immunotoxins CAT-5001 (formerly SS1P) and amatuximab (previously known as MORab-009) were promising[16–18], and therefore progressed to clinical trials. CAT-5001, administered to mesothelioma patients, among other cancer types, showed only modest clinical responses[17,18]. Amatuximab failed to demonstrate any radiological responses in a phase I trial in mesothelioma and other cancer types[19], however preclinical studies demonstrated significant anti-tumor efficacy using combination of amatuximab and chemotherapy treatment[20] justifying a multicenter phase II clinical trial utilizing cisplatin/pemetrexed with amatuximab in mesothelioma patients. This trial has been completed and results are expected soon. More recently a phase I study of SS1(dsFv) PE38, a recombinant antimesothelin immunotoxin was commenced which is ongoing at this moment (ClinicalTrials.gov Identifier: NCT00575770).

Another method of passive immunotherapy uses adaptive transfer of (autologous or allogeneic) antigen specific effector cells (like T cells and NK cells) that can be expanded and/or activated *ex vivo* and subsequently administered to the patient to attack the tumor[21]. This approach showed the potential to reconstitute host immunity against pathogens, like Epstein-Barr virus (EBV) in immune suppressed patients, but more importantly also provides evidence that adaptive T cell transfers can prevent the induction of EBV-associated lymphomas[22]. This led to the concept that antigen specific T cell transfer can be used as an anti-tumor therapy to eradicate established tumors. The approach of adaptive T cell transfer to eradicate malignancies is challenging[23].

*Active immunotherapy*

Active immunotherapeutic approaches aim at inducing or boosting immune effector cells *in vivo* against tumor cells, through the administration of immune mediators capable of activating the immune system.

Several cytokines are capable of activating and recruiting specific immune cells that can enhance anti-tumor immunity (e.g. IL-2, IL-12, IL-15, TNF-$\alpha$, GM-CSF). These cytokines can be used as single agent or in combination with other immunotherapeutic strategies.

Defined TAA epitopes have been used to vaccinate cancer patients[24]; however this approach is limited by the relatively low number of identified specific peptides and by the requirement of MHC typing. By using the whole TAA protein for immunization, the need of peptide identification can be circumvented. These proteins can be taken up by APCs and endogenously processed into epitopes for presentation to T cells. Adjuvants need to be added to induce APCs activation and avoid tolerance induction[25]. DNA sequences coding for specific TAAs can be directly injected into the skin. DNA then needs to be taken up, transcribed into mRNA, translated into a protein and processed into peptides by APCs.

In mesothelioma, the TAA's mesothelin and Wilms tumour-1 (WT-1) are highly expressed and thought to be physiologically relevant to this tumor type[26]. In the Memorial Sloan-Kettering Cancer Center a phase I peptide vaccination clinical trial in mesothelioma patients is ongoing (ClinicalTrials.gov Identifier: NCT01265433). In these patients, inoculation with WT-1 peptide elicited WT-1-specific CD4 and CD8 T-cell responses, with minimal toxicity[26]. TroVax® has been shown to stimulate an immune response to a particular protein widely found on mesothelioma cells called 5T4, a clinical trial testing the effectively of TroVax® is currently active in the Wales Cancer Trials Unit (ClinicalTrials.gov Identifier: NCT01569919).

An important restriction of this method is the relatively inefficient delivery into APCs. Viruses engineered to express TAAs can be injected directly into the patient. The virus then infects the host cell, leading to cell death and presentation of antigenic epitopes to the immune system. A wide variety of viral vectors are available. Currently, a trial using intrapleural administration of a vaccine with a measles virus strain is performed at the Mayo Clinic (ClinicalTrials.gov Identifier: NCT01503177). However there are

concerns regarding the immune-dominance of viral antigens over TAAs, resulting in a strong anti-virus response leading to virus eradication and attenuation of the anti-tumor immune response[27].

DCs have emerged as the most powerful initiators of immune responses. In the natural activation of the adaptive immune system against tumor cells, DCs play a crucial role since they are capable to engulf tumor antigens and activate lymphocytes in an antigen specific manner. Therefore, the application of DCs to therapeutic cancer vaccines has been prompted[28].

The research group of Dr. Robinson published a very interesting trial, in which they used an autologous tumor lysate vaccine that was manufactured from surgically resected mesothelioma material and administered subcutaneously together with granulocyte-macrophage colony stimulating factor (GM-CSF). GM-CSF facilitates APCs recruitment and survival *in vivo*, which in turn may generate tumor-specific immunity after uptake of the TAA from the lysate. Twenty-two patients were enrolled onto this trial. Of these, five developed positive delayed type hypersensitivity skin tests and five showed evidence of altered antibody specificities by western blotting, proving that GM-CSF could induce tumor specific immunity, both cellular and humoral responses. 32% of the patients developed at least one type of anti-MM immune response. Furthermore, the therapy was safe and was associated with stable disease, however no major tumor regressions were observed[29].

While this study showed potential for GM-CSF as immunotherapeutic approach, *in vivo* stimulation of APCs is also a very attractive method. Sipuleucel-T is an active cellular immunotherapy consisting of autologous peripheral-blood mononuclear cells (PBMCs), including APCs. Recently, Kantoff *et al.* published a phase III trial where they used *ex vivo* activated Sipuleucel-T with a recombinant fusion protein (PA2024). PA2024 consists of a prostate antigen, prostatic acid phosphatase that is fused to GM-CSF, an immune-cell activator. Sipuleucel-T prolonged survival among men with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer[30], providing evidence for cell-based immunotherapeutic agents in solid tumors.

In mesothelioma, the source of the TAA for DC loading remains a critical issue that will determine the efficacy of the DC-based vaccination. A careful identification and

characterization of antigenic epitopes is needed when peptides want to be used. However, the ideal source of TAAs may be the tumor itself, since it expresses all the TAAs that can be targeted.

Incubating DCs with dead tumor cells (necrotic or apoptotic cells) leads to a diversified immune response involving cytotoxic T lymphocytes (CTLs) as well as CD4+ T cells. Dead tumor cells exposed DCs to a full array of antigenic peptides that rapidly gain access to both MHC Class I (cross-presentation) and MHC Class II pathways. This was shown in a pioneering article by the research group of Dr. Gregoire. In their paper they successfully demonstrated *in vitro* culture and antigen loading in a human mesothelioma model, resulting in a specific CTL response[31].

One of the advantages of an *ex vivo* culture model is that DCs can be generated in large amounts, and pulsed with tumor antigens under optimal conditions. In mesothelioma, we previously investigated the effect of DC-based immunotherapy on the outgrowth of mesothelioma in a murine model[32]. We established that DC-based immunotherapy induced strong tumor-specific CTLs responses leading to prolonged survival in mice. The efficacy of immunotherapy was dependent on the tumor load; most beneficial effects were established at early stages of tumor development.

On the basis of these preclinical animal studies, we have performed the first clinical trial in which autologous tumor lysate–pulsed DCs were administrated in mesothelioma patients [33]. Patients were eligible for the study when sufficient tumor cells could be obtained from pleural effusion or tumor biopsy material at the time of diagnosis. DC-immunotherapy was planned after completion of the cytoreductive therapy provided that during chemotherapy no major side effects occurred and there was no progressive disease. Patients received three immunizations with mature DCs, loaded with autologous tumor lysate. Each immunization, consisting of 50 x 10e6 cells, was administered intradermally and intravenously (figure 2). Overall, the vaccination regimen with loaded DCs was well tolerated and a successful immune reaction was induced by the DC vaccinations.

The University Hospital of Antwerp has started a similar protocol in mesothelioma and several other solid tumors, but is using WT-1 as antigen loading for the DCs (ClinicalTrials.gov Identifier: NCT01291420), circumventing the need for patient's tumor material. However, this approach limits the anti-tumor response to a single

peptide, making it obligatory for the tumor to significantly express this peptide in order for the immunotherapy to be effective.

Another method to load DCs is to make use of measles virus infected mesothelioma cells. It was shown that this method cells induced DC spontaneous maturation and that priming of autologous T cells by DCs loaded with measles virus infected mesothelioma cells led to a significant proliferation of tumor-specific CD8 T cells[34].

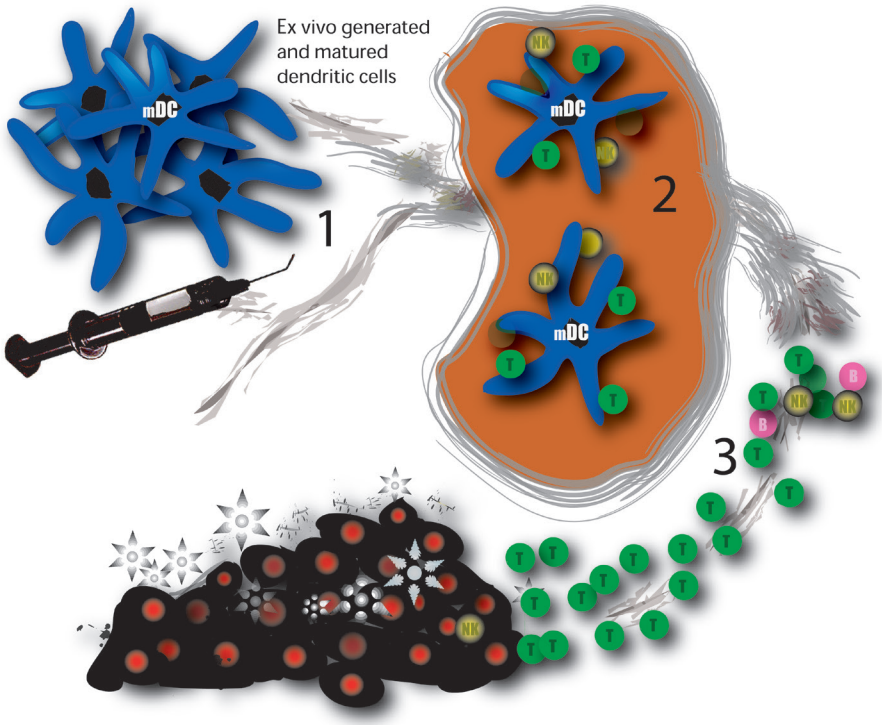


**Figure 2.** Schematic drawing showing the administration of *ex vivo* cultured mature dendritic cells into a patient (1), resulting in antigen presentation in the lymph node (2) and a specific anti-tumor cytotoxic anti-tumor response (3). Tumor cells are depicted as dark cells.

## IMPROVING IMMUNOTHERAPY

While immunotherapy was proven safe and feasible, it has not established its place yet in mesothelioma treatment. Partly, this is due to the presence of immunosuppressive cells in peripheral blood, lymphoid organs and within the tumor environment that hamper immunotherapeutic treatments. Several strategies have been performed or are currently tested that target the immunosuppressive cells aiming to improve the efficacy of immunotherapy. In the following sections, we will focus on three populations of suppressive cells, the MDSCs, Tregs and M2 TAMs that are increased in most cancer patients. It is becoming increasingly clear that these populations contribute to the impaired anti-tumor responses frequently observed in cancer patients. Therefore, combating immunosuppression through modulation of these cell types will be an important key to increase the efficacy of immunotherapy, and should lead to better prognosis for cancer patients.

*Myeloid-derived suppressor cells*

MDSCs are a heterogeneous population of bone marrow-derived myeloid cells, comprising of immature monocytes/macrophages, granulocytes, and DCs at different stages of differentiation[35]. A subset of MDSCs, mononuclear MDSCs (MO-MDSCs) is mainly found at the tumor site while polymorph nuclear MDSCs (PMN-MDSCs) subset is found in blood, lymphoid organs and at the tumor site. They express a number of surface markers, that are on themselves not unique but in combination can define MDSCs. MDSCs are increased in cancer patients and it is anticipated that they play a suppressive role during the innate and adaptive immune responses to cancer, but have also been described in the course of other pathologic processes such as thermal injury, various infectious diseases, sepsis, trauma, after bone marrow transplantation and in some autoimmune disorders.

Activation of MDSCs not only requires tumor-derived factors (e.g. tumor-derived prostaglandin E2 (PGE2)), but also IFN-γ produced by T cells and factors secreted by tumor stromal cells (like IL-1β, IL-4, IL-6, IL-10, IL-13). Activation of cytokine receptors on MDSCs leads to activation of STAT-signaling pathways, resulting in the production of immune suppressive substances (like TGF-β, reactive oxygen species (ROS) and nitric oxide synthetase (NOS)).

MDSCs can inhibit the anti-tumor immune response in several ways;

- MDSCs are capable of producing reactive oxygen species (ROS) and peroxynitrite, which is responsible for most of the adverse effects on T cells, linked to ROS. Changes caused by nitration of the T cell receptor makes T cells incapable of interacting with the MHC complex on APCs, which is necessary to obtain T cell specific stimulation[36,37].
- MDSCs can inhibit the anti-tumor response in an antigen non-specific manner by the high expression of the enzyme inducible nitric oxide synthetase (iNOS), leading to the generation of NO. NO can suppress T cell function though various mechanisms including the inhibition of the cell signaling pathways and inducing DNA-damage to T cells.
- Arginase-I activity by MDSCs depletes L-arginine from the environment, contributing to the induction of T cell tolerance by downregulating the CD3ζ-chain expression of the T cell receptor[38,39].
- MDSCs block T-cell activation by sequestering cysteine and thus limiting the availability of the essential amino acid cysteine[40].
- MDSCs can inhibit T cell proliferation by producing IL-10 and TGF-β[41].
- Anti-tumor cells, like NK- and NKT-cells, can be inhibited by MDSCs via TGF-β1 depending mechanisms. MDSC can bind to the TGF-β receptor on target cells via membrane bound TGF-β, leading to activation of intracellular pathways resulting in down regulation of NK specific receptors[41].
- The plasma membrane expression of enzyme ADAM17 on MDSCs cleaves L-selectin on naïve T cells, decreasing their ability to home to sites where they could be activated[42].
- MDSCs can indirectly enhance immune suppression via the induction of Tregs[43–45].
- MDSCs differentiate under certain biological conditions into mature functionally competent macrophages or to DCs influencing tumoral responses[46].

*Targeting MDSCs*

Both gemcitabine and 5-fluorouracil (5FU) have shown to be selectively cytotoxic on MDSC in murine tumor models[47]. The treatment of tumor-bearing mice with 5FU led to a decrease in the number of MDSC in the spleens and tumor beds of

animals whereas no significant effect on T cells, NK cells, DCs, or B cells was noted. 5FU showed a superior efficacy over gemcitabine to deplete MDSC and selectively induced MDSC apoptotic cell death[47].

Gene expression profile analysis of multiple tumor types identified SCF (c-kit ligand) as a candidate tumor factor involved in MDSC accumulation. Inhibiting c-kit using the tyrosine kinase inhibitor sunitinib resulted in a decrease of the number of MDSC and Treg in advanced tumor-bearing animals[48].[12] J. Ozao-Choy, G. Ma, J. Kao, G.X. Wang, M. Meseck and M. Sung, *et al.* The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. *Cancer Res*, **69** 6 (2009), pp. 2514–2522. | View Record in Scopus | | **Full Text** via CrossRef

The production of ROS by MDSCs, which is responsible for most of the adverse effects on T cells, is highly depending upon cyclooxygenase-2 (COX-2) enzyme activity[49]. The inducible COX-2 enzyme is essential in the biosynthesis of prostaglandins. Celecoxib is a selective COX-2 inhibitor. Therefore, we investigated the effect of celecoxib treatment on the four MDSC subsets that were identified in the spleen of tumor-bearing mice[50]. When combining DC-based immunotherapy and celecoxib treatment, a significant improvement of the immunotherapy was seen in comparison to no or single modality treatment. Treatment of tumor-bearing mice with dietary celecoxib prevented the local and systemic expansion of all MDSC subtypes and also their suppressive function was impaired. At the National Cancer Institute, allogeneic tumor cell vaccine is combined with celecoxib and metronomic oral cyclophosphamide as adjuvants in thoracic malignancies (ClinicalTrials.gov Identifier: NCT01143545); the rationale for using cyclophosphamide is discussed further in this article.

*Tumor-associated macrophages*

Macrophages are a major component of the leukocyte infiltrate in the tumor micro-environment[51] and have even been described as key orchestrators of cancer-related inflammation[52]. Classically activated (M1) macrophages, following exposure to IFN-γ, have anti-tumor activity and tissue destructive activity. In response to IL-4 or IL-13, macrophages undergo alternative (M2) activation. M2 macrophages are oriented to tissue repair, tissue remodeling and immune regulation and display a defective NF-κB activation in response to different pro-inflammatory signals[53].

TAM recruitment in tumors is mediated by several cytokines, of which CCL2 seems to be the main player, other chemokines involved in monocyte recruitment are CCL5, CCL7, CXCL8, and CXCL12, as well as cytokines such as VEGF, PDGF and the growth factor M-CSF[52]. It has been shown that MO-MDSCs are capable of differentiating towards M2 TAMs. Therefore, similar recruitment factors are described that contribute to the infiltration of TAMs and MDSCs into tumor tissue[54]. In addition, dynamic changes of the tumor microenvironment occur during the transition from early neoplastic events toward advanced tumor stages resulting in local hypoxia, low glucose level and low pH. These events in the tumor microphysiology drive the switch from a M1 macrophage toward the M2 type.

M2 TAMs are able to suppress the adoptive immune response through various mechanisms and contribute to angiogenesis and tumor invasiveness:

- M2 TAMS are able to produce immune suppressive cytokines, like CCL17, CCL18, CCL22, IL-1β, IL-6, IL-10 and TGF-β. IL-10 in combination with IL-6 can lead to upregulation of molecules in TAMs, which are implicated in suppression of tumor-specific T cell immunity[55].
- M2 TAMs express the enzyme indoleamine 2,3-dioxygenase (IDO), a well-known suppressor of T cell activation. IDO catalyzes the catabolism of tryptophan, an essential amino acid acquired for T cell activation[56].
- M2 TAMs contribute to immune suppression via indirect ways. Secretion of CCL18 leads to recruitment of native T cells. Attraction of naive T cells into the tumor microenvironment is likely to induce T cell anergy[57]. Besides CCL18, CCL17 and CCL22 are abundantly expressed. These cytokines interact with CCR4 receptor, expressed by Tregs and induces T-helper 2 polarization[58]. Via expression of VEGF, M2 TAMs can block antigen uptake by APCs and attract MDSCs, which can function as M2 TAM precursors but are also actively suppressing T cell function. MDSC are depending on prostaglandin E2 (PGE2) for their function. PGE2 is secreted by many types of cancer; however, M2 TAMs are also capable of producing PGE2 and therefore assist MDSC function[59].
- In tumor stroma, M2 TAMs produce matrix metalloproteases (MMPs) and other proteases, leading to degradation of the extracellular matrix. During this process several cytokines, chemokines and growth factors are released from the matrix

that promotes and facilitates endothelial cell survival and migration and thereby enhances angiogenesis[60].

- Besides indirect mechanisms, angiogenesis is also directly stimulated by M2 TAMs. M2 TAMs can produce proangiogenic factors like vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β and platelet derived growth factors (PDGF). The release of these factors leads to the neovascularization, especially in hypoxic regions within the tumor[52,61].
- In addition to angiogenesis, M2 TAMs are also strongly involved in lymphangiogenesis, a process mediated by a number of factors including VEGF-C and VEGF-D via VEGFR3[52].
- Outside the scope of the tumor microenvironment, but a pivotal step in general tumor biology; M2 TAMs cooperate on tumor dissemination by promoting invasion characteristics. One of the main factors involved significantly is TNF-β: coculture of neoplastic cells with macrophages enhances invasiveness of malignant cells through TNF-dependent MMP induction by macrophages[52].

*Targeting M2 TAMs*

There is accumulating evidence supporting the hypothesis that effects on TAMs may contribute to the anti-tumor effect of bisphosphonates[62]. We investigated the effect of zoledronic acid (ZA) in mesothelioma-inoculated mice. Our data showed that the addition of ZA to macrophage-inducing culture conditions significantly inhibits the upregulation of F4/80, MHCII and CD11c. In addition, these data reveal that adding tumor supernatant leads to polarization of the macrophage phenotype towards M2 subtype, and that ZA can prevent this polarization *in vitro*, leading to a significant reduction in the CD206 expression on macrophages cultured in the presence of ZA. *In vivo*, however, no significant differences on tumor progression and survival could be observed between untreated mice and mice treated with ZA, because the reduction in TAMs was associated with an increase in MDSC[63].

IL-6 stimulates tumor macrophage infiltration in ovarian cancer and recently is has been shown that this action can be inhibited by the neutralizing anti-IL-6 antibody siltuximab in preclinical and clinical studies[64].

A recent study revealed that activation of macrophages by the infusion of antibodies against CD40 may induce macrophage-mediated tumor regression in 30% of cases in both a mouse model for pancreatic cancer and in patients with pancreatic cancer[65,66].

Since TGF-β is responsible for skin tumor infiltration by macrophages enabling the tumors to escape immune destruction[67], TGF-β seems to be a major player in the formation of the suppressive tumor microenvironment. Blockade of TGF-β has been shown to enhance tumor vaccine efficacy, but at this moment the exact mechanism has not been unraveled yet[68]. Since CCL2 plays a major role in the recruitment of TAMs, anti-CCL2 would be a logical step in preventing this recruitment. However, in a study on anti-CCL2 it was found that anti-CCL2 does not prevent the influx of TAMs[69]; this could be due to the inability to reach an adequate dosage of anti-CCL2 in the tumor microenvironment to counteract the influx of TAMs.

*Regulatory T cells*

Tregs are a population of CD4+ T cells with a central role in the prevention of autoimmunity and the promotion of tolerance via their suppressive function on a broad repertoire of cellular targets[70]. Characteristic of human Tregs is the expression of CD25 (IL-2 receptor-α chain), forkhead box P3 (Foxp3) transcription factor, glucocorticoid-induced TNF-receptor-related-protein (GITR), lymphocyte activation gene-3 (LAG-3), cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), and a down regulation of CD127 (IL-7R), however, all these markers are not truly Treg-specific[71]. Tregs can be divided into natural Tregs and adaptive Tregs. Natural Tregs are important in the suppression of auto-reactive T cells that slip through the selection processes and therefore natural Tregs maintain peripheral tolerance against self-antigens preventing autoimmunity. In humans, these cells represent 2-5% of total circulating CD4+ T cells in peripheral blood[72]. Adaptive Tregs arise from naive T cells and are triggered by suboptimal antigen stimulation and stimulation with TGF-β. Adaptive Tregs can be subdivided into IL-10 secreting Tregs type I (Tr1 cells); TGF-β producing Tregs (Th3 cells) or IL-35 secreting Tregs (iTr35 cells). These cells are characterized by the secretion of immune suppressive cytokines directly inhibiting T cells and converting DCs into suppressive APCs[73]. This contagious spread

of suppressive capacity, mainly mediated by IL-35 from Tregs, to other T cells is called infectious tolerance[74].

Tregs infiltrate human cancers and their prevalence in tumor-infiltrating lymphocytes is much higher than their proportion in peripheral blood, constituting 20% or more of tumor-infiltrating CD4+ lymphocytes[75]. Elevated levels of Tregs have been identified in blood of cancer patients, compared with normal individuals, and their presence predicts for poor survival [76]. In mesothelioma patients, elevated levels of Tregs have also been identified in pleural fluid, with a clear patient-to-patient variability[77].

Natural Tregs are derived in the thymus and migrate into the periphery. It has been proposed that Tregs need to be activated and/or expanded from periphery and bone marrow if needed. Since 25% of CD4+ T cells in the bone marrow function as Tregs, it has been suggested that the bone marrow plays an active role in humoral and cellular immune regulation.

TAA-specific Tregs accumulate in the peripheral lymphoid organs and at the tumor side. However TAA-specific Tregs are also found in the bone marrow, suggesting that after activation Tregs can migrate back to the bone marrow and induce T cell tolerance before these cells enter the circulation[78]. Although exact mechanisms are not fully explored, it has been shown that CCR4+ (receptor for CCL22) Tregs migrate toward tumor microenvironments expressing CCL22[12]. Also CD62L and CCR7 have been described as important homing markers on Tregs[79]. CD62L is critical for the migration of Tregs to draining lymph nodes. CCR7 is expressed by a majority of Tregs and is essential in homing to lymphoid organs and microenvironments expressing CCL19 (the ligand for CCR7)[80].

As MDCSs and TAMs, Tregs have several pathways that limit anti-tumor responses:

- Direct cell-cell interaction between Tregs and target cells is important for tolerance induction by Tregs[81]. These target cells include CD4+ and CD8+ effector cells, B cells, NK, T cells, DCs and monocytes/macrophages. The cell-cell binding leads to apoptosis by activation of programmed cell death-ligands (PDL), the release of perforin[82] and granzyme-A or B[36] and by reducing the proliferation through upregulation of intracellular cyclic AMP[83,84].
- Tregs produce themselves or induce other cells to secrete immunosuppressive cytokines such as IL-10, IL-35, and TGF-β to blunt immune responses[85], but also

other molecules produced by Tregs like carbon monoxide[86] and galectins[87] are reported to play roles in suppression. However, the relative importance of the individual inhibitory factors is dependent on the target disease and experimental model.

- Tregs can inhibit antitumor effector NK and NK T cells via membrane bound TGF-β[88]. The binding of membrane-bound TGF-β on Tregs to TGF-β-receptor on target cells leads to the activation of intracellular pathways, which eventually leads to the down regulation of the NKG2D-receptor on NK and NKT cells.
- CTLA4+ Tregs induce the expression of indoleamine 2,3-dioxygenase (IDO) in APCs, a potent regulatory molecule mediating the catabolism of the essential amino acid tryptophan into the pro-apoptotic kynurenine, which is toxic to neighboring T cells[89].
- Tregs are forming aggregates around DCs to prevent contact between DCs and T cells and in this way disturb the induction of the adaptive immune response by preventing proper antigen presentation[90,91].
- Treg aggregation leads to decreased upregulation of CD80 and CD86 on immature DCs and down regulation of these molecules on mature DCs [92]. These phenomena are antigen specific and dependent on lymphocyte function-associated antigen 1 (LFA-1) and CTL-associated protein 4 (CTLA-4)[22].
- Tregs induce B7-H4 expression by APCs, a member of the B7 family that negatively regulates T-cell responses[93].
- Expression of both ectoenzymes CD39 and CD173 on Tregs can hydrolyse pericellular ATP/AMP into the cAMP or the immunosuppressive nucleoside adenosine[94].
- Binding of lymphocyte activation gene 3 (LAG3) on Tregs to the MHC class II molecules expressed on immature DC suppresses DC maturation[95].
- Activated Tregs, which express more high-affinity IL-2R than conventional T cells, may absorb IL-2 from the microenvironment and therefore starve effector T cells that need IL-2 to survive[96].

However, none of these mechanisms can explain all aspects of suppression. It is probable that various combinations of several mechanisms are operating, depending on the milieu and the type of immune responses.

*Targeting Tregs*

Owing to the significant role of Tregs in the failure of immune surveillance and immunotherapy, many attempts to deplete Tregs or inhibit their function in cancer patients have been studied. Many of the strategies to reduce Tregs target CD25, which makes up the alpha-subunit of the IL-2R, that is present on the surface of Tregs and activated cells. An engineered recombinant fusion protein of IL-2 and diphtheria toxin (denileukin diftitox [Ontak]) and other CD25-directed immunotoxins (daclizumab, LMB-2, RFT5-SMPT-dgA) have been investigated for Treg depletion, which seems to kill selectively lymphocytes expressing the IL-2 receptor. However, early human trials have not proven that this approach results in tumor regression and have shown that these strategies may not adequately deplete Foxp3+ Tregs, and may also deplete antitumor effector cells[97–100]. Other possible approaches to reduce immunosuppression of Tregs is via CTLA-4 blockade (e.g. ipilimumab)[101,102], anti GITR agonism[103], and vaccination against Foxp3[104] and some other suggested approaches, such as the inhibition of IDO, TGF-β, ectonucleotidase (expressed by Tregs and generates immunosuppressive adenosine), or the activation of other agents such as OX40 or Toll-like receptor 8 have not yet proven to be beneficial. IL-7 administration was shown to increase T cell numbers and decrease of the Treg fraction in humans[105], on the contrary, other reports have shown that IL-7 leads to the development of Tregs[106,107]. In conclusion, there are many conflicting results in abrogating the action of Tregs, and thus it is unclear which approach holds promise for cancer treatment.

Low-dose cyclophosphamide (CTX) prevents the development and functionality of the Tregs[108], the mechanism behind this effect, however, is not completely understood. We investigated the effect of CTX on immune-suppression and the combination of CTX and DC-based immunotherapy was studied in a murine MM model[109]. Our data showed that metronomic administration of low-dose CTX has a strong immune-modulating effect *in vivo*. This is currently tested in a clinical trial in mesothelioma patients (ClinicalTrials.gov Identifier: NCT01241682). Tregs can be significantly reduced in mice with anti-murine CCL2/CCL12 monoclonal antibodies, resulting in significant reductions in Treg cells in the spleens and tumors. Using these antibodies, the tumor microenvironment was also drastically altered. This resulted in a significant improvement of immunotherapy[68]. Sorafenib has been proven cytotoxic for Tregs, although the pathway is not fully understood. Sorafinib treatment is

associated with a decrease in frequency of Treg cells without influencing the function of peripheral immune effector cells[110]. Recently, p300 was found to be an important target for modulation of host Foxp3+ Treg functions and a inhibition of p300 using a small molecule inhibitor, C646 (p300i), impaired Foxp3 acetylation and inhibited Treg function[111].

*Immune-adjuvant therapies*

An alternative approach to immunotherapy is to enhance the intrinsic activity of the immune system. In this field, ipilimumab was proven to be active in metastatic melanoma[112]. Ipilimumab is a monoclonal antibody against cytotoxic T-lymphocyte antigen (CTLA)-4. It is normally expressed at low levels on the surface of naïve effector T cells, but is up regulated on the cell surface when there is a long-lasting and strong stimulus via the T cell receptor (TCR). CTLA-4 then competes with CD28 for CD80/CD86 on APCs, effectively shutting off TCR signaling and thereby serves as a physiologic "brake" on the activated immune system[113]. Ipilimumab prevents this feedback inhibition, resulting in an unabated immune response against the tumor. The side effects of this therapy, however, can be significant due to the down regulation of tolerance to patient's own normal tissue and colitis is often seen in patients[114]. In mesothelioma, preclinical models have been well described and a phase II trial is currently ongoing in Italy[26].

Other, preclinical approaches are the Toll-like receptor (TLR) ligands to activate DCs[115] or TLR7 agonist to induce systemic, CD8+ T-cell and type-I IFN anti-tumor responses[116].

## NEED FOR REVISING RESPONSE EVALUATION IN IMMUNO-THERAPY

Immunotherapy represents a new class of agents in the treatment of mesothelioma. As seen for Sipuleucel-T in prostate cancer and ipilumumab in melanoma, improvement in the overall survival of patients was seen, however, the agents did not change initial disease progression. Even, a transient worsening of disease manifested either by progression of known lesions or the appearance of new lesions can be seen, before disease stabilizes or tumor regresses.

Commonly accepted treatment paradigm, however, suggests that treatments should initially decrease tumor volume, which can be measured using CT-scan. Also, progression-free survival is increasingly used as an alternative end-point of studies. This seems to be unfortunate for immunotherapy, which may initiate an immune response that ultimately slows the tumor growth rate, resulting in longer survival, but not a decrease in tumor volume on CT or an increased progression free survival (figure 3). Future trials are currently planned to investigate these hypotheses, however, clinicians at this moment may need to reconsider how to measure success of their immunotherapeutic approach[117].

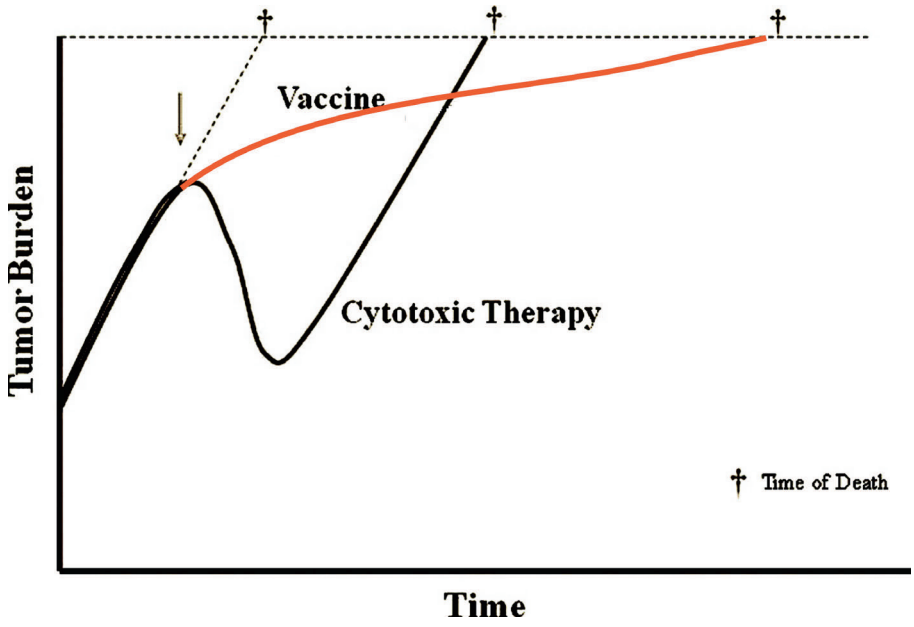


**Figure 3.** Tumor growth is a dynamic biologic process; that is the net result of cells dividing and other cells dying. Intrinsic tumor biology, as well as extrinsic factors such as therapies, affects the tumor's growth rate. However, chemotherapy only affects the tumor growth rate while it is being administered, which may result in a dramatic but transient response. Following discontinuation of chemotherapy, the growth rate returns to its pre-treatment slope, driven by the underlying biology of the tumor. Immunotherapy (red line), on the other hand, can alter the biology of the host by inducing an active antitumor immune response including a memory response. This may not cause an immediate or dramatic change in tumor burden, but continued cumulative slowing pressure on tumor growth rate, especially if started early in the disease course, may lead to substantially longer overall survival. The arrow indicates the initiation of treatment; cross indicates time of death from cancer[117]. (Figure used with permission from author)

## SUMMARY

In conclusion, the role of the immune system in mesothelioma is vast. The tumor uses villainous tricks to evade immune surveillance and harnesses itself against the immune system. Immunotherapy tries to modulate this immune system to strengthen the anti-tumor effect, which is unfortunately hampered by these defense mechanisms from the tumor. At this moment, MSDCs, M2 TAMs and Tregs seems to be the key players in this process, but undoubtedly extended research will eventually unravel this complex interplay of cells and will reveal more cell types and/or subtypes. Targeting these defense mechanisms could be the key to fully unleash the potential of immunotherapy. Since several cell types are responsible for tumor survival, probably combination therapy targeting multiple cell types will be necessary. It is thrilling that the immunotherapy has been established in several tumor types as a proven therapy in recent years and that many trials are ongoing with promising results. In mesothelioma, the first steps have been made and using the accumulating knowledge, immunotherapy will hopefully prove to be an effective treatment.

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# Chapter III

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

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*PLoS One. 2014 Sep 5;9(9):e10674*

## ABSTRACT

*Backgound*: 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 and 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 both 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 found 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 l 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).

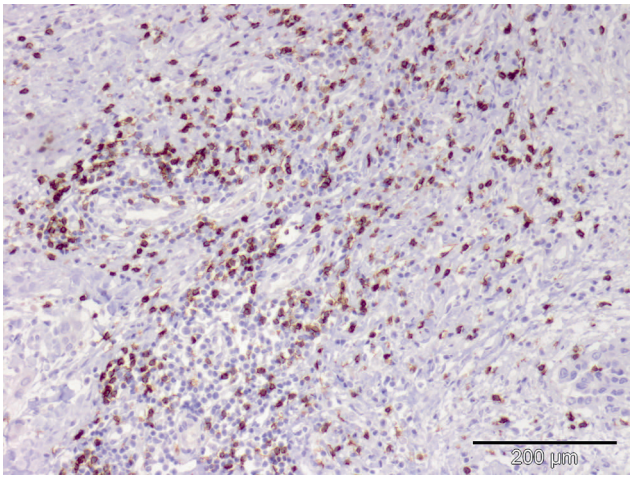



**Figure 1**
Representative image of CD8 staining in the tumor biopsy of one MPM patient.

*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]).

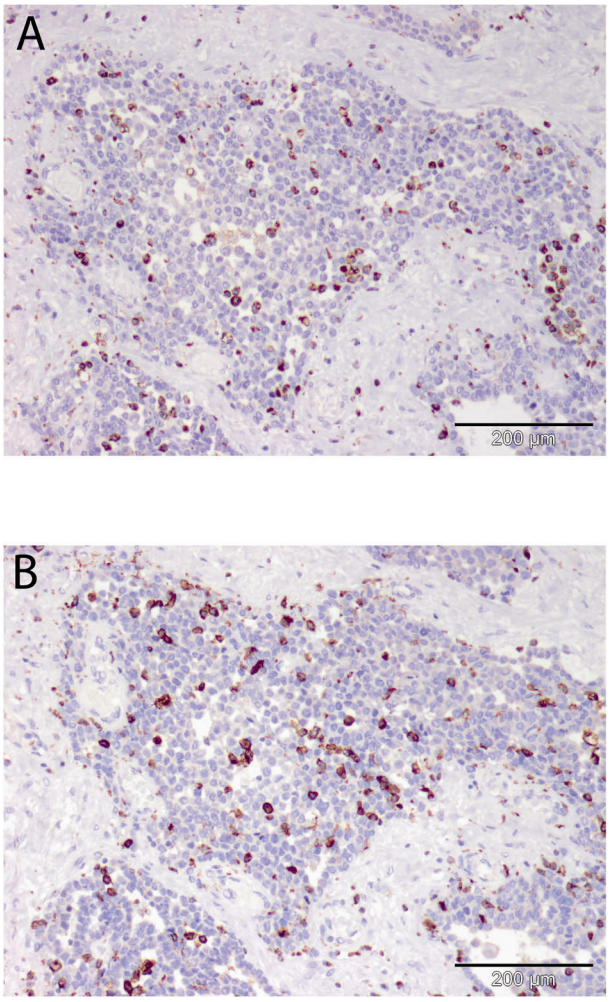



**Figure 2**
Representative images of CD68 (a) and CD163 (b) staining in the tumor biopsy of one MPM patient.

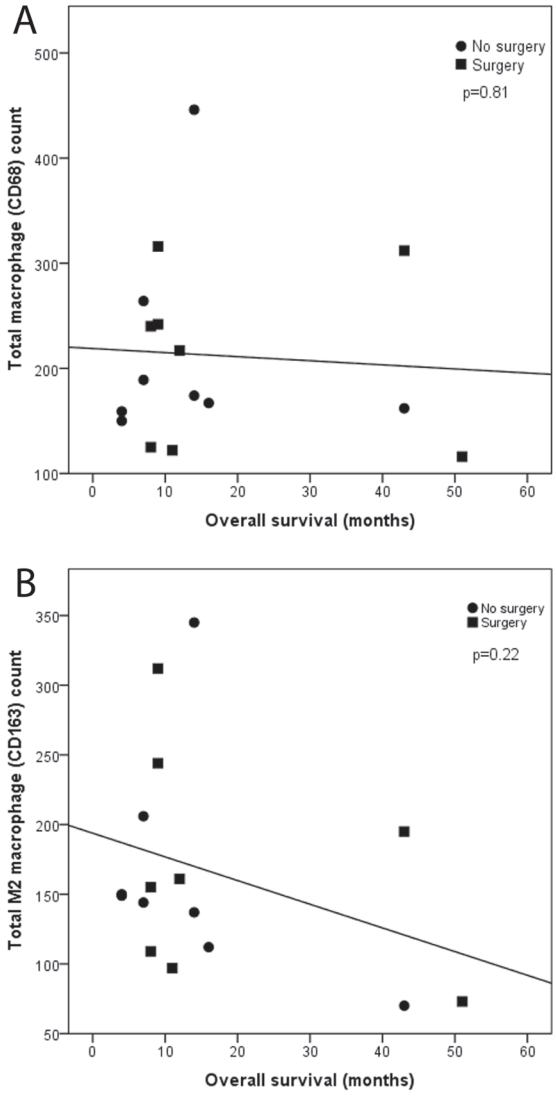



**Figure 3**
Correlation between CD68 (a) count or CD 163 (b) count and OS in both surgery and non-surgery groups. The CD68 count does not correlate with OS (Pearson r -0.07, p=0.81), the CD163 count shows an inverse trend with OS (Pearson r -0.33, p=0.22).

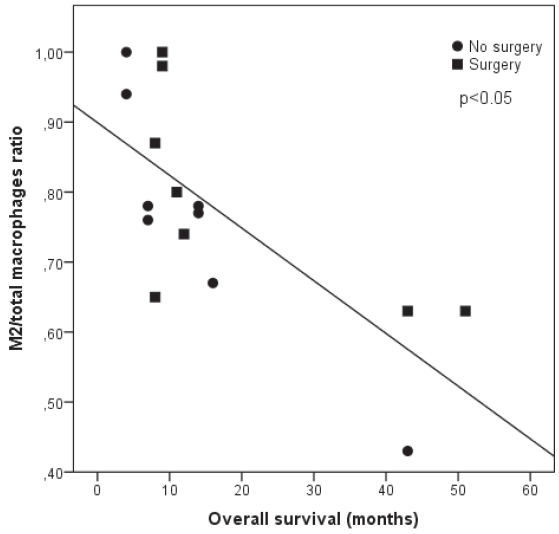
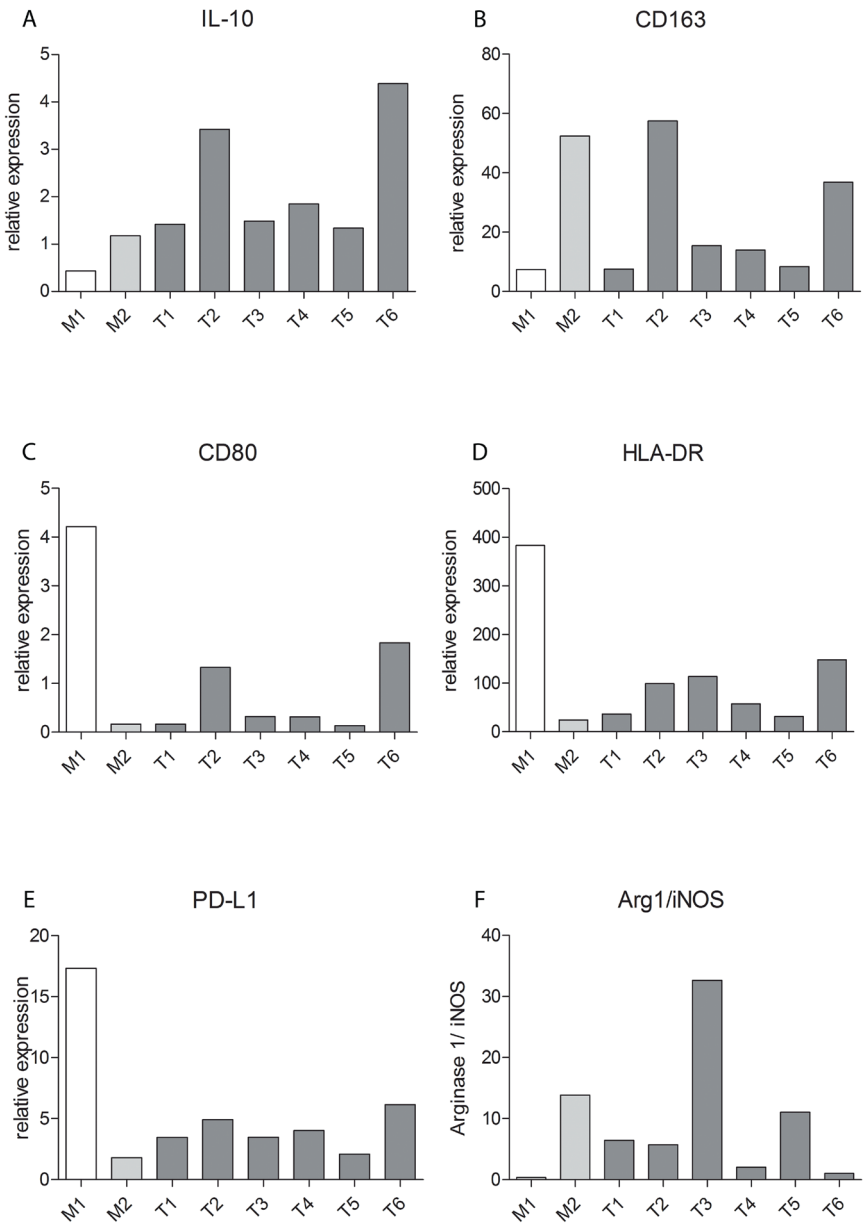



**Figure 4**
Correlation between CD163/CD68 ratio in tumor in both surgery and non-surgery patients and OS. This ratio is significantly negatively correlated with OS in the total patient group (Pearson r -0.72, $p<0.05$)

*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.




**Figures 5 a-f**

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 [8,9]. 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.

**Acknowledgments**: We would like to thank Lisette de Vogel of the Department of Pathology for the technical assistance with the immunohistochemical techniques.

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# Chapter IV

## 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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*Accepted for publication in Lung Cancer pending minor revisions*

## ABSTRACT

*Background*: 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.

*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: 30/0.025 cm2 (range 2-60) in the LTO group and 140/0.025 cm2 (range 23-314) in the patients without LTO ($p<0.01$).

*Conclusions*: 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.

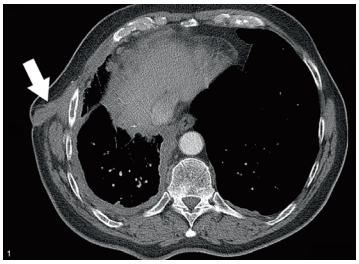

**Figure 1: LTO on CT scan.**
CT scan of a patient with LTO after thoracoscopy on the right side. Outgrowth of tumor is seen in the thoracoscopy tract (white arrow).

**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) |



*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 ($p<0.001$).

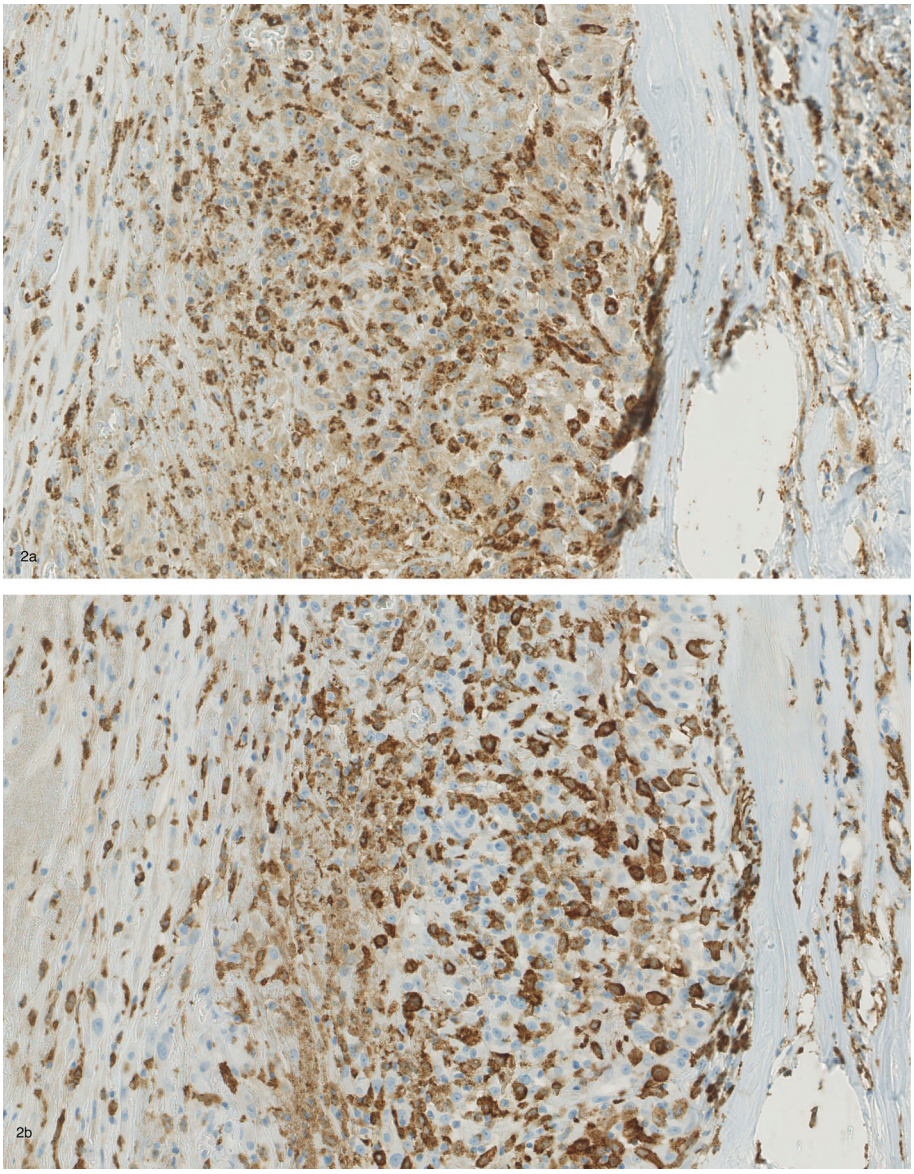


**Figure 2a and 2b: Immunohistochemical stainings of CD68 (2a) and CD163 (2b) in the same microscopic field of a mesothelioma tumor specimen.**
The brown color represents the CD68 staining (2a) and the CD163 staining (2b) in mesothelioma tumor specimens. The blue colored cells are CD68 or CD163 negative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

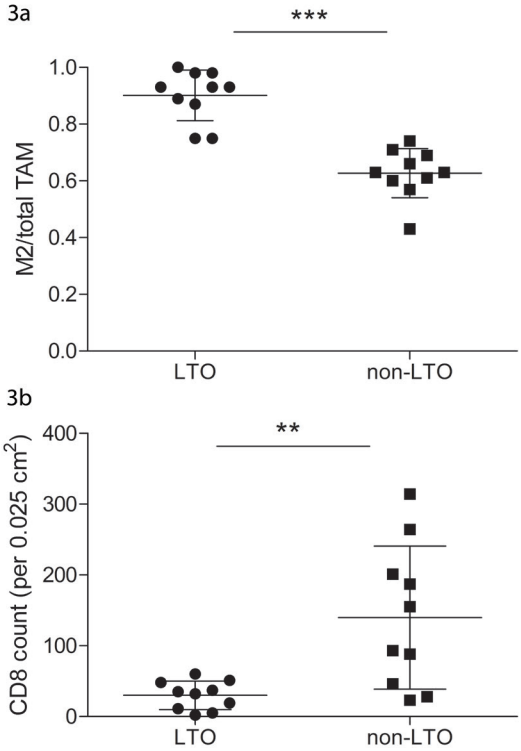


**Figure 3a and 3b: Increased proportions of M2 TAMs and educed numbers of CD8+ cells in diagnostic biopsies from in diagnostic biopsies of mesothelioma patients who develop LTO.**

(a) Ratio of CD163 positive cells (M2) and CD68 cells (all macrophages) of patients who developed LTO 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.

*Tumor infiltrating lymphocytes in MPM*

A representative image of an immunohistochemical staining of TILs is shown in figure 4. 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).

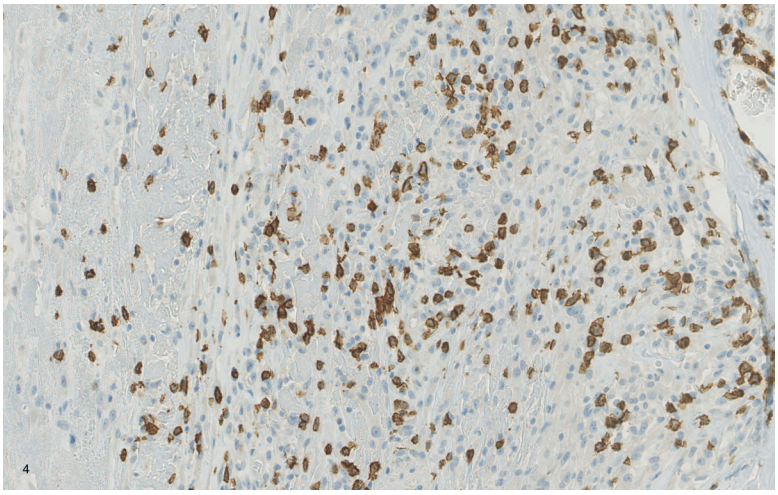

**Figure 4: CD8+ Immunohistochemical staining in a mesothelioma tumor specimen.**
CD8 staining is shown in brown. The blue colored cells are CD8 negative cells.

*CD8 and M2/total TAM ratio*

The correlation between the CD8 TIL count and the M2/total TAM ratio is shown in figure 5. 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.

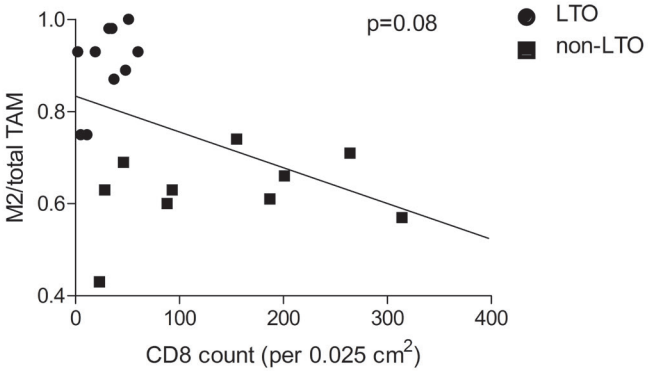


**Figure 5: Correlation between M2/total macrophage ratio and CD8 lymphocyte count.**
Squares are patients with local tumor outgrowth, circles without local tumor outgrowth. A near significant correlation was found between the M2/total macrophage ratio and the CD8 TIL count (Spearman's rho -0.40, p=0.08).

## 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.



## ACKNOWLEDGEMENTS

We thank Stichting Asbestkanker Rotterdam and the Mesothelioma Applied Research Foundation (MARF) – Larry Davis Memorial Grant for their financial support.

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# Chapter V

## Pleural effusions of malignant mesothelioma patients polarizes monocytes towards immunosuppressive macrophages of the M2 phenotype

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## ABSTRACT

Insights into tumor-infiltrating immune cells are pivotal to optimize immunotherapeutic approaches. Malignant pleural mesothelioma (MPM) is a rare thoracic malignancy in which the immune infiltrate predominantly consists of tumor-associated macrophages (TAMs) of the M1 or M2 phenotype, and T-cell subsets. Pleural effusion (PE) often accompanies MPM and is considered to be an easy-access opportunity to investigate the tumor environment. In the present study, we investigated the influence of PE of MPM patients on macrophage phenotype and interaction with T-cells *in vitro* and *in vivo*. We demonstrate that PE of MPM patients induces a M2 phenotype in macrophages, which was confirmed by the suppressive activity of macrophages on T-cell proliferation when co-cultured in the presence of PE *in vitro*. In addition, we show that PE of MPM patients is rich in M2-skewing cytokines, like IL-6 and TGF-β. The presence of these cytokines can, at least in part, be attributed to tumor cells as was shown in the comparison of cytokine concentrations between PEs and accompanying MPM tumor cell line supernatants (n=6). The capability of PE to induce an immunosuppressive M2 phenotype in vitro was confirmed in an in vivo study in which we investigated TAMs and T-cells in the PE of 30 MPM patients. We demonstrated that TAMs are abundantly present in PE of MPM patients and are predominantly of the M2 phenotype. In addition, we found a negative correlation between these TAMs and T-cells in the PE of MPM patients (n=30, $p<0.001$). These findings demonstrate that TAMs are key players in the immunosuppressive environment of MPM and emphasize their potential as a therapeutic target. In addition, the presence and phenotype of TAMs in PE should be taken into consideration in the application of (immuno)therapies in MPM patients. Therefore, these findings may have important consequences for studies investigating intrapleural anti-tumor treatments.

## INTRODUCTION

Malignant pleural mesothelioma (MPM) is a highly aggressive cancer with currently limited treatment options. The pleural cavity is an interesting compartment to administer different treatment modalities, and different options are now under investigation. 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 and a low serum concentration [1]. Also intrapleural gene transfer was shown to be feasible [2]. Recently, intrapleural delivered CAR-therapy was shown as a promising treatment option in a murine model of mesothelioma [3].



The immune system is considered to play a major role in the pathogenesis, prognosis and, potentially, in the treatment of this devastating disease [4-7]. Studies have shown promise for the use of immunotherapy in MPM patients [7-9].

Despite encouraging results responses are hampered by local and systemic immunosuppressive mechanisms [10,11]. Therefore, attention is focusing on the cellular and molecular mechanisms, which play a role in the immunosuppressive tumor environment.

Especially, intrapleural therapies will be influenced by local pleural conditions, like interaction with plueral effusion (PE). PE accompanies mesothelioma in approximately 70% of the cases [12]. PE consists of varying amounts of tumor cells and numerous types of immune cells (Figure 1) [13,14].

Immune cells invade both the tumor and PE of MPM patients [18-20]. These infiltrating immune cells can exert either beneficial or detrimental effects, depending on their phenotype [21]. Tumor-associated macrophages (TAMs) are a major component of the immune cell infiltration of the tumor microenvironment in mesothelioma patients [22]. Under the influence of various stimuli within the tumor microenvironment, TAMs can develop into a tumor-inhibitory (M1) or tumor-promoting (M2) phenotype [23,24]. M1 TAMs are characterized by their cytotoxic activity, expression of high levels of reactive oxygen species, and their capability for antigen-presentation. TAMs of the M2 phenotype are known to exert tumor-promoting and immunosuppressive

functions in the tumor microenvironment, through the production of different cytokines and proteases, e.g. interleukin (IL)-10 and matrix metalloprotease-9 (MMP-9). Furthermore, M2 TAMs are capable of directly inhibiting T-cell activation, the key players in anti-tumor immunity [22,25,26] The presence and M2 phenotype of TAMs in MPM tumor biopsies was found to be related to a worse survival [27,28]. Vice versa, the infiltration of CD8 T-cells has been associated with a favorable prognosis in MPM [29]. As a thoracocentesis is often performed either for diagnostic purposes or to relieve dyspnea, PE could provide an easy-access opportunity to examine the tumor environment.

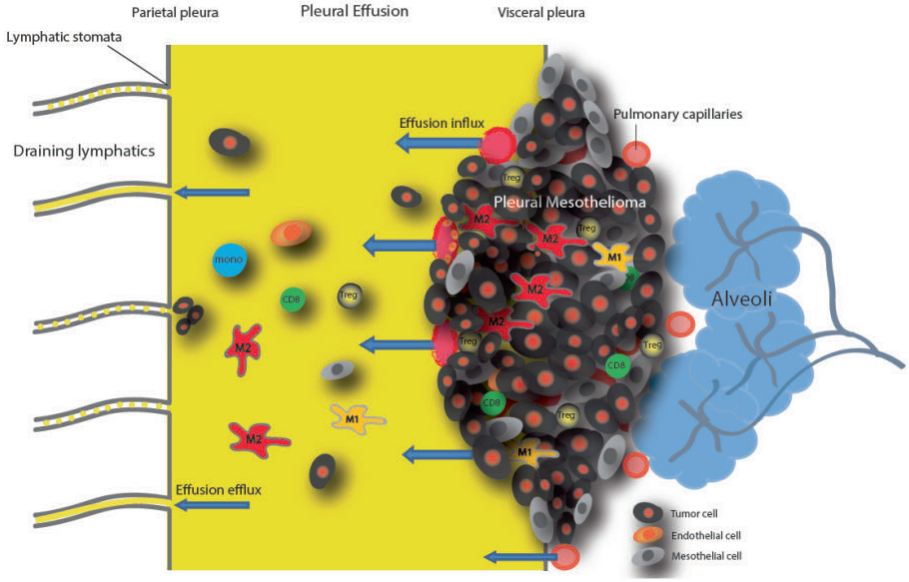



**Figure 1.** Schematic representation of pleural effusion in MPM. MPM can develop from both the visceral pleura and the parietal pleura. Pleural effusion accumulates in the pleural space when influx outweighs efflux. Increased production occurs due to excessive plasma leakage through hyperpermeable intratumoral vessels. In addition, blockade of the pleuropulmonary lymphatics by tumor cells results in reduced absorption [14]. Pleural effusion of mesothelioma patients can comprise different cell types and soluble factors, which can be derived directly from the tumor and its environment or from the vasculature. Immune cells like T-cell subsets (e.g. CD8 T-cells and regulatory T cells) and macrophage subsets (M1 or M2 macrophages) can be present in malignant pleural effusions [15-18].

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 TAMs and T cell subsets.

## MATERIALS AND METHODS

*Subjects*

MPM patients were selected from the patient databank which was set up for our immunotherapy trials [6]. The study was approved by the institutional Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands (NL24050.000.08). All participants provided written informed consent. 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.

*Collection and processing of pleural effusions*

Thoracocentesis was performed using fine-needle aspiration inserted into the pleural cavity and the pleural fluid 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 [30]. Leucocytes were cryopreserved in 10% DMSO and stored at -150 °C. PE supernatants were stored at -80ºC. 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 earlier [18].

*Cytokine measurements*

The levels of 12 cytokines were measured by a magnetic bead-based multiplex assay (11-plex and single plex (transforming growth factor beta (TGF-β)) Bio-Plex Pro™ Magnetic Cytokine Assay, Bio-Rad) in the six PE supernatants used for the *in vitro* experiments. The same assay was performed for conditioned media of accompanying mesothelioma cell lines, which were originally derived from the six used PEs. MPM cell line conditioned media was harvested at 80% confluency in all cell lines. The levels of the following cytokines were determined: IL-6, TGF-β, vascular endothelial growth factor (VEGF), IL-12, IL-10, IL-13, IL-17, tumor necrosis factor alpha (TNF-α), IL-4, IL-2, IL-1β and interferon gamma (IFN-γ).

*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 following monoclonal antibodies were used in the first panel: anti-human CD3ε (APC-Cy7, clone UCHT1, eBioscience), CD4 (FITC, clone RPA-T4, BD Biosciences), CD8a (APC-eFluor450, clone RPA-T8, eBioscience), CD25 (PE-Cy7, clone M-A251, BD Biosciences), CD127 (PE, clone M21, BD Biosciences) FoxP3 (APC, clone PCH101, eBioscience), and a Live/Dead® Fixable Aqua dead cell stain in Amcyan (Invitrogen). The following monoclonal antibodies were used in the second panel: CD14 (PE Texas Red, clone TuK4, Invitrogen), CD16 (Pacific Blue, clone 3G8, BD Biosciences), CD68 (biotin, clone Y1/82A, Biolegend) streptavidin APC-Cy7 (BioLegend), CD163 (PE, GH1/61, eBioscience), CD206 (PerCP-Cy5.5, clone 19.2, eBioscience), CD11c (APC, clone S-HCL-3, BD Biosciences), human leukocyte antigen (HLA)-DR (PE-Cy7, clone L243, BD Biosciences), CD45 (FITC, clone HI30, eBioscience), and a Live/Dead® Fixable Aqua dead cell stain in Amcyan (Invitrogen). Co-receptor expression of cultured macrophages was measured using the following monoclonal antibodies: CD80 (PerCP-ef710, clone 2D10.4, eBioscience), CD86 (BV421, clone FUN1, BD Biosciences), programmed cell death ligand 1 (PD-L1) (APC, clone M1H1, eBioscience), immunoglobulin-like transcript (ILT) 3 (FITC, clone B56, R&D systems, T cell immunoglobulin mucin (TIM3) (PE, clone F38-2E2, BioLegend) and HLA-DR (APC-Cy7, clone L243, BD Biosciences). The analysis was performed using FlowJo software (Tree Star Inc.).

*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 [30]. Monocytes and T-cells were isolated with MACS® separation using a Monocyte Isolation Kit followed by a Pan T-cell Isolation Kit (all Miltenyi Biotec). Purity of the isolated fractions was confirmed using flow cytometry (>97% pure, data not shown).

*Macrophage cultures*

For all conditions, healthy monocytes 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) in RPMI-1640 medium containing L-glutamine and sodium bicarbonate. Subsequent polarization to the M1 or M2 phenotype occurred in the presence of lipopolysaccharide (LPS) (100 ng/ml, Sigma-Aldrich) and IFN-γ (20 ng/ml, R&D systems) for M1 or IL-10 (40ng/ml, R&D systems) 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 [28]. In short, cells were harvested followed by mRNA isolation using a RNeasy micro kit (Qiagen) and subsequent cDNA preparation using a First Strand cDNA synthesis kit (Thermo Fisher). Quantitative real time PCR reactions were performed using a 7300 real time PCR system (Applied Biosystems). 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.

**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 |

*T-cell co-culture and proliferation assay*

Autologous T-cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) as previously described [30]. CFSE labeled T-cells were stimulated using anti-CD3/anti-CD28 beads (Invitrogen) and co-cultured in a 1:1 ratio with macrophages that were differentiated during 6 days from monocytes isolated from the same healthy donor as described earlier. T-cells and macrophages were co-cultured during 4 days in either 10% normal healthy AB serum, 10% PE supernatants (n=6) or 30% MPM cell line conditioned media (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 [31].

*Statistical analysis*

Data are expressed as mean ± standard deviation. Paired data were tested 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.

## RESULTS

*Pleural effusions polarize monocytes towards a M2 macrophage phenotype*

The influence of PE supernatants on the phenotype of healthy monocyte-derived macrophages was investigated *in vitro*. Standard M1 or M2 polarizing culture conditions were used as controls. The standard macrophage marker CD68 was used

to confirm proper macrophage maturation (Figure 2A). Overall, PE supernatants induced a M2 phenotype with a typical high expression of scavenger receptor CD163 (Figure 2B and IL-10 (Figure 2C) and low expression of the activation markers CD80 (Figure 2D), PD-L1 (Figure 2E) and the typical pro-inflammatory marker HLA-DR (Figure 2F).

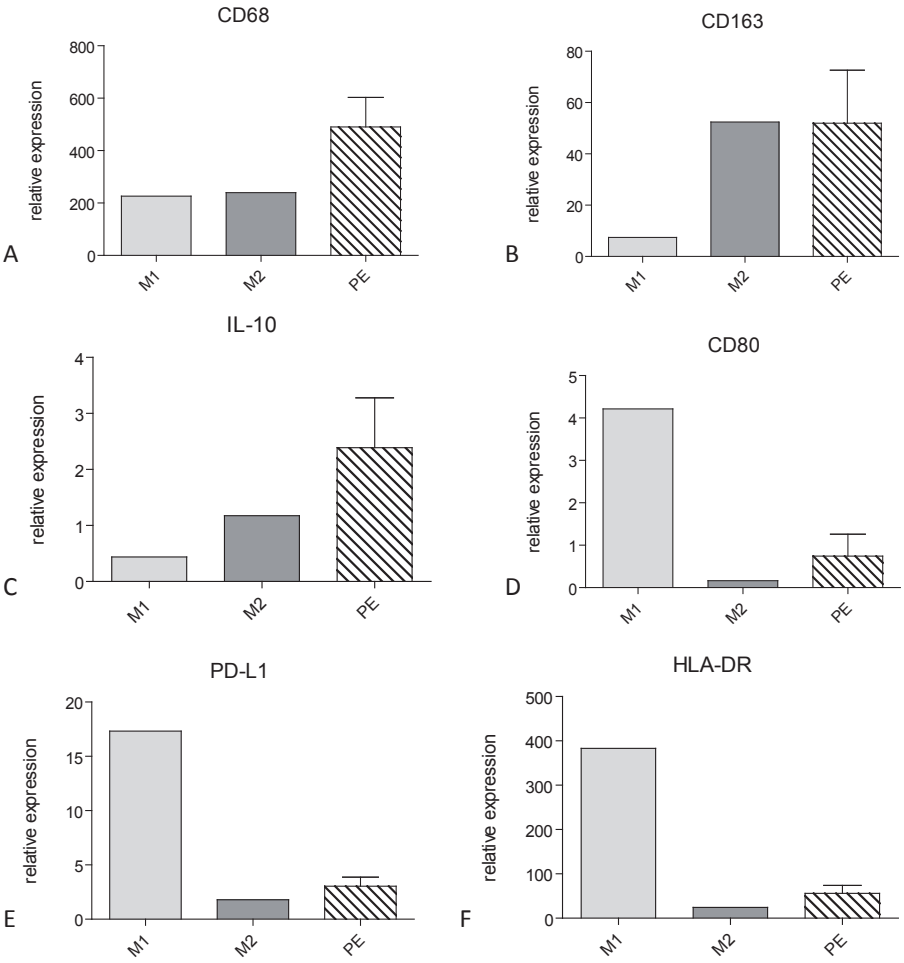



**Figure 2.** Expression of signature macrophage phenotype-related genes after culture under standard M1 or M2 condition or in the presence of PE supernatant (n=6). Panel A shows the expression of the general macrophage marker CD68, panel B and C show the expression of the specific M2 markers scavenger 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.

*Pleural effusions and accompanying MPM tumor cell line supernatants comprise similar patterns of macrophage polarizing cytokines*

In order to investigate the presence and level of cytokines with known potential to induce a M1 or M2 macrophage phenotype, we performed a magnetic bead-based multiplex assay on the 6 PE supernatants used for the macrophage cultures. Figure 3A demonstrates that IL-6 and TGF-β, both associated with a M2 phenotype of TAMs [32], were at the highest level amongst the measured cytokines in the PEs. In addition, the pleiotropic cytokine VEGF could be measured at relatively high levels. IL-12, TNF-α and IFN-γ are associated with a M1 phenotype skewed milieu, although IFN-γ was undetectable and TNF-α could only be measured at low levels, the relatively high concentration of IL-12 illustrates the complexity of the local interactions in PE. The classical type 2 immune response cytokines IL-10, IL-13 and IL-4 could all be detected in PE at relatively low levels. Figure 3B demonstrates that the measured cytokines could be in part directly tumor cell derived. The cytokines measured in conditioned media of the 6 corresponding MPM cell lines show a similar pattern compared to the original PEs from which the MPM cell lines were developed.

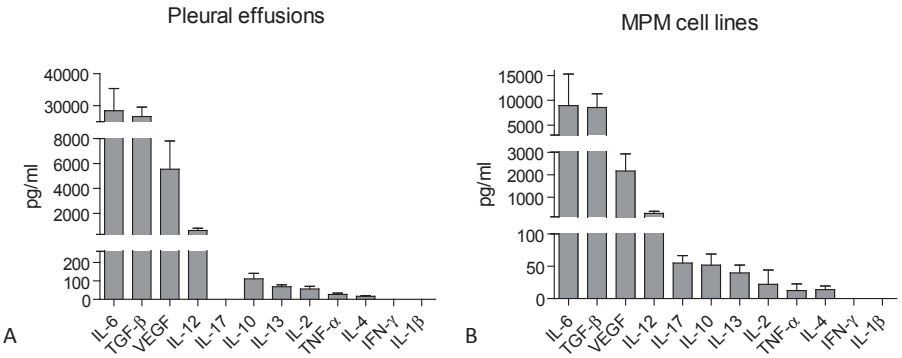



**Figure 3.** Concentrations of selected cytokines in PE supernatants and corresponding MPM cell lines conditioned media (n=6). Cytokine levels were measured using a magnetic bead-based multiplex assay.

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

To investigate the immunosuppressive function of macrophages cultured in PE (10%, n=6) and corresponding MPM cell line conditioned media (CM, 30%, n=6), we co-cultured CM and PE 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. Normal human AB serum was used as a control. Figure 4 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 CM, 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 although the cytokines determined in our study present in CM and PE are very similar, other, cytokines or soluble factors that were not measured could attribute to the difference in macrophage function compared to phenotype.



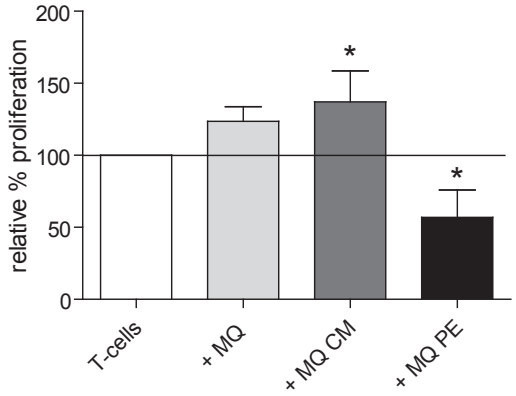



**Figure 4.** Macrophages suppress T-cell proliferation in the presence of PE. The percentage proliferation of only T-cells (control, in conditioned medium (CM) or in PE) was set at 100% and the T-cell proliferation during co-culture with macrophages was calculated relative to the basic proliferation of only T-cells. Co-culture with macrophages under control conditions (n=3, 10% NHS) did not show a statistically significant increase or decrease in T-cell proliferation. Co-culture with macrophages in the presence of CM demonstrated an increase in T-cell proliferation (n=6, $p<0.05$). Co-culture with macrophages in the presence of PE induced a suppression of T-cell proliferation (n=6, $p<0.05$, paired Wilcoxon test).

*PE-cultured macrophages demonstrate a relative decreased expression of (co-) activation markers and increased expression of Tim-3 during co-culture with activated T-cells*

In order to further investigate the immunosuppressive function of macrophages on T cells cultured in the presence of PE, the expression of different co-activation and co-inhibitory receptors by macrophages were measured at baseline and after 24 hours and 96 hours of co-culture. As a control, these markers were measured on standard M1 and M2 macrophages co-cultured with T-cells. Only the 96 hour time point is depicted here as results were comparable at baseline and 24 hours (data not shown). Results shown are representative data of two independent experiments performed with the same PE. This PE was selected based on its profound suppressive effects in the earlier experiments. Macrophages cultured in the presence of PE and M2 condition showed a similar lower expression of (co-) activation markers compared to standard M1 macrophages (Figure 5A-D). Expression of the co-inhibitory receptor ILT3 was not increased on PE or M2 macrophages (Figure 5E), TIM3 expression showed a moderate increase (Figure 5F).

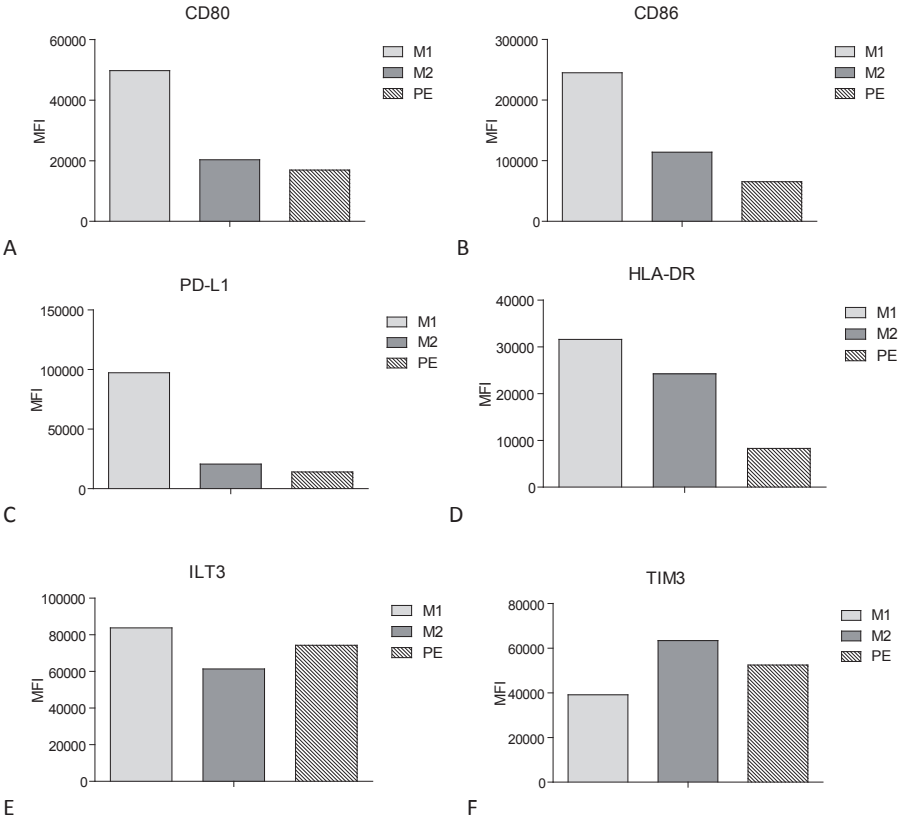



**Figure 5.** Macrophages cultured in the presence of PE and M2 condition show a decreased expression of the (co-) stimulatory markers CD80, CD86, PD-L1 and HLA-DR compared to M1 macrophages (panel A-D). Expression of the co-inhibitory marker ILT3 was highest on M1 macrophages (panel E), TIM3 expression was slightly increased on M2 and PE macrophages (panel F).

*PE-cultured macrophages have a robust suppressive phenotype*

Previous experiments indicated that potentially an increased expression of TIM3 could be a mechanism of the suppressive phenotype of PE-cultured macrophages. However, addition of a TIM3 blocking antibody did not result in recovery of T-cell proliferation during co-culture with M1, M2, or PE-cultured macrophages (data not shown). As macrophages are known for their potential to metabolize pivotal nutrients, e.g. tryptophan, we added 10% NHS to the 10% PE culture condition to investigate whether nutrient depletion could explain the suppression of T-cell proliferation. This

addition of 10% NHS to the 10% PE culture condition did not result in an increase of T-cell proliferation (data not shown). Furthermore, the addition of IFN-γ and LPS (M1 condition) to PE did not result in a recovery of T-cell proliferation, indicating the robust effects of PE (data not shown).

*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. Patient characteristics are described in table 2. 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.

**Table 2** Patient characteristics

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

*Macrophages and T- cells in pleural effusion 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 6 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. 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) [15,18].

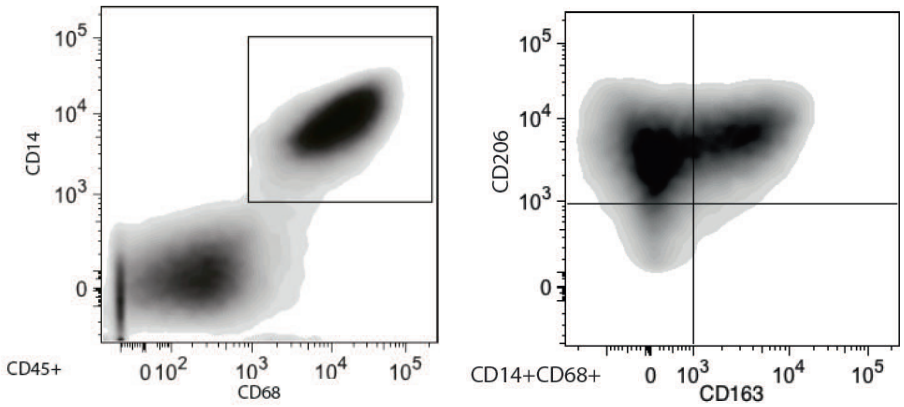



**Figure 6** Flow cytometric analysis of TAMs in PE. TAMs were characterized as CD45+CD14+CD68+ cells. These TAMs were further classified depending on their CD206 and CD163 expression. All TAMs which expressed either CD206, CD163 or both, were classified as M2 TAMs.

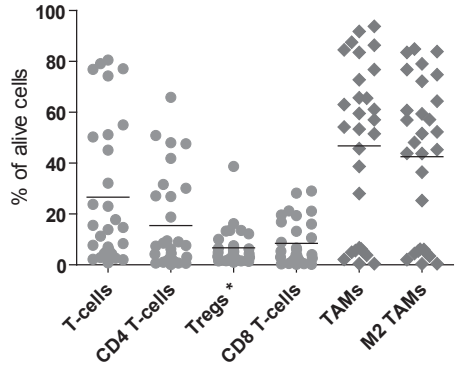
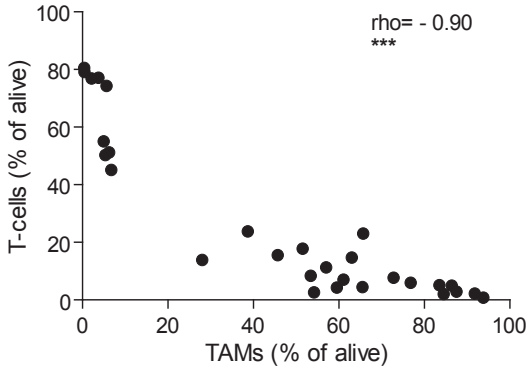
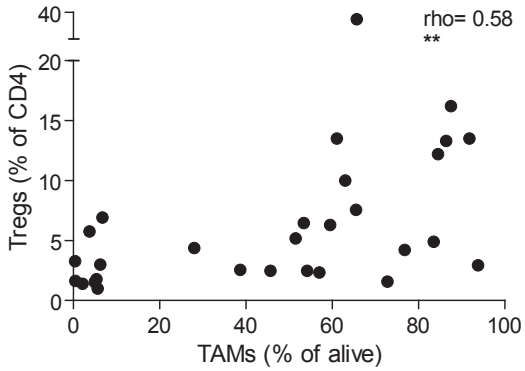



**Figure 7** T-cell subsets and TAMs in PE of 30 MPM patients. * Tregs are depicted as percentage of CD4 T-cells. All other cell populations are depicted as percentages of total alive cells. Tregs were classified as CD4+CD25+CD127-FoxP3+ cells. TAMs are CD45+CD14+CD68+, M2TAMs are CD206+, CD163+ or CD206+CD163+ TAMs.

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 8A, 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 8B, 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, $p=0.75$).




A




B

**Figure 8** Correlation between T-cells and TAMs in PE of MPM patients Panel A shows the correlation between all TAMs and T-cells. T-cells are all CD3+ cells, TAMs are all CD45+CD14+CD68+ cells. Spearman's rho -0.90, p<0.001. Panel B 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.

## DISCUSSION

In the present study, we investigated the immunosuppressive properties of PE of 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 of MPM patients exhibited a M2 phenotype and suppressive function *in vitro*. In contrast, macrophages cultured in MPM cell line conditioned media did not suppress T cell proliferation. 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*.

We have demonstrated earlier that MPM cell line supernatants are capable of inducing macrophages with the M2 phenotype [28]. The current study showed that the PEs, which were the original source of the MPM cell lines, induce a much stronger M2 phenotype. In addition, both MPM cell lines supernatants and PEs contained comparable patterns of measured 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 greatly. Macrophages cultured in the presence of PE suppressed T-cell proliferation whereas MPM cell line conditioned media did not exert this effect. Although some of the measured cytokines are known for their M2-skewing effect, these results indicate that these cytokines are not responsible for the suppressive function of macrophages in the current study. In order to further investigate the mechanism behind the suppressive function of the macrophages we determined the expression of a selection of co-receptors. The co-inhibitory receptor TIM-3 has been described earlier to be expressed on myeloid cells and exert an immunosuppressive effect [33-35]. However, blockade of this receptor did not result in recovery of T-cell proliferation in our current study. In addition, suppletion of normal serum to the culture condition had no effect, indicating that nutrient depletion caused by e.g. IDO expression of the macrophages is not responsible for the effect [36]. Therefore, the main mechanism by which macrophages suppress T-cell proliferation in the presence of PE *in vitro* remains to be elucidated. Nevertheless, by demonstrating a negative correlation between TAMs and T-cells in PE of MPM patients we have provided an indication that this interaction 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 in literature as pivotal human M2 markers [26].

Recently, Scherpereel *et al* showed that there is a defect in the recruitment of CD8 T-cells in malignant PE of various cancer patients [15]. Our current data demonstrate a potential role for TAMs regarding this T-cell inhibition. Although we were not able to reveal the specific mechanisms involved, we identified TAMs as a pivotal target to improve the immunosuppressive environment in MPM.

Among the measured cytokines in PE and conditioned media of corresponding MPM cell lines, IL-6 and TGF-β were detected at the highest level. Both these cytokines have been associated earlier with immunosuppression and a worse prognosis in cancer patients. IL-6 is a multifunctional cytokine that is known to play a role in the regulation of cell proliferation, survival, and metabolism and IL-6 signaling has also been implicated in carcinogenesis [37,38]. Elevated levels of serum IL-6 have been associated with worse survival and poor treatment response in lung cancer patients in earlier studies [39-41]. Furthermore IL-6 has been described as a M2 polarizing factor [42] and its potential as a therapeutic target in cancer has been investigated [43]. TGF-β is known for its potential to stimulate angiogenesis, alter the stromal environment, and cause local and systemic immunosuppression via e.g. the induction of M2 macrophages [44,45]. Furthermore, MPM cell lines have been described earlier to produce TGF-β, and high levels in the tumor microenvironment of MPM patients have been reported [20,46]. These observations have led to the recent clinical trial with a TGF-β blocking antibody in MPM patients [47].

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 could greatly enhance the potential of these approaches. More specifically, in MPM we identified TAMs as an important suppressive component and therapeutic target in the pleural cavity.

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.

## AUTHORS'CONTRIBUTIONS

L.L. contributed to the design of the study, performed the experiments, analyzed the data and drafted the manuscript. R.C. contributed to the design of the study, the data analysis and drafting of the manuscript. M.L. and K.B. collaborated in performing the experiments and contributed to drafting of the manuscript. J.H. and J.A. contributed to the design of the study and critical editing of the manuscript. All authors read and approved the final manuscript.

## ACKNOWLEDGEMENT

The authors thank the Mesothelioma Applied Research Foundation – Larry Davis Memorial Grant for their financial support.

## CONFLICT OF INTEREST STATEMENT

None declared.

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# Chapter VI

## Dendritic cell-based immunotherapy in mesothelioma

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***Immunotherapy*** *(2012)* ***4****(10), 1011–1022*

## ABSTRACT

Mesothelioma is a rare thoracic malignancy with dismal prognosis. Current treatment options are scarce and clinical outcomes are rather disappointing. Due to the immunogenic nature of mesothelioma, several studies have investigated immunotherapeutic strategies to improve the prognosis of patients with mesothelioma. In the last decade, progress in knowledge on the modulation of the immune system to attack the tumor is remarkable, but the optimal strategy for immunotherapy has yet to be unraveled. Because of their potent antigen-presenting capacity, dendritic cells are acknowledged as a promising agent in immunotherapeutic approaches in a number of malignancies. In this review, we give an update and provide a future perspective in which immunotherapy may increase the outcome of mesothelioma therapy.

## INTRODUCTION

Asbestos was named by the Ancient Greeks, its name meaning "inextinguishable". It has been said that the Greeks also noted its harmful effects in the first century AD: "sickness of the lungs" was described 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"[1]. 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 an expanding number of articles appeared in medical journals[2–4]. Some authors already suggested a link between inhalation of asbestos fibers and carcinogenesis[5,6]. The term "mesothelioma" was secured in the medical literature in 1931 when Klemperer and Rabin described the distinct features of this diffuse pleural neoplasm[7]. It was, however, not until 1960 that the link between asbestos fibers and mesothelioma became incontrovertible with an article published in *The Lancet* entitled "Primary Malignant Mesothelioma of the Pleura" by Eisenstadt and Wilson[8]. 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[9–12]. Inhaled asbestos fibers within the lung translocate to the pleural space where they cause infiltration of circulating macrophages, which in turn try to phagocytize the inhaled foreign bodies[13]. In the effort to clear these asbestos fibers, reactive oxygen species are generated, causing DNA damage to nearby cells. Subsequently, inflammatory cytokines and recruitment of immune cells to sites of inflammation within the pleura are induced[14–17]. 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 [17], a process often called ''frustrated phagocytosis"[18]. In addition to this pro-carcinogenic and pro-inflammatory substance release, asbestos fibers can sometimes directly penetrate the cells and damage chromosomes. Also, the retained asbestos fibers may adsorb other carcinogens on their surface[19–23]. As a

result DNA alterations occur, such as inactivation of *p16INK4a/p14ARF*, *NF2/Merlin,* and *LATS2*, and the activation of *YAP*[24,25].

In contrast to the increase in knowledge of the etiology of mesothelioma, the treatment options for mesothelioma are still scarce and prognosis is poor, with a median survival of only 9-12 months after diagnosis.

Surgery for mesothelioma is a very controversial subject, with the number of randomized controlled trials being small. Most thoracic surgeons would agree that a macroscopic complete resection for mesothelioma is only possible in a limited number of patients. Whether there is a role for surgery in the other patients is not known. Also, conflicting opinions regarding the optimal surgical procedure exist; in effect, extrapleural pneumonectomy or various forms of pleurectomy/decortication, with the current trend towards more localized resections [26,27].

Chemotherapy is the only treatment that improved survival in randomized controlled trials in mesothelioma patients. This is based on a publication by Vogelzang and colleagues in 2003 in which they compared cisplatin alone to a combination of cisplatin and pemetrexed that resulted in a survival advantage of 3.3 months in the combination therapy arm[28]. 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 cisplatin and raltitrexed compared to cisplatin alone, confirming that a combination of cisplatin and an antifolate is superior to cisplatin alone in patients with mesothelioma[29]. Whether the antifolate/cisplatin combination is the most effective chemotherapeutic option remains uncertain, since no head-to-head chemotherapeutic comparison has been performed in mesothelioma, for example the comparison between the current standard regimen of cisplatin/pemetrexed to cisplatin/raltitrexed, gemcitabine/cisplatin, mitomycin, vindesine/cisplatin or vinorelbine. However, for every individual 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[30,31]. However, most likely due to absence of

sensitizing mutations in the EGFR tyrosine kinase domain, the results of these clinical trials were disappointing[32,33]. Remarkably, in peritoneal mesothelioma, there were reports of novel EGFR mutations with a possible sensitivity to erlotinib[34], but this has been contradicted by others[35]. Among the anti-angiogenic agents, thalidomide is the most extensively studied drug. After numerous previous trials the phase III NVALT 5/MATES (Maintenance Thalidomide in Mesothelioma Patients) trial with thalidomide as switch-maintenance in non-progressive patients after first line pemetrexed chemotherapy could unfortunately not prove a survival advantage[36]. Phase II clinical trials of vascular endothelial growth factor (VEGF) tyrosine kinase inhibitors have shown, at best, modest activity in mesothelioma[37,38]. Bevacizumab, a humanized anti-VEGF antibody, is currently being studied for its use in mesothelioma in addition to chemotherapy in France and Belgium in a phase III trial (ClinicalTrials.gov Identifier: NCT00651456), following several phase II trials with variable results[39]. An increasing amount of preclinical data highlighting the effectiveness of histone deacetylase inhibitors in mesothelioma cell lines and mouse xenograft models has led to a number of early phase clinical trials in patients with mesothelioma[40]. The results of these efforts have led to a multicenter, randomized, placebo-controlled phase III study of the histone deacetylase inhibitor vorinostat in patients with advanced mesothelioma which did not improve survival compared with placebo as second-line therapy for mesothelioma[41]. In conclusion, there are no promising chemotherapeutic or targeted agents at the horizon for patients with mesothelioma. Clearly, there is a need for new approaches in the treatment of mesothelioma.

Sporadically, a mesothelioma patient has a tumor that regresses spontaneously. This observation is ascribed to the immune system that may evoke a clinical response in mesothelioma patients under some circumstances[42–44]. Mesothelioma is indeed an immunogenic cancer and can induce immune recognition, immune cell infiltration, and immune-mediated killing, the extent of which may define disease prognosis. As early as in 1982, the impact of T cell infiltration on survival in mesothelioma patients was demonstrated, showing a positive correlation between T cells and increased survival[45]. More recently, subtyping of T cells showed that high frequencies of CD8+ tumor infiltrating T cells had a positive effect on progression-free and overall survival,

while increased frequencies of CD4+CD25+ regulatory T cells and CD45RO+ memory T cells tended to be negative prognostic indicators[46]. Higher frequencies of infiltrating CD3+ T cells correlated with worse overall survival in patients having mesothelioma with sarcomatoid or biphasic histology[47]. In addition to the immunogenic characteristics of the tumor, exposure to asbestos fibers also has significant negative direct effects on several components of the immune system[48]. These findings indicate that people exposed to asbestos fibers possess reduced tumor immunity, making them more sensitive to cancer development. In summary, understanding the immune system and developing mechanisms to activate it or to overcome the immune suppression could prove beneficial to the patient; a therapeutic strategy called immunotherapy. In this review, we discuss the progress of immunotherapy in mesothelioma over the years and focus on dendritic-cell based immunotherapy, since the stimulation of these powerful antigen presenting cells appears to be a very effective method of inducing an anti-tumor response.

*Immunotherapy in mesothelioma*

The first attempts to activate the immune system in mesothelioma were published 30 years ago with the Bacillus Calmette-Guérin (BCG) vaccine trial that favored a non-specific activation of the immune response[49,50]. The use of interleukin-2 to stimulate the immune response was investigated more than 10 years later[51,52], and other activators of the immune response, like granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-α2a and interferon-γ, followed[53–55]. However, these therapeutic approaches have been abandoned because of lack of efficacy or unacceptable toxicity.

Due to its location in the pleural cavity, the possibility of local vector administration to apply immunotherapy via gene transfer appears to be an attractive strategy in mesothelioma. In a recently published pilot and feasibility trial using an adenovirus vector expressing a homologous type 1 human interferon gene (IFN−a2b), antitumor humoral immune responses against mesothelioma cell lines were seen in seven of the eight subjects evaluated. Furthermore, evidence of disease stability or tumor regression was seen in the remaining five patients, including one partial tumor regression at sites not contiguous with vector infusion[56].

Preclinical studies targeting mesothelin, a differentiation antigen present on normal mesothelial cells and overexpressed in several human tumors, including mesothelioma, as well as ovarian and pancreatic adenocarcinoma[57], with immunotoxins CAT-5001 (formerly SS1P) and amatuximab (previously known as MORab-009) were promising[57–59]. Unfortunately, in clinical trials CAT-5001 showed only modest clinical responses in mesothelioma patients[58,59] and amatuximab failed to demonstrate any radiological responses in a phase I trial in mesothelioma and other cancer types[60]. Preclinical studies demonstrated significant anti-tumor efficacy using a combination of amatuximab and chemotherapy treatment[61], justifying a multicenter Phase II clinical trial utilizing cisplatin/pemetrexed with amatuximab in mesothelioma patients. The preliminary outcomes of this trial were recently presented and showed that amatuximab in combination with chemotherapy resulted in 90% of patients having an objective tumor response or stable disease[62]. However, progression-free survival was not significantly different from historical results of patients treated with chemotherapy only. More recently, a phase I study of SS1(dsFv) PE38, a recombinant antimesothelin immunotoxin was commenced which is on going at this moment (ClinicalTrials.gov Identifier: NCT00575770).



In addition to the agents mentioned above, it is also possible to use immune cells for immunotherapy in mesothelioma. One approach is to make use of lentiviral or retroviral vectors to transduce T cells with modified T-cell receptors engineered to attack specific tumor antigens[63]. Preclinical results of this method are promising and this approach will proceed to a clinical trial at the University of Pennsylvania (USA)[64]. Adoptive transfer of lymphocytes with tumoricidal properties can, in theory, bypass the daunting task of breaking tolerance to tumor antigens and generating a high frequency of high-avidity effector T cells. In a preclinical mesothelioma model, tumor-reactive T cells expressing chimeric antigen receptors (CAR) were found to mediate regression of the tumor[65].

Another approach is to stimulate the antigen presenting cells, which in turn can induce a specific T cell anti-tumor response. In this field dendritic cell-based therapy has proven itself very promising. The present authors have recently published the results of a clinical with dendritic cell based immunotherapy in mesothelioma[66].

*Dendritic cells*

Dendritic cells (DCs) were first described by Steinman, a discovery for which he was rewarded the Nobel Prize in 2011[67]. These cells are widely acknowledged as the central surveillance cell type and play a pivotal role in the initiation and programming of tumor-specific T-cell responses[67–70]. DCs are the most potent antigen-presenting cells specialized in inducing activation and proliferation of $CD8^+$ cytotoxic T lymphocytes (CTLs) and helper $CD4^+$ lymphocytes. DCs originate from bone marrow precursors and migrate to peripheral tissues, where they differentiate into immature DCs (iDCs). iDCs capture tumor-associated antigens (TAAs) and start migrating via lymphatic vessels to regional lymphoid organs. This migration is coordinated by chemokines and their receptors, matrix molecules and adhesion molecules on DCs, as well as the surrounding tissues. DCs mature en route; activating their ability to convert antigens to 10- to 15-mer peptides bound to major histocompatibility complex (MHC) class I and class II molecules. Mature DCs upregulate production of surface co-stimulatory molecules (e.g. CD80 and CD86) and cytokines needed to stimulate lymphocytes in the tumor-draining lymph nodes. DCs present tumor antigens to naive CTLs amongst other cells, inducing anti-tumor immune responses[71–74].

However, tumors induce a microenvironment that interferes with the natural development and function of DCs by a number of mechanisms (figure 1).

- A growing tumor may outgrow its blood supply, leaving portions of the tumor milieu deprived of adequate oxygen supply (hypoxia). Under these conditions the expression of matrix-metalloproteases and the migratory activity of DCs is suppressed[75,76].
- Tumor metabolites such as lactic acid, arachidonic acid metabolites (prostanoids), and gangliosides contribute to DC dysfunction[77–79].
- The list of tumor-derived cytokines and chemokines is constantly growing and includes, but is not limited to, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-15, VEGF, TFG-β, TNF-α, EGF, FGF, HGF, MIP[80], of which IL-10 and TGF-β seem to be the best characterized tumor-derived cytokines with well-defined immunosuppressive function.

- Besides the induction of defective DC function, tumor-induced factors can also skew the differentiation of monocytes/DCs toward alternatively activated macrophages and endothelial-like cells[81–83].
- Tregs - immune cells that are abundantly present in the tumor microenvironment - can impede DC function.

Cumulatively, these mechanisms result in DCs that express substantially lower levels of MHC class II molecules, adhesion molecules, and costimulatory molecules than under normal conditions, and which are consistent with the phenotype of nonactivated DCs[84,85]. These tolerogenic DCs (tDCs) are impaired in their ability to phagocytose antigen and to stimulate T cells. They also contribute to the recruitment, expansion, and function of Tregs, leading to a defective induction of antitumor responses[86].

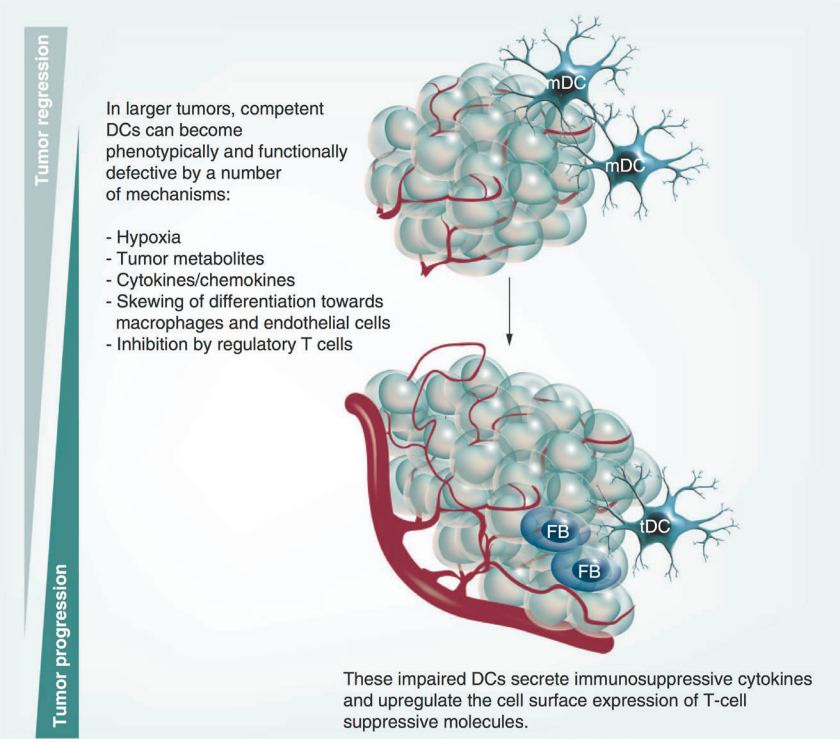


**Figure 1: Impairment of dendritic cell activity by tumor environment**
mDC are capable of inducing an antitumor response in small tumors. However, when tumors grow and establish a tumor microenvironment, several factors impair the functions of the tDCs.
DC: Dendritic Cell; FB: Fibroblast; mDC: Mature dendritic cell; tDC Tolerogenic dendritic cell.

*First preclinical DC-based immunotherapy*

In 2004, the research group of Gregoire published a pioneering article in which they used DC based immunotherapy in a human mesothelioma model[87]. By using dead cells (necrotic or apoptotic lysate) for the loading of DCs, the cells were exposed to a full array of antigenic peptides that rapidly gain access to both MHC Class I (cross-presentation) and MHC Class II pathways, therefore leading to a diversified immune response involving CTLs as well as $CD4^+$ T helper cells. In their paper, the authors successfully demonstrated *in vitro* culture and antigen loading of DCs in a human mesothelioma model, resulting in a specific CTL response. Heat shocking the tumor cells before apoptosis induction was required to induce potent cross-priming of CTLs with antitumor activity.

In 2005, the present authors' group published the first trial on DC-based immunotherapy of mesothelioma in a murine model[88]. This was a peritoneal tumor model using the AB-1 tumor cell line. DCs were cultured and loaded with tumor lysate and vaccinations were given at different time points in relation to tumor inoculation. Immunization with tumor lysate–pulsed DCs before a lethal tumor implantation prevented mesothelioma outgrowth; mice receiving tumor lysate–pulsed DCs were protected for months and even resisted a secondary challenge with tumor, illustrating the induction of long-lived immunity by using DC-based immunotherapy. Also, immunization with tumor lysate–pulsed DCs after tumor implantation reduced mesothelioma growth, depending on the method of DC-maturation and tumor load. In contrast with the curative effect when tumor lysate–pulsed DCs were given before or 1 and 8 days after tumor challenge, immunization with tumor lysate–pulsed DCs at the day of tumor implantation promoted mesothelioma outgrowth and poor prognosis occurred. The observation of a paradoxical tumor-enhancing effect of simultaneous administration of DCs may be caused by several factors. First, high levels of cytokines or soluble mediators produced by mesothelioma cells could down regulate cellular immune responses induced by DCs. Next, tumor cells might cluster with DCs, which, through their highly motile nature, might lead to more widespread dissemination and attachment of cancer cells to the mesothelial surface. Finally, if DCs are mixed with tumor cells *in vivo* it has been shown that DCs can transform into endothelial cells, thus enhancing tumor vasculogenesis and

tumor growth[89]. Successful tumor lysate–pulsed DC immunotherapy was associated with cytotoxic T-cell induction and even transfer of splenocytes or $CD8^+$ T cells from surviving mice receiving DC immunotherapy transfers tumor protection for tumor-bearing mice. DC vaccinations had a better outcome when DCs were injected early in tumor development, indicating that tumor load played an important role in survival. Although the potency of immunotherapy treatment decreased when DCs were injected later, mice still showed an improved prognosis compared with no treatment, but eventually tumors escaped immune surveillance and all mice died. It is now well established that larger tumor mass is associated with an immunosuppressive milieu that has the capacity to suppress the effector arm of the anti-tumoral immune response (CTL response inside the tumor) and the inductive arm of the immune response (i.e., the potential of antigen-presenting DCs to induce CTL responses)[90].

*First clinical trial*

The research group of Robinson published a trial in 2006, in which they used an autologous tumor lysate vaccine that was manufactured from surgically resected tumor and administered subcutaneously together with GM-CSF. GM-CSF stimulates antigen-presenting cells (APC) *in vivo*, which in turn presents TAAs and thereby generate tumor-specific immunity. A total of 22 patients were enrolled in the trial. Of these, five developed positive delayed type hypersensitivity skin tests and five showed evidence of altered antibody specificities by western blotting, proving that tumor lysate plus GM-CSF could induce tumor specific immunity, both cellular and humoral. Of these patients, 32% developed at least one type of immune response against mesothelioma tumor cells[91]. *In vivo* stimulation of APCs is an attractive method; however, it remains important to determine whether the activation signals might actually polarize the DCs in the desired manner. For example, engaging dendritic cell asialoglycoprotein receptor induces DCs to secrete IL-10, which polarizes T cells into IL-10-secreting suppressor T cells, which in turn might negatively affect tumor-specific effector T cells[92]. Furthermore, the tumor microenvironment interferes with the stimulation of DCs, as is discussed above. Therefore, *ex vivo* culture and antigen-loading of DCs, while demanding more labor, seems preferable. In this way DCs can be cultured and matured *in vitro* without the immunosuppressive effect of the tumor.

Also, the loading of the TAAs can be performed in a controlled situation to make use of the full potential of these cells.

On the basis of their preclinical animal studies, the present authors have performed the first clinical trial in which autologous tumor lysate–pulsed DCs were administrated to mesothelioma patients[66]. In this clinical trial, patients were eligible for the study when sufficient tumor cells could be obtained from pleural effusion or tumor biopsy material at the time of diagnosis. DC-immunotherapy was planned after completion of the cytoreductive chemotherapy provided that during chemotherapy no major side effects occurred and there was no progressive disease. DCs were obtained using concentrated leukocyte fractions that were generated through peripheral blood leukapheresis[93]. Large numbers of DCs were generated *ex vivo*, in the absence of the suppressing tumor milieu, and subsequently loaded them with a preparation of autologous tumor antigens and keyhole limpet hemocyanin (KLH) as positive control. Mature DCs were injected three times with a 2-week interval both intravenously (distribution to the liver, spleen and bone marrow) and intradermally from where they migrate to the regional lymph nodes (figure 2). In this way, these DCs could maximally stimulate cytotoxic T cells, B cells, T cells, NK cells and NKT cells that are essential for tumor lysis.

Overall, the vaccination regimen with loaded DCs was well tolerated in all patients and no common toxicity criteria grade 3 or 4 toxicities were reported. A local skin rash occurred at the site of the intradermal injection after the first vaccination in 8 of the 10 patients. Subsequent vaccinations (second and third) gave a quicker and increased induration and erythema in all patients suggesting that some form of immunity was induced. Most patients developed mild-to-moderate flu-like symptoms after the vaccination, particularly fever, muscle aches, chills, and tiredness, these symptoms normalized after one day. Since it was a principle-of-proof study, no conclusions can be drawn regarding improvement of the progression-free survival or overall survival. To assess the T-cell capacity for cell lysis, flow cytometric detection of $CD3^+CD8^+$ T cells expressing granzyme B per $CD8^+$ T cell was used. Nine patients showed a significantly increased percentage of granzyme $B^+$ $CD8^+$ T cells after vaccination and the granzyme B expression per $CD8^+$ cell was increased in most patients. Furthermore, radioactive

chromium release assays were performed in 6 of 10 patients from whom pleural fluid was obtained. In four patients a clear induction of cytotoxicity against autologous tumor cells was measured. The cytotoxicity levels of one patient increased after every vaccination; for the other three patients three vaccinations were necessary to induce cytotoxicity. In addition, serum samples from all patients showed a significant increase of e antibodies reactive to KLH postvaccination, both of the immunoglobulin (Ig)G and IgM isotype. The response remained at the same level for several months after the last DC injection and gradually decreased after 6-12 months. This proves that a successful immunoreaction was induced by the DC vaccinations. In conclusion, administration of DCs loaded with autologous tumor cell lysate to patients was safe and feasible and no significant adverse effects were observed.

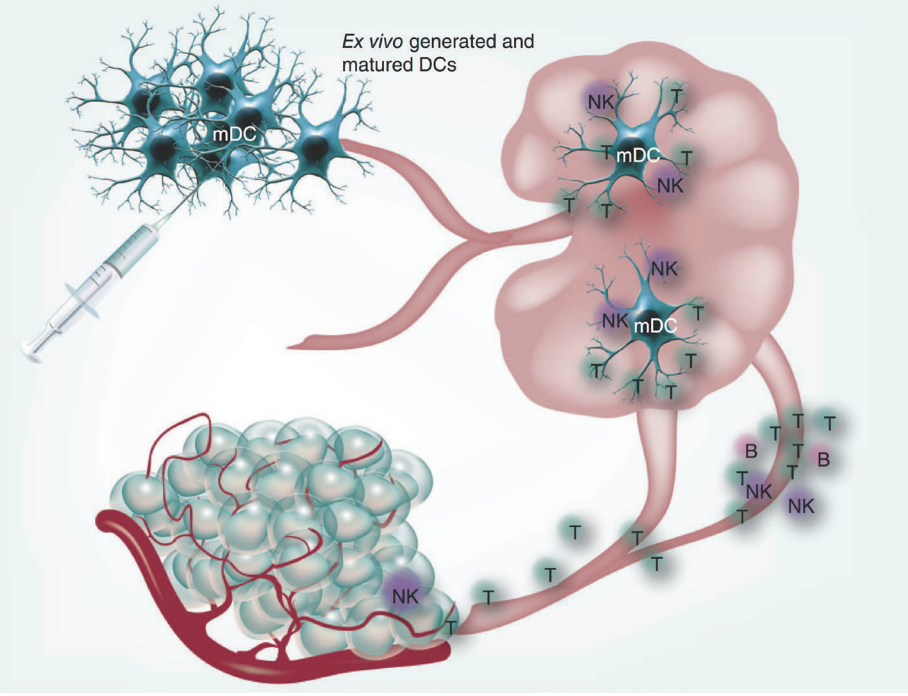


**Figure 2** administration of *ex vivo* maturated autologous DCs into a patient, resulting in antigen presentation in the lymph node and a specific anti-tumor cytotoxic anti-tumor response.
B: B cell; DC: Dendritic cell; mDC: Mature dendritic cell; NK: Natural killer cell; T: cytotoxic T cell

*Future developments in DC-based immunotherapy*

There is still room for improvement in DC production, either *ex vivo* or *in vivo*. The most commonly used approach to harvest DCs for immunotherapy is to use the differentiated DCs from peripheral blood mononuclear cells (PBMCs) obtained from whole blood or by a leukapheresis procedure. However, DCs can also be propagated from $CD34^+$ precursors. $CD34^+$ precursors are first mobilized from the bone marrow by treatment of patients with GM-CSF prior to leukapheresis procedures[94]. In addition, DCs can also be directly isolated from circulating DCs. Circulating DC subsets comprise less than 1% of PBMCs. *In vivo* expansion of these rare cells can be achieved by administration of hemopoietic growth factors such as Flt3L followed by leukapheresis[95]. For a more elaborate description of DC subsets, the review by Liu and Nussenzweig is recommended[96]. All of these methods for generating DCs are currently used in clinical trials, but there is no consensus on the optimal method of generating DCs for immunotherapy use[97,98].

A novel strategy for loading antigens involves the direct targeting of antigens to DCs *in vivo* to induce tumor-specific immune responses[99]. Although the limitations have been mentioned above, *in vivo* targeting of DCs represents an option for DC immunotherapy as it bypasses the expensive and labor-intensive *ex vivo* DC generation process. Vaccines may be produced on a larger scale and at a lower cost than an *ex vivo* cultured DC vaccine. *In vivo* targeting also allows for the stimulation of natural DC subsets at multiple sites *in vivo*. Newer approaches involve the targeting of DC-specific molecules. Candidate receptors for stimulation and maturation of DCs include Fc receptors, CD40 and C-type lectin receptors (CLRs)[97]. However, further studies are still required to translate this new strategy to clinical applications in humans.

Improving maturation of DCs also has the potential to improve the efficacy of the immunoresponse; recently, it has been shown that *in vitro* sequential DC maturation can be beneficial[100]. This method tries to mimic the *in vivo* situation in which DCs exposed at the periphery to maturation stimuli migrate to lymph nodes, where they receive secondary signals from $CD4^+$ T-helper cells. It was shown that a sequential activation with activated $CD4^+$ T cells dramatically increased the maturation of DCs in terms of their phenotype and cytokine secretion com- pared with DCs activated

with maturation stimuli delivered simultaneously[100]. Furthermore, this sequential maturation led to the induction of CTLs with long-term effector and central memory phenotypes.

Either *ex vivo* or *in vivo*, the most optimal method of DC production has to be established, the question remains whether efficacy will be enhanced due to optimization of this method, because immunomonitoring of the present authors' clinical trial and those using other DC vaccines have demonstrated that these cells are now sufficiently powerful to be used in clinical trials[101].

Besides DC production, the method of antigen loading is one of great debate; the ideal target for cancer immunotherapy would be a tumor associated antigen (TAA) that is exclusively expressed in all tumor cells, but not in normal tissues in order to avoid potential induction of autoimmunity. In addition, the TAA should be important for tumor growth and survival, so that downregulation to escape the immunotherapeutic effect of the vaccine is impossible. Most TAAs are self-derived proteins and thus *in vivo* poorly immunogenic, certainly keeping in mind the concept of the immunosuppressive environment of the tumor. Nevertheless, DCs loaded with these antigens can be used to initiate antigen-specific T-cell responses. In recent years a large number of strategies have been developed to deliver TAAs to DCs, using defined epitopes, specific TAAs, apoptotic whole-cell suspensions, necrotic cell lysates or cellular DNA or mRNA and employing both viral and nonviral techniques[102,103].

In the present authors' study and in others in mesothelioma whole-cell material is used. The need for patients' tumor material for antigen loading of the DCs unfortunately results in patients being excluded from these trials if there is an inability to collect sufficient tissue samples. The University Hospital of Antwerp (Belgium) has started a trial of DC-based immunotherapy in mesothelioma and several other solid tumors, using WT-1 as antigen loading for the DCs (ClinicalTrials.gov Identifier: NCT01291420), circumventing the need for patients' tumor material. In the present authors' view, this approach limits the antitumor response to a single peptide, making it obligatory for the tumor to significantly express this peptide in order for the immunotherapy to be effective. In addition, it is becoming clear that most tumors consist of different clones of tumor cells expressing different TAAs. Elimination of one clone does not prevent outgrowth of another.

But even when the preferable method of DC preparation and antigen-loading has been established, immunotherapy has to overcome an immunosuppressive environment caused by the tumors' recruitment of suppressive cell types that inhibit an effective antitumor response, among which are myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) of M2 phenotype, and Tregs. Dampening of this immunosuppression through various methods of cell modulation might be an important key to increase the efficacy of DC-based immunotherapy.

MDSCs are increased in cancer patients and play a suppressive role in the innate and adaptive immune responses to cancer. The present authors have recently shown the role of MDSC in DC immunotherapy and several strategies are being studied to counteract this immunosuppression, for example gemcitabine, 5-fluorouracil (5FU), celecoxib or sunitinib[104–106].

TAMs are a major component of the leukocyte infiltrate in the tumor micro-environment and are described as key orchestrators of cancer-related inflammation[108]. Evidence is accumulating on their role in tumor initiation, progression and metastasis[109]. M2 TAMs are considered as 'alternatively activated' macrophages and have a different phenotype compared to the 'classically activated' macrophages. Classically activated macrophages (M1 macrophages) are characterized by the expression of high levels of pro-inflammatory cytokines and reactive oxygen species and have anti-tumor activity. By contrast, alternatively activated macrophages (M2 macrophages) are considered to be involved in tissue remodeling and wound healing and are able to suppress the adoptive immune response through various mechanisms and contribute to angiogenesis and tumor invasiveness[110]. Suppressing these M2 TAMS might prove crucial to improve the efficacy of immunotherapy; zoledronic acid, the anti-IL-6 antibody siltuximab, antibodies against CD40 and antagonists of CSF-1 receptor (CSF-1R) are candidates for suppression of these TAMs[111–115].

Tregs are a population of $CD4^+$ T cells with a central role in the prevention of autoimmunity and the promotion of tolerance via their suppressive function on a broad repertoire of cellular targets and have several pathways that limit anti-tumor response[116]. An engineered recombinant fusion protein of IL-2 and diphtheria toxin and other CD25-directed immunotoxins, low-dose cyclophosphamide (CTX) inhibition of p300 function using a small molecule inhibitor, sorafinib and anti-CCL2/

CCL12 monoclonal antibodies have been investigated for Treg depletion[117–124].

Another method that is being extensively studied is to enhance the antitumor immune response by the blockade of immune checkpoints. Immune checkpoints refer to a plethora of inhibitory pathways hardwired into the immune system that are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues in order to minimize collateral tissue damage. It is now clear that tumors co-opt certain immune-checkpoint pathways as a major mechanism of immune resistance, particularly against T cells that are specific for tumor antigens. Because many of the immune checkpoints are initiated by ligand–receptor interactions, they can be readily blocked by antibodies or modulated by recombinant forms of ligands or receptors. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) antibodies were the first of this class of immunotherapeutics to achieve a survival benefit in a phase III trial in melanoma[125], but several blockers of additional immune checkpoint proteins, such as programmed cell death protein 1 (PD1), are now being studied[126].



## CONCLUSION

The role of the immune system in mesothelioma is vast. In malignant diseases, progress in modulating the immune system has been slow at first, but more recently, immunotherapy has taken a flight. In mesothelioma, multiple strategies are currently being tested and many combinations of therapeutic options await research, with DC-based therapy being one of the most exciting options in our view.

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# Chapter VII

## Sustained tumor control after dendritic cell vaccination with low dose cyclophosphamide as adjuvant treatment in patients with malignant pleural mesothelioma

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*Submitted for publication*

## ABSTRACT

*Background*: Earlier we have demonstrated that autologous tumor lysate-pulsed dendritic cell-based immunotherapy in malignant pleural mesothelioma (MPM) patients is feasible, well-tolerated, and capable of inducing immunological responses to tumor cells. However, to our opinion, the full therapeutic potential of immunotherapy has not yet been fully exploited in cancer. Reducing the initial tumor burden and overcoming the tumor-induced immune suppression may open up two exciting new options to improve immunotherapy and increase the efficacy for patients. The aim of this study was to assess the immunological responses induced by the administration of tumor lysate-pulsed dendritic cells in consolidation therapy of MPM patients ministered under reduced tumor-induced immune suppression conditions.

*Methods*: Five MPM patients after chemotherapy and five patients after chemotherapy followed by pleurectomy/decortication received metronomic cyclophosphamide (100 mg/day alternating for four weeks) and three to five vaccinations of clinical-grade autologous dendritic cells intradermally and intravenously at two-week intervals. Each vaccine was composed of $50x10^6$ mature dendritic cells pulsed with autologous tumor lysate and keyhole limpet hemocyanin (KLH) as surrogate marker. Delayed-type hypersensitivity activity to tumor antigens and KLH was assessed, both in vivo and in vitro. Peripheral blood mononuclear cells during the treatment were analyzed for immunological responses.

*Results*: Administration of dendritic cells pulsed with autologous tumor lysate with cyclophosphamide in MPM patients for consolidation therapy was safe with moderate fever as the only side effect. No related grade 3 or 4 toxicities associated with the combination treatment or any evidence of autoimmunity was detected. The addition of CTX decreased he percentage of $^{CD3+CD4+CD25+FoxP3+CD127-}$Treg of total CD4 cells from 9.43 (range 4.34-26.10) before CTX to 4.51 (range 0.27-10.30) (P=0.02), independent of initial Treg numbers. DC vaccination therapy combined with CTX resulted in radiographical disease control in eight of the ten patients. Overall survival was promising, with seven out of ten patients having a survival of ≥ 24 months and two patients still alive after 44 and 60 months.

*Conclusions*: This study demonstrated that consolidation therapy with autologous tumor lysate-pulsed dendritic cell-based therapy and simultaneously reducing the tumor-induced immune suppression is well-tolerated, and capable of inducing immunological response to tumor cells in MPM patients. This combination resulted in sustained tumor control in the majority of patients.

## INTRODUCTION

Malignant pleural mesothelioma (MPM) is a highly lethal neoplasm with limited treatment options. Despite aggressive treatments like combination treatment with surgery, chemotherapy, and radiotherapy, it is almost inevitably accompanied with recurrences. However, remarkably, long-term survivors do occur either with or without any treatment[1,2]. Recently the role of the immune system in mesothelioma has regained new insights, which may explain the differences in behavior of the disease but also open new potentials for treatment[3].

Earlier we performed a phase I clinical trial using active immunotherapy in ten patients treated with chemotherapy followed by three vaccinations of autologous tumor lysate-pulsed monocyte-derived dendritic cells (DC)[4]. Results showed that these vaccines were well tolerated without systemic toxicity and radiographical tumor responses were established. We were also able to determine distinct immune and cytotoxic T lymphocyte antitumor activity in the peripheral blood of the patients.

It is now more recognized that the efficacy of immunotherapy is influenced by the immunosuppressive environment created by the tumor[5]. Harnessing the potency of the immune system by immunoactivating treatments is hampered by the presence of this immunosuppressive environment. A number of immunosuppressive cytokines and cells have been described in malignant diseases. We, among others, have determined that regulatory T-cells (Tregs) play a major role in mesothelioma[6,7]. Regulatory T-cells contribute to an impaired T cell function.

Clinical studies have shown that low dose cyclophosphamide (CTX) induces beneficial immunomodulatory effects in the context of active or adoptive immunotherapy[8,9]. One of the mechanisms underlying these modulations is by reducing Tregs numbers and their functionality. We found that prolonged (metronomic) low-dose CTX augmented the anti-tumor effects of DC vaccines and increased survival via a reduction in the number of Treg in our murine model of mesothelioma[10].

In this study, we investigated whether these findings could be confirmed in patients. Ten patients with MPM previously treated with chemotherapy or chemotherapy and debulking surgery were treated as a maintenance treatment with CTX combined with DC-based immunotherapy. The aim was to define the

safety and toxicity of the maintenance therapy in these two groups and to observe and document anti-cancer activity by laboratory and clinical evaluation. We found that CTX combined with DC-immunotherapy was feasible and safe. The addition of metronomic CTX reduced regulatory T-cells. Significant increases in immunoactivity against the tumor were established and also overall survival was encouraging with seven patients surviving ≥24 months.

## MATERIALS AND METHODS

*Study design*

The study was approved by the institutional ethical committee of the Erasmus MC (MEC-2008-109) and the Central Committee on Research involving Human Subjects (CCMO; NL24050-000-08) as defined by the WMO (Medical Research Involving Human Subjects Act). Procedures followed were in accordance with the ethical standards of these committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The study is registered at http://www.clinicaltrials.gov with identifier NCT01241682.

Patients with suspicion of MPM or treatment naive patients who have already been diagnosed with MPM, with a medical need for a pleurodesis, gave informed consent to isolate and store pleural effusion cells. Patients underwent standard treatment consisting of 4-6 courses of chemotherapy with either pemetrexed-cisplatin or pemetrexed-carboplatin. Response assessment was done according to modified RECIST criteria[11]. In case of progressive disease after chemotherapy, patients were excluded from participation and treated according to clinicians and patient's decision. It is general practice in our reference center to consider patients for a pleurectomy/decortication (P/D) only after completion of chemotherapy, in case of persistent complaints, e.g. dyspnea, related to tumor load. P/D is only performed in patients fit for operation in which a macroscopic debulking is considered feasible according to published guidelines. Patients with either partial or stable disease were evaluated for study treatment with DC.

DC-immunotherapy in combination low dose CTX was planned 8 to 10 weeks after completion of the cytoreductive therapies, thus either chemotherapy or

surgery (figure 1). From all participants, blood and serum samples were taken at regular intervals during immunotherapy. Blood was tested for immunological responses, liver, and renal functioning. In addition, serum samples were screened for the development of auto-immunity. Overall survival was stated as survival after date of diagnosis. Final survival data was gathered in Februari 2014.

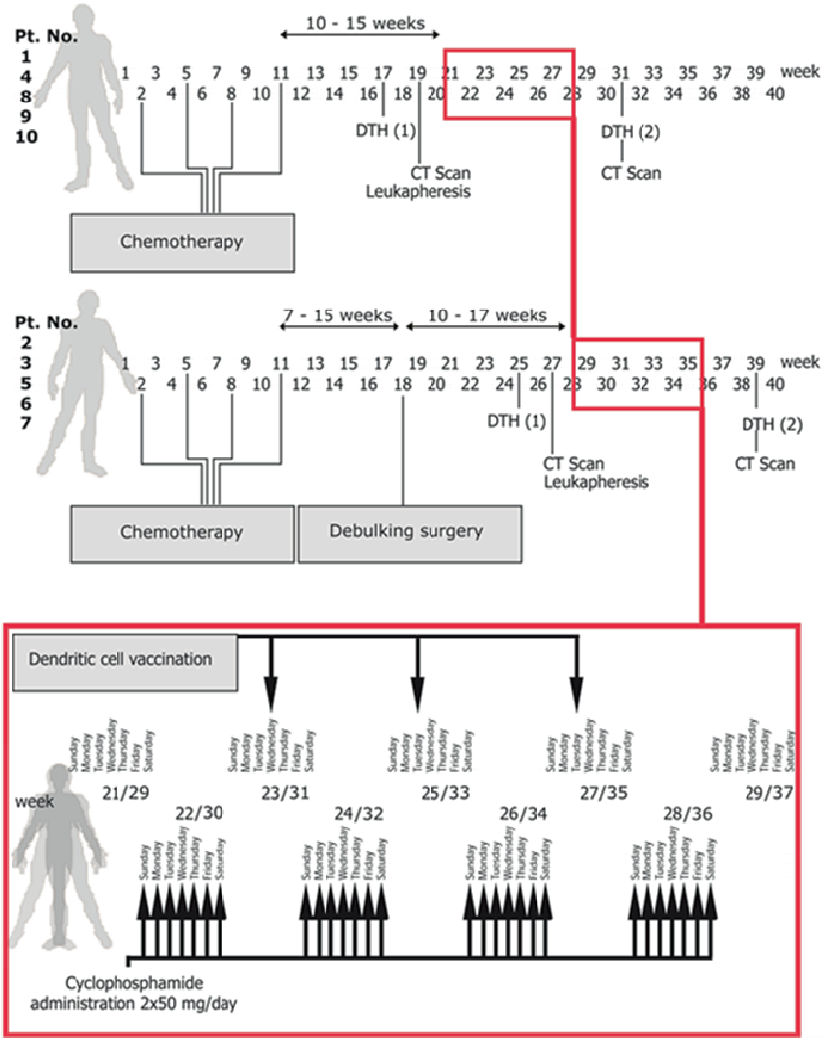


**Figure 1:** Schematic representation of the treatment procedure. A 4th and 5th vaccination, after 6 or 12 months after the last DC vaccine, was given if enough dendritic cells were available.

The study endpoints were (1) to assess the effect of cyclophosphamide on immunological parameters (cytotoxic T cells and regulatory T cells in the blood of patients) and (2) to assess safety, toxicity, and efficacy of the combination therapy of DC vaccinations and CTX in MPM patients.

*Patient eligibility*

Signed written informed consent was obtained from each patient. At the time of diagnosis, patients with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, with acceptable vital organ functions were included. Patients were eligible for the study when sufficient (> $150x10^6$) tumor cells could be obtained from pleural effusion or tumor biopsy material at the time of diagnosis. Serum of all patients was tested negatively for infectious agents (human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV) I&II, hepatitis B virus (HBV), and hepatitis C virus (HCV)). No signs of autoimmune disease were detectable in these patients by testing for antinuclear antibodies (ANA), extractable nuclear antigens (ENA), and rheumatoid factors (RF). Delayed type hypersensitivity (DTH) skin tests were performed to investigate if the chemotherapeutic agents still exert their influence on the patients' immune system. A solution of 3.75 Lf of purified tetanus toxoid (NVI, Bilthoven, The Netherlands) was used as positive control and 25 µl 0.9% saline as negative control. Reactivity to tetanus toxoid was measured in all ten patients 48 hours after subcutaneous injection (induration > 5 mm) and was considered as evidence for cellular immunocompetence.

*Preparation of tumor lysate for DC loading*

Isolation and preparation of autologous tumor lysate for DC loading was prepared as described earlier[4]. Tumor tissue (cases 2, 3, 5, 6, and 7) was removed by debulking surgery (pleurectomy/decortication) and placed in phosphate-buffered saline (PBS) in sterile containers and transported immediately to the cleanroom facility. Representative tumor pieces were embedded in Tissue-Tek II optimum cutting temperature ([OCT] Miles, Naperville, IL, USA), snap-frozen and stored at –80 °C. Remaining tumor tissue was dispersed to create a single cell suspension. In case of pleural effusions (cases 1, 4, 8, 9, and 10), fluid was aspirated and collected in

sterile flasks without anticoagulant or other additives. Effusions were transported immediately to the cleanroom facility and centrifuged at 400xg for 15 min at room temperature (RT). If necessary small or large amounts of red blood cells in the cell pellet were removed by hypotonic lysis using sterile water or Ficoll-Paque PREMIUM, respectively. Cells were counted and resuspended at a concentration of $50x10^6$ / ml in PBS. Cytospin preparations and/or tumor sections were prepared and examined for the presence of tumor cells using the following antibodies: cytokeratin 5/6, cytokeratin 19, thrombomodulin, N-cadherin, vimentin, HBME-1, calretinin, and Wilms' tumor 1 (WT-1) protein (all DAKO, Glostrup, Denmark). Total amounts of malignant cells exceeded $150x10^6$ cells and the percentage was at least 30% of total cells (inclusion criteria) or in the case of biopsy material, total wet weight was at least 0.2 gram showing >30% positivity for tumor markers. Remaining cells were lysed by six cycles of freezing in liquid nitrogen and thawing at room temperature followed by 100 Gy of irradiation. Large particles were removed by centrifugation (5 min, 200xg), and supernatants were passed through a 0.45 µm filter. The resulting tumor lysates were stored in aliquots at –80°C until use.



*Dendritic cell culture*

We used our previously described method to generate clinical-grade mature dendritic cells in conformity with Good Manufacturing Practice (GMP) guidelines[4]. In brief, concentrated 120 to 150 ml leukocyte fractions were generated through a 4-h restricted peripheral blood leukapheresis, processing on average 9L of blood (COBE Spectra Apheresis System, Gambro BCT, Zaventem, Belgium). Peripheral blood mononuclear cells were then enriched using counter-flow centrifugal elutriation (Elutra, Gambro BCT, Zaventem, Belgium) as described by *Berger et al*.[12]. In 60% of the cases the percentages of contaminating granulocytes in the enriched monocyte fractions after counter-flow centrifugal elutriation were below 10%. A density gradient centrifugation was routinely performed on all samples to remove the granulocytes before the culturing process. In this way, the percentages of granulocytes at the start of the procedure were always below 8% for all preparations. Monocytes were resuspended at a concentration of $5x10^6$ cells/ml in XVIVO-15 (Cambrex Bio Science, Verviers, Belgium) supplemented with 2% pooled human AB serum (DC-culture

medium [DC-CM]). The next day, half of the medium was removed and replaced by the same volume of DC-CM supplemented with 1000 IU/ml interleukin (IL)-4 (CellGenix, Freiburg, Germany) and 1600 IU/ml granulocyte macrophage-colony stimulating factor (GM-CSF; CellGenix). After 5 days of culture, semi-adherent and non-adherent cells were harvested by pipetting. Cells ($1x10^6$) were seeded per well of a 6-well plate in fresh DC-CM supplemented with tumor cell lysate (1 tumor cell equivalent to 3 DC), 500 IU/ml IL-4, 800 IU/ml GM-CSF, and 10 µg/ml keyhole limpet hemocyanin (KLH) (Calbiochem, La Jolla, CA, USA). The co-loading with the protein KLH, a foreign protein that stimulates T-helper responses, was used to monitor the immune competence. On day 8, the maturation cocktail was added (prostaglandin E2 [PGE2 Prostin E2; 10 µg/ml Pharmacia&Upjohn, Puurs, Belgium], tumor necrosis factor-alpha [TNF-α, 20 ng/ml), interleukin (IL)-1β [5 ng/ml], and IL-6 [15 ng/ml; all CellGenix]). Cells were harvested at day 10 and $50x10^6$ cells used for immediate vaccination; remaining cells were cryopreserved in DMSO for later vaccinations ($55x10^6$ cells per vial) and for DTH skin testing.

*Flow cytometric analysis of clinical-grade DC*

An aliquot of the vaccine preparation was retained to examine the expression of extracellular markers. The following monoclonal antibodies were purchased from BD Biosciences / BD Pharmingen (Erembodegem, Belgium): FITC-conjugated CD86 and CD195, PE-conjugated CD83 and CD-95, CD80 - PE-Cy5, CD209 - PerCP-Cy5.5, CD11c - APC, and APC-Cy7 conjugated HLA-DR. The specificity of the antibodies was checked using equivalent concentrations of fluorochrome- and isotype-matched negative control immunoglobulins. Cells were washed with FACS buffer (PBS supplemented with 0.25 % BSA, 0.5 mM EDTA, and 0.05% sodium azide) and counted. At least $0.4x10^6$ cells in 100 µl were stained with appropriate dilutions of antibodies. Cells were incubated on ice for 30 min in the dark, washed twice with FACS buffer and analyses by LSR flow cytometry (BD Biosciences). Release criteria for each batch of DC were sterility testing (negative for aerobic or anaerobic microorganisms), viability (>80% viable by flowcytometry and propidium iodide or 7-aminoactinomycin D [7-AAD]), purity (>95% $CD11c^+$ MHC class $II^+$), maturation (>60% of CD80 expression) and stability after freezing.

*Dendritic cell vaccination and cyclophosphamide intake*

Loaded dendritic cells are defined as an advanced therapy medicinal product (ATMP) and released for vaccination after thorough check by accredited qualified person according to the Clinical Trials Directive (2001/20/EC). These include consistency and quality in the processing steps and final products and check of the manufacturing facility, among others. The vaccine was routinely analyzed for DC purity and tested for infectious agents before administration to patients. Several quality control tests were performed before the cellular vaccine was released. Patients received at least three immunizations with mature DC loaded with autologous tumor lysate and KLH with a 2-week interval (Figure 1). Six and twelve months after the third DC vaccination, a revaccination to boost the immune system was given (if enough dendritic cells were available [$4^{th}$ / $5^{th}$ vaccination]) (Table 2). Each immunization, consisting of $50x10^6$ cells, was administered intradermally (i.d.) and intravenously (i.v.). Dosage was divided 1/3 i.d. in the forearm and 2/3 though i.v. route by mixing the components in 100 ml of normal saline drip. Constant monitoring of blood pressure, body temperature, and oxygen saturation was done till 2-h after the administration of vaccine therapy.

Patients were treated with 2 times 50 mg tablet/day of CTX (Endoxan; Baxter B.V., Utrecht, The Netherlands) day -7 to the day of every vaccination (followed by a week interval (figure 1)). The patients were asked to take the medication 2 hours after breakfast and dinner and to increase their fluid intake by extra drinking water or other non-caffeinated beverages during the day. Urine of patients was routinely checked for signs of hematuria.

*Delayed type hypersensitivity skin test*

DTH skin testing was performed by the intradermal application of tetanus toxoid (positive control) and a physiological salt solution (negative control) on the ventral surface of the forearm one week before the apheresis to assess the patient's immunocompetence. If no response to tetanus was obtained that could be related to the effects of chemotherapy, apheresis was postponed and the skin DTH test was repeated two weeks later. If the second test was also negative the patient was excluded from participation in the study. A DTH skin test was also performed two weeks after the third vaccination; further completed with autologous tumor lysate

(10 µg), KLH (5 µg), tumor lysate loaded DC with or without KLH (both $5x10^6$ cells). DTH responses were evaluated after 48 h.

*Immune response assessment against KLH*

Serum samples were collected into SST serum separation tubes (BD biosciences) before, during, and in the lifelong clinical follow-up of the patients. After allowing the serum 30 min to clot, tubes were centrifuged 10 min at 1000 x g. Serum was collected, aliquoted and stored at –80 °C until use. Humoral responses to KLH were measured in the serum of patients by ELISA. Microtiter plates (96 wells) were coated overnight at 4 °C with 25 µg/ml KLH in PBS per well. After blocking the plates with 1% powdered milk in PBS, different concentrations of patient serum (range, 1 in 100 to 1 in 500,000) were added for 1 h at room temperature. After extensive washing, specific anti-human IgG or anti-human IgM conjugated with horseradish peroxidase were allowed to bind for 1 h at room temperature. Peroxidase activity was revealed with the use of 3,3'5,5-tetramethyl-benzidine (TMB) as substrate and absorbance was measured in a microtiter plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

*Treg analysis*

Blood samples obtained before the treatment protocol, at the first vaccination (after induction cyclophosphamide), and two weeks after the third vaccination were analyzed. Before immune staining, the cells were stained for viability using LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen life technologies) in PBS. Subsequently the cells were washed in FACS buffer and incubated 30 min at 4ºC with APC-eF780 labeled anti-CD3 (Clone UCHT1; eBioscience), AF700-labeled anti-CD4 (Clone RPA-T4; eBioscience), PE-Cy7-labeled anti-CD25 (Clone MA251; BD Biosciences), V450-labeled anti-CD127 (Clone hIL7R-M2; BD Biosciences), PE-TexasRed-labeled anti-CD45RA (Clone MEM-56; Invitrogen life technologies), FITC-labeled anti-CCR7 (Clone 150503; R&D Systems, Abingdon, UK) and BV605-labeled anti-CCR4 (Clone 1G1; BD Biosciences). Thereafter the cells were washed, fixated, and permeabilized using the eBioscience FoxP3 kit and the cells were stained intracellularly with PE-labeled anti-FoxP3 (Clone 236A/E7; eBioscience), APC-labeled anti-Ki-67 (Clone 20Raj1;

eBioscience) and PerCP-eF710-labeled anti-CTLA-4 (Clone 14D3; eBioscience). Before analysis, the cells were washed with FACS buffer. The samples were measured on a LSR-II flow cytometer (BD Biosciences) and data analysis was performed using FlowJo software.

*Statistical analysis*

Mean Treg percentage was compared before and after CTX administration and p values were calculated with the Wilcoxon signed-rank test. Statistical calculations were performed using IBM SPSS Statistics 21. Statistical significance was established at the $p < 0.05$ level, and analysis was two-sided.

## RESULTS

*Patients*

Ten patients with advanced MPM and stable disease or response after chemotherapy were enrolled in the study between September 2009 and November 2011. Patient characteristics are summarized in table 1. Nine patients commenced treatment within 6 weeks of diagnosis, one patient opted for delayed start of treatment (5 months). Five patients underwent an additional pleurectomy/decortication (table 2) before immunotherapy. One patient, patient 7, was included although pretreated with more lines of therapy. After pleurectomy/decortication was performed, he was treated in the study protocol. Due to this deviation in pre-treatment, the survival post-surgery is used in this study and his survival after diagnosis is only given as supplement in table 2.

*Clinical activity*

Radiographical responses determined after the third vaccination are shown in table 2. Of the five non-surgical patients, four had SD and one had CR after DC therapy. In the surgically treated patients, the disease could not be evaluated in three patients because surgery led to a macroscopic CR. However, two surgical patients presented with new lesions after DC therapy and therefore had PD. Therefore, in total, disease control (no remaining evaluable disease, CR, PR and SD) was achieved in eight out

of ten patients. In addition, the overall survival of the patients is shown in table 2 with a follow up time of 6 years. Seven out of ten patients had a survival of ≥ 24 months (table 2 and figure 2). Two patients are still alive with one patient in complete remission (60 months after diagnosis) and the other with very slow progressive disease (44 months after diagnosis).

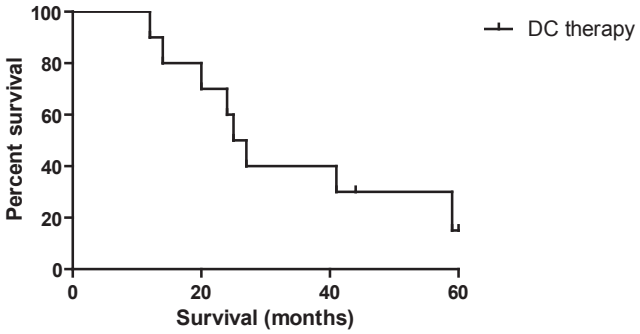


**Figure 2.** Kaplan-Meier plot of overall survival for all evaluable patients in the study. As of last follow-up on Februari 2014, two of ten patients are still alive.

**Table 1.** Characteristics of the ten MPM patients at time of diagnosis included in the trial.

| Patient No. | Gender | Age [a] | Tumor subtype [b] | TNM stage | ECOG ps [c] |
|---|---|---|---|---|---|
| 1 | Male | 62 | Epithelioid | T1bN2M0 | 0 |
| 2 | Female | 55 | Biphasic** | T2N0M0 | 0 |
| 3 | Male | 63 | Epithelioid | T1bN0M0 | 1 |
| 4 | Male | 71 | Epithelioid | T2N0M0 | 0 |
| 5 | Female | 35* | Epithelioid | T1bN0M0 | 0 |
| 6 | Male | 58 | Biphasic** | T1bN0M0 | 1 |
| 7 | Male | 48 | Epithelioid | T3N0M0 | 0 |
| 8 | Male | 78 | Epithelioid | T4N2M0 | 1 |
| 9 | Male | 55 | Epithelioid | T1b0M0 | 0 |
| 10 | Male | 75 | Epithelioid | T1aN0M0 | 1 |

[a] Patient age in years; [b] Histological tumor subtype by microscopic examination; [c] WHO ps = World Health Organisation performance status * Exposed as a child to high concentrations of asbestos by playing on an asbestos paved farmyard.** Biphasic mesothelioma subtype is made up of both epithelioil cells and sarcomatoid cells.

**Table 2.** Radiographical responses on chemotherapy and DC-based immunotherapy treatment, number of DC vaccinations and overall survival.

| Patient No. | Chemotherapy | Response on chemotherapy | Surgical resection | Number of DC vaccinations | Response on DC-based immunotherapy | Overall Survival (months) |
|---|---|---|---|---|---|---|
| 1 | 4xCDDP-PEM | SD | No | 4 x | SD | 24 DOD |
| 2 | 4xCDDP-PEM | PR | Yes | 6 x | NA | 59 DOD |
| 3 | 4xCBDCA-PEM | SD | Yes | 3 x | PD | 20 DOD |
| 4 | 4xCBDCA-PEM | SD | No | 6 x | SD | 25 DOD |
| 5 | 4xCDDP-PEM | SD | Yes | 5 x | NA | 60 AWD |
| 6 | 4xCDDP-PEM | PR | Yes | 3 x | PD | 12 DOD |
| 7 | 6xCDDP-PEM* | PR | Yes | 3 x | NA | 41** DOD |
| 8 | 4xCBDCA-PEM | SD | No | 4 x | SD | 14 DOD |
| 9 | 4xCDDP-PEM | PR | No | 3 x | CR | 44 AWD |
| 10 | 4xCDDP-PEM | SD | No | 3 x | SD | 27 DOD |

CDDP = cisplatin, CBDCA = carboplatin, PEM= pemetrexed
DOD = Died of disease; AWD = Alive with disease (as of February 2014)
*Additional previous therapy prior to pleurectomy/decortication consisted of zoledronic acid, intrapleural gene therapy[29], gemcitabine with and without cisplatin alternating with cyclophosphamide.
** Survival post pleurectomy/decortication, OS after diagnosis was 83 months.



Figure 3 illustrated the response in patient 9, a 55-year-old male with pleural mesothelioma with a partial response on chemotherapeutic treatment with cisplatinum-pemetrexed. Adjuvant DC immunotherapy was administered at three intervals. A complete response was seen. Patient remains in follow-up and is clinically in excellent condition. CT scan 3 years after completion of the DC immunotherapy shows minimal progression of disease.

Figure 4 shows the CT scans of a surgically treated patient, a 35-year-old women with asbestos exposure during childhood (patient 5). She presented with two pleural masses with no radiographical response to cisplatin-pemetrexed treatment. A successful pleurectomy/decortication was performed, pathological evaluation of the tumor revealed a largely vital tumor with only 20% signs of necrosis, compatible with the radiographical finding of SD after chemotherapy. Furthermore, the tumor extended into the resected margins. Five injections of adjuvant DC therapy were given and no disease recurrence is seen yet, 4 years after DC treatment.

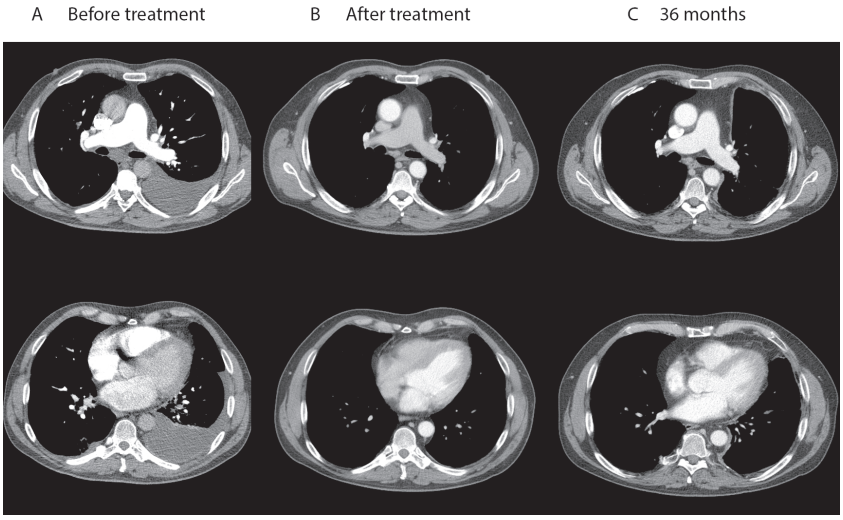


**Figure 3.** Prolonged tumor response in a non-surgical patient. (A) The large pleural effusion and enlarged mediastinal node N7 normalized after chemotherapeutic and DC immunotherapeutic treatment (B). 36 months later, only a slight pleural thickening is seen (C).

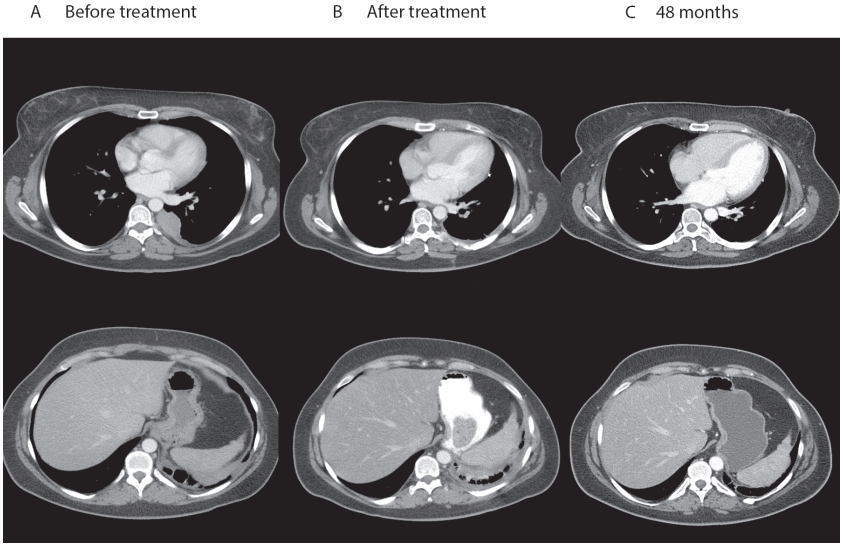


**Figure 4.** Sustained response in a surgical patient. (A) Before treatment two pleural masses are seen on the CT scan; one dorsally next to the spine and the other on the diaphragm. (B) After pleurectomy/decortication, the cranial lesion is not seen and slight post-surgery abnormalities are seen. Caudal, due to post-surgery changes, it is difficult to assess any tumor. (C) 48 Months after DC treatment, no pleural masses are seen both cranial and caudal.

*Safety and toxicity*

None of the ten patients withdrew from the study. There were no logistic problems of practical problems in the preparation process so all vaccinations were given as planned. The safety and toxicity of the combination of a low dose CTX (orally) and tumor lysate-pulsed DCs injected intradermally and intravenously in patients with MPM were defined. No related grade >3 toxicities were found in the patients. A summary of the main adverse events not related to tumor progression is presented in table 3. Injection of DCs was well tolerated without systemic toxicity, except of transient fatigue and low-grade fever on the day of the injection. These symptoms normalized after one day. A local skin reaction in the form of erythema without induration was seen in more than half of the subjects after DC injection. Subsequent vaccinations (second and third) gave a quicker and increased induration and erythema in all patients suggesting that some form of immunity was induced. None of the study participants developed any clinical evidence of autoimmunity. One patient developed a cardiomyopathy 18 months after DC vaccination. This was deemed to be related to the previous cisplatin treatment.



**Table 3.** Adverse events

| | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
|---|---|---|---|---|
| Fever | 4 | 1 | 0 | 0 |
| Nausea | 4 | 0 | 0 | 0 |
| Rash | 3 | 0 | 0 | 0 |
| Thoracic pain | 3 | 0 | 0 | 0 |
| Lethargy | 3 | 0 | 0 | 0 |
| Dyspnoea | 2 | 0 | 0 | 0 |
| Depression | 0 | 1 | 0 | 0 |
| Diarrhea | 1 | 0 | 0 | 0 |
| Cardiomyopathy | 0 | 0 | 1 | 0 |
| Abdominal pain | 1 | 0 | 0 | 0 |

*Treg numbers*

The percentage of $^{CD3+CD4+CD25+FoxP3+CD127-}$Treg of total CD4 cells significantly decreased from 9.43 (range 4.34-26.10) before CTX to 4.51 (range 0.27-10.30) after the first dosage of metronomic CTX at the first DC vaccination (P=0.02)(figure 5a). The Treg of

total CD4 cells percentage per patients are shown in figure 5b. The initial percentage of Tregs was elevated in four patients (two surgical, two non-surgical), of whom one non-surgical patient showed an exceptional high level of Tregs, 28% of all CD4 cells. Levels of Tregs decreased in three of these four patients. There was no correlation between initial Treg levels or decrease with radiological response or survival.

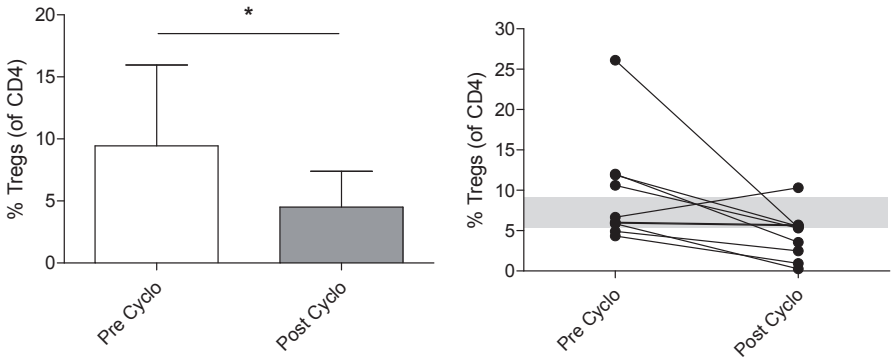


**Figure 5a and B.** Tregs (CD3+CD4+CD25+FoxP3+CD127- cells) were measured in blood samples of patients before CTX treatment and after the first week of metronomic CTX showing a significant decrease of the mean Treg percentage of total CD4 cells (P=0.02) (A). (B) The individual Treg differences before and after the first CTX administration, showing a decrease in nine of ten patients. The grey bar indicates the normal value of Tregs as a percentage of CD4 in healthy controls.

*Immune responses*

Serum samples from all patients showed a significant increase of pre-vaccine versus post-vaccine antibodies reactive to the model antigen KLH, both of the immunoglobulin (Ig)G and IgM isotype. No or very low amounts of antibodies were detected in undiluted serum of all patients before vaccination, illustrating the suitability of this antigen to determine the immunocompetence of the vaccine. Responses against the model antigen increased after the initial 3 vaccinations were completed. In our previous trial KLH response was already present after the first vaccination[4]. This difference is likely due to the suppressive effect of CTX on B cells[13]. After the cycles of CTX administration were stopped, KLH response was evidently present. In the patients in whom it could be determined, the response remained at the same level for several months after the last DC injection and gradually decreased

after 6 to 12 months. This proves that a successful immunoreaction was induced by the DC vaccinations (Figure 6).

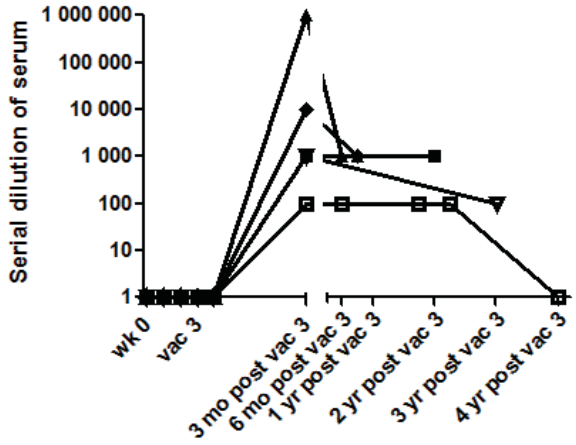


**Figure 6.** KLH-specific IgG increases after DC-based immunotherapy. Kinetics of IgG responses against KLH was measured in serially diluted serum of all patients, with a follow-up of four years maximally.



*Skin DTH testing*

The DTH skin test that was performed after the third vaccination was evaluable in nine patients. There were no skin reactions to saline as the negative control. All patients revealed a positive test to the positive control tetanus as well as to KLH (5 µg), and to DC with KLH ($5x10^6$ cells). This test proves that an effective immune response to the positive control KLH can be induced by DC vaccination. Seven patients out of these nine patients revealed a positive test to tumor lysate loaded DC ($5x10^6$ cells).

In three patients, there was insufficient material left for a post vaccination skin test with solely autologous tumor lysate (10 µg), i.e. on six patients this test was performed. Two patients revealed a positive test. Interestingly, the two patients that revealed a negative skin test to both tumor lysate and tumor lysate loaded DCs were the patients with progressive disease after DC vaccination and the shortest surviving patients of these nine evaluable patients.

## DISCUSSION

In this study, we show that combination treatment CTX and DC immunotherapy was safe and feasible as a maintenance treatment in patients with mesothelioma after chemotherapy. The endpoint of the study was met with a significant decrease of Treg percentage of CD4 cells. DC therapy in combination with CTX was associated with an increase in immunoactivity against the tumor. Radiographical tumor response could not be established in all patients. In the five surgical patients no measurable disease was present at inclusion of the trial. In the five non-surgical patients one response was found. Overall disease control was found in eight of the ten patients, with two patients showing progression of disease. Overall survival was promising given the poor prognosis of this patient population.

The role of surgery in mesothelioma is under debate[14,15]. This is due to disease recurrences occurring after surgery. Therefore, an effective adjuvant treatment is of upmost importance. It has been shown in animal models that by resection of an established primary tumor the tumor-associated immune suppressive environment is decreased[16]. This theoretically supports the combination of surgery and immunotherapeutic strategies. Our data support that this combination is feasible and should be studied further.

The addition of CTX resulted in a decrease in the percentage Tregs of total CD4 cells in most patients during CTX treatment, independent of initial Treg percentage. The reduction of CTX on Tregs is also found in previous studies. However, the dosing schedule of CTX is of importance with continuous and metronomic CTX resulting in a reduction of CTX[17,18]. In contrast, single dose and twice-a-week dosing of CTX did not show an effect on Treg numbers[19,20]. Whether this decrease in Treg percentage caused an increased immune response compared to without Treg depletion, cannot be concluded from these data, as no control group is included. The results found in this study are similar to our earlier murine experiments with DC immunotherapy and CTX[10]. In that study we did found survival to be increased when Treg were reduced.

The fact that we did found normal Treg percentage in a significant number of patients may be a reflection of patient selection whilst we selected patients who were non-progressing or responding to chemotherapy. It can however not be excluded

that in the patients with normal Treg other immunosuppressive mechanisms are more prominent like for instance M2 tumor-associated macrophages (TAMs) or myeloid derived suppressor cells (MSDCs). This finding could thus be of importance for personalizing immunotherapy.

In this small study no correlation could be made for initial Treg percentage or decrease in Tregs to radiological response and overall survival. This could be explained by a number of mechanisms: The circulating Tregs could not be indicative of the Treg numbers in the tumor. Also, Tregs subpopulations have recently been described[21], although an evident decrease of Tregs was found a shift in the subpopulation of the Treg may be of interest.

The DTH skin test proves that an effective immune response to KLH can be induced by DC vaccination, but that this test was not positive for tumor lysate and/or tumor lysate loaded DCs in all patients. Although the number of patients is too small to draw any definite conclusion, the two patients that experienced progressive disease after DC vaccination and had the shortest survival showed a negative skin test for both tumor lysate and tumor lysate loaded DCs. Therefore, the skin test should be evaluated in a larger cohort of patients as a marker for outcome of DC vaccination therapy.



One of the subjects for further research is the impact of the immunosuppressive environment created by the tumor. This has now been shown to be both complex and subject to changes over time. Immune checkpoint inhibitors like CTLA-4 and PD-1 pathways have been described to negatively influence the immune response and anti-CTLA4 antibodies show clinical efficacy in patients with mesothelioma[22]. There may be a role of combining DC immunotherapy with these antibodies. However, others and we have also shown that immunosuppressive cells, like M2 TAMs and MDSCs, apart from Tregs negatively impact the immune system. These immunosuppressive cells negatively interact with checkpoint inhibitory therapy and with DC treatment[23,24]. Theoretically decreasing the number of these cells may enhance efficacy of checkpoint inhibition and DC treatment.

This study has some limitations. First, only radiographic CT scanning was performed. No PET-scanning was done. In an earlier trial with immunotherapy it was shown that a decrease in tumor FDG uptake was found[25,26]. We hypothesize that the

immune responses generated with our trial could also increase FDG-activity via the influx of immune cells, as has been suggested recently[27]. However PET-imaging could have yielded additional information.

Second, the autologous tumor lysate used for antigen loading of dendritic cells originated from treatment-naïve patients in the non-surgery group while in the surgery group the tumor lysate was prepared from resected material. These patients received chemotherapy as an induction to their surgery and thus the tumor was already treated. Three patients in the surgical group showed a prolonged survival, but selection bias should be considered. In addition, the two other surgical patients showed PD. The numbers of our study are too small to draw conclusions on this subject, but this will be addressed in future studies.

Third, the Treg values were measured in peripheral blood to assess the efficacy of CTX on the suppression of Tregs. While this is the most convenient method for the patient to study Tregs, it might not be representative to the Treg density at the tumor[21,28]. However, in mesothelioma, repeat biopsies are only feasible in selected patients.

Fourth, although unlikely, we cannot exclude that leukapheresis has a temporary influence on the Treg numbers. Baseline values of Tregs were obtained at screening, before leukapheresis, while the Treg samples after CTX treatment were obtained after leukapheresis. To our knowledge, the impact of leukapheresis on Treg count is unknown. In four patients treated with DC vaccination, a total blood count including leucocyte differentiation was available at the 2 time points at which Tregs were measured and these showed no change in leucocyte count or shift in leucocyte differentiation. Also, CTX was able to decrease Treg counts in previous studies without leukapheresis attributing the Treg decrease to alone CTX[8,18].

## CONCLUSIONS OF THE STUDY

The addition of CTX significantly decreased the mean Treg percentage of the total CD4 cells in peripheral blood, independent of initial Treg numbers. However, no correlation with response or overall survival could be detected. In addition, we found that the percentage of Tregs of the CD4 cells was elevated in four of the ten patients

at start of the study. DC vaccinations in combination with low dose metronomic CTX administrated to patients with mesothelioma was safe and feasible and no significant adverse effects were observed. Also, DC vaccination therapy proved to be feasible after debulking surgery. DC vaccination therapy combined with CTX resulted in radiographical disease control in eight of the ten patients. Overall survival was promising, with seven out of ten patients having a survival of ≥ 24 months and two patients still alive after 44 and 60 months.

## ACKNOWLEDGEMENTS

We thank Eric Braakman and Anita Schelen from the Department of Hematology for their excellent support on facility and ATMP regulations. Meriam Grootes and others are thanked for apheresing the patients and Femke Muskens for his technical assistance during DC culturing. Chris Vermaat is thanked for his expert-advices and quality control of the DC vaccines. This study was financially supported by Stichting Asbestkanker Rotterdam (www.asbestkanker.nl), Stichting Coolsingel, Stichting NutsOhra and the Mesothelioma Applied Research Foundation (MARF).

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# Chapter VIII

## From autologous tumor cell lysate to allogeneic tumor cell lysate dendritic cell-based immunotherapy in humans

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## ABSTRACT

Autologous tumor lysate loaded dendritic cell (DC) therapy has shown to be feasible in patients with malignant pleural mesothelioma (MPM). The need for fresh autologous tumor material resulted in only a minority of the patient participating. Allogeneic tumor lysate could be used as an alternative for autologous material. This method has several advantages over autologous tumor material. In a murine model, allogeneic tumor lysate loaded DC therapy was shown to be equally effective as autologous tumor lysate loaded DC therapy. In this chapter, the clinical trial is outlined using allogeneic tumor lysate loaded DCs therapy in patients with MPM.

## INTRODUCTION

We previously reported our results of a novel treatment for malignant pleural mesothelioma (MPM) with dendritic cell (DC) immunotherapy[1]. In that study, 10 patients with MPM were treated with autologous DCs loaded with autologous tumor cell lysate sequential to standard chemotherapeutic treatment. These DCs were *ex vivo* cultured from peripheral monocytes that were obtained through leukapheresis. During culture, these cells were loaded with irradiated and freeze-thawed autologous tumor cells in combination with a cytokine cocktail to stimulate maturation and major histocompatibility complex (MHC) expression. The tumor cells were obtained earlier from either pleural fluid obtained by pleurocentesis or resection or biopsy obtained by a surgical procedure. Then, the autologous tumor lysate-loaded DCs were injected into the patient intravenously and intradermally. Using this form of therapy, a specific cytotoxic T-cell (CTL) response was induced through DC-based stimulation, giving way to a new form of anti-tumor treatment in mesothelioma.

The second trial that was performed included 10 patients and added cyclophosphamide to the treatment to reduce the number of regulatory T cells, (T-regs). Reduction of Tregs results in suppression of the immunosuppressive environment and this should enhance the effect of the immunotherapy, as is discussed in **chapter 7**. A second change from the first study was that some patients were included after debulking surgery, which was shown to be feasible.



## LIMITATIONS OF AUTOLOGOUS DC LOADING APPROACH

Only fresh frozen material can be used to load the DCs when using autologous whole tumor lysate. This type of DC loading is hampered by the need of sufficient autologous tumor material, both in quality and in quantity. This was only the case in about 10% of the patients screened for the trial. Most patients referred were already diagnosed with mesothelioma and had no indication for further biopsies or pleural taps. In addition, in treatment naïve patients presenting with pleural effusion the ability of obtaining sufficient amount of tumor cells for the tumor lysate was limited

in part of patients. As was shown in our second trial, post chemotherapy surgical specimens could successfully be used as source for tumor lysate. However, surgery is only possible in 10-15% of patients. Therefore, only a minority of the referred patients could participate in our trial. Furthermore, the process of autologous tumor material processing for DC loading is laborious, expensive, risk for infections etc. (Table 1). Therefore, an alternative method of DC-loading would be preferable.

**Table 1:** Disadvantages of autologous lysate and advantages of allogeneic lysate to pulse DC

Autologous lysate to pulse DC has many limitations:

- > 90% of patients are excluded because of insufficient amount or low quality of tumor material
- patients' distress and pain to resect tumor material for DC loading
- disease progression during processing autologous lysate
- quality control on each sample is laborious
- heterogeneous mixture, leading to dilution of TAA

Allogeneic lysate to pulse DC has many advantages:

- access to a sustained and virtually limitless source of TAA
- allows standardization and large-scale production
- constant quality and composition of the vaccines and reliable comparative analysis of clinical outcome facilitated
- "off-the-shelf", no impact on disease progression
- simple logistics
- less laborious production process
- increases cost-effectiveness
- most patients can be included in immunotherapy by this method

## ALTERNATIVE METHODS OF DC LOADING

An alternative method to direct the DCs towards an anti-tumor response could be to load the DCs with one of the characterized tumor antigens. DC-based vaccines with defined peptides or recombinant proteins from tumor-associated antigens (TAA) are widely used in tumor immunotherapy. In mesothelioma, Wilm's tumor suppressor gene 1 (WT-1), mesothelin, calretinin, fibroblast activation protein (FAP), telomerase and different cancer testis antigens (CTA) such as melanoma-associated antigen (MAGE), cancer/testis antigen cancer-associated gene (GAGE) and synovial sarcoma

X (SSX) gene families, have been described as TAA[2–10]. However, these proteins are not expressed on the membranes of all MPM tumors. Currently, it is uncertain which antigen is the best target for immunotherapeutic treatment. More importantly, the use of this single target strategy has more disadvantages; the efficacy of vaccination against a single or a few TAA is limited by peptide restriction to a given human leukocyte antigen (HLA) type and the induction of CTL. Furthermore, the propensity of tumors to down-regulate antigens, and so escape immunological detection, is a major disadvantage when using the single target approach[11]. Therefore, it has now been described that preferably multiple antigens need to be targeted to obtain a long-lasting effective tumor-specific T-cell response[12]. This strategy decreases the possibility of tumor escape by eliciting a broader immune response[13].

Polyvalent therapeutic strategies, aimed at targeting many antigens at once, may overcome these problems. One such strategy is to load DC with tumor cell lysates, either from autologous or allogeneic background. This can even be done without further defining the antigens[14]. Tumor cells, by definition, express all relevant candidate TAAs, and this rich source of antigens contains epitopes of both CD8+ CTL and CD4+ T helper cells. Tumor lysates might be advantageous in providing the full antigenic repertoire of the tumor and, particularly, unique tumor antigens, which will theoretically decrease the ability of tumors to evade the immune response by down regulation of a single antigen[15]. Therefore, it diminishes the chance of tumor escape compared to using single epitope vaccines.



## ALLOGENEIC DC LOADING IN MICE

In mice, a distinctive immune response was observed when autologous tumor lysate loaded DC therapy was given after injection of tumor cells[16]. In order to overcome the problem of the necessity of sufficient amounts of autologous tumor material, we tested whether allogeneic tumor lysate-loaded DCs were able to improve survival in mice as good as autologous tumor lysate-loaded DCs. Therefore, we performed an *in vivo* experiment using our murine model for MPM. BALB/c mice were injected with a lethal dose of AB1 mesothelioma tumor cells (syngeneic to BALB/c mice) and CBA/j

mice were injected with a lethal dose of AC29 (syngeneic to CBA/j mice). Seven days following tumor inoculation, BALB/c mice and CBA/j mice received unloaded DC, autologous tumor lysate loaded-DC, or allogeneic tumor lysate loaded-DC. The key observation from this study is that when loaded onto DCs, allogeneic tumor lysate was as efficient as autologous tumor lysate in improving the survival of mesothelioma-bearing mice (*Hegmans et al., submitted for publication*).

## ALLOGENEIC DC LOADING IN HUMANS

Allogeneic whole tumor cell lysates are already developed for several tumor types and are typically composed of one to three irradiated cell lines[17–19]. These are injected intradermally, with or without an immunostimulant, to activate the immune system. These trials show that an effective immune response can be established by using this method, however clinical benefit is limited[20]. The antigen injection requires the antigen to be recognized by dendritic cells *in vivo* for antigen presentation. This process is known to be suppressed in most tumor bearing hosts[21].

For mesothelioma, no allogeneic tumor lysate has been described yet. To develop a strategy that could open up avenues to treat more MPM patients in a better and more standardized manner, we want to investigate the use of a batch of allogeneic tumor material from multiple MPM cell lines. This allogeneic lysate consists of tumor cell derivatives from five well-characterized MPM cell lines and is intended to be used to load DC *in vitro*, which will then be used to vaccinate patients.

These five cell lines were chosen based on clinical data and growth pattern and a range of invasive and aggressive properties of the tumor. The MPM cell lines can be provided in a potentially inexhaustible supply in cell factories and the quality can be easily assessed and monitored. We anticipate that this cross-presentation by optimally stimulated DC is a more powerful tool to induce anti-tumor responses *in vivo*.

*The allogeneic lysate*

Acceptability of cell lines for the allogeneic lysate production was based on the following criteria:

- Patient's consent for allogeneic use of their tumor material,
- Diagnosis of MPM was cytological or histopathological proven by pathologists,
- No risk factors for disease transmission based on medical history by clinical study coordinator,
- Guarantee for long term culture (at least 35 passages were established for all 5 cell lines),
- Negative results for tests of sterility, mycoplasma, and adventitious viruses (hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV type I & II), human T-cell lymphotropic virus (HTLV type I&II), and treponema pallidum (syphilis)),
- Diversity of the cell lines in patient, clinical, and cellular characteristics (age and sex of the donor, medical history, treatment response, progression-free survival, overall survival, and histological subtype).

Informed consent and patient characteristics, culture history of each cell line, including methods used for the isolation of the cells from which the cell line was derived, passage history and quality control results are documented in the individual cell line dossiers. The cell lot of these allogeneic primary cells has been appropriately characterized (i.e. cell growth pattern, tumor marker expression, tumorigenicity (by culturing cells in methylcellulose), growth pattern in culture, karyotyping of the cells, and chimerism analysis). The allogeneic lysate consists of a lysate of these five cell lines in equal cellular amounts. Every batch is tested for sterility, stability, quality, and potency.

## CLINICAL PHASE I SAFETY STUDY USING ALLOGENEIC LYSATE PULSED DCS IN PATIENTS

*Aim of the study*

The aim of this phase I study is to assess the toxicity and safety of allogeneic lysate pulsed DCs in MPM patients. Secondary end-points include the establishment of an immune response against TAA and keyhole limpet hemocyanin (KLH). Read-out parameters are the side effects, immune responses, anti-tumor response, and survival of this DC-based immunotherapy both *in vivo* and *in vitro*.

*Patient eligibility*

Main inclusion criteria include:

- Patients with histological or cytological confirmed diagnosed MPM, who are non-progressive after at least 4 cycles of cisplatin/carboplatin and pemetrexed containing chemotherapy or patient without disease progression without chemotherapy in whom chemotherapy is postponed or patients refusal for chemotherapy.
- Measurable disease in two dimensions by a radiologic imaging study.
- Patients must have normal organ function and adequate bone marrow reserve: absolute neutrophil count > 1.0 x $10^9$/l, platelet count > 100 x $10^9$/l, and Hb > 6.0 mmol/l.

Main exclusion criteria include:

- Patients on steroid therapy (or other immunosuppressive agents) are excluded on the basis of potential immune suppression. Patients must have had 6 weeks of discontinuation and must stop of any such treatment during the time of the study. Prophylactic usage of dexamethason during chemotherapy is excluded from that 6 weeks interval.
- No prior malignancy is allowed except for adequately treated basal cell or squamous cell skin cancer, superficial or in-situ cancer of the bladder or other cancer for which the patient has been disease-free for five years.

Statistical Considerations and number of patients

In the phase I study, a 3x3 design will be applied and 3 different dose levels of allogeneic lysate pulsed DCs ($10*10^6$ cells, $25*10^6$ cells and $50*10^6$ cells). If no dose-limiting toxicity (DTL) is encountered among the first three evaluable patients treated at a particular dose level, the next dose level can be opened. In case there is one DLT among the first three patients at a certain dose level, then 3 other patients will be treated at this dose-level. If in these total 6 patients, no other DLTs are seen (so in total 1/6 patients with a DLT), the next dose level can be opened. If there are more than 2 DLTs in a particular dose level, this level is considered to exceed the maximum tolerated dose and the dose level one level lower will be considered as the recommended dose for further studies. This approach implies that in the phase I part at least three and maximum 18 patients are needed.

Study design

After chemotherapy a leukapheresis is performed of which the monocytes are used for differentiation to DCs using specific cytokines. The procedure to grow DCs *in vitro* and pulse them with tumor lysate is performed according to our former DC-immunotherapy protocols that were approved by the ethics committee (METC-2008-109, CCMO NL24050.000.08).

Pulsed autologous DCs are re-injected every two weeks. Quality control tests will be performed before the cellular vaccine is released. After the third injection with allogeneic lysate pulsed DCs, revaccinations to boost the immunsystem are given in a 3 monthly interval until unacceptable toxicity.

*Treatment evaluation (Objectives)*

Treatment evaluation includes safety and toxicity, immunological response and clinical response

Safety and toxicity

The phase I part of this trial is designed to define the safety and toxicity of the allogeneic lysate pulsed DCs injected intradermally and intravenously in patients with MPM. Toxicities will be scored according to CTC criteria version 4.03. The following

toxicities occurring during 8 weeks after the first vaccination, will be considered as dose-limiting:

- *Hematological:*

Thrombocytopenia grade 3 during longer than 7 days or grade 4
Neutropenia grade 3 during longer than 7 days or grade 4

- *Non-hematological:*

Any grade 3/4 toxicity except for diarrhea, nausea, vomiting, hypertension if not adequately treatable, skin toxicity.

- *Immune related:*

Any grade 4 except for rash and (drug related) fever
The feasibility of the method and possibilities to expand to a phase II will be investigated when phase I proves successful.

Immune responses
To determine if this immunization results in a detectable immune response, DTH tests will be performed twice; before and after DC-immunotherapy:

1) With tetanus toxoid within 10 days before the start of treatment, at the inner side of the forearm.
2) Unpulsed DCs, DCs pulsed with allogeneic lysate, DCs pulsed with KLH and allogeneic lysate will intradermally be injected with approximately $10*10^4$ DC/site.

All DTH results will be recorded in the patient's chart. Digital color photographs of the skin DTH response will be taken and all results are stored confidentially according to Dutch law ("Wet Bescherming Persoonsgegevens").

Clinical responses
To observe and document the clinical response in MPM patients who receive the complete treatment, as measured by the development of evaluable and measurable disease lesions (as objectivized by clinical examination, radiologic and scintigraphic evaluation) in response to the treatment. Assessments will be performed according to immune related modified Response Evaluation Criteria In Solid Tumors (RECIST)[22].

Timeline

The first patient received treatment with allogeneic lysate pulsed DCs ($10*10^6$ cells) in January 2015. The accrual of patient that will be included in this phase I study is expected to complete within several months.

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# Chapter IX

**Within the tumor's microenvironment:**

The Good, the Bad and the Ugly

Summary, General Discussion and Conclusions

## INTRODUCTION

In spite of asbestos bans in most developed countries, the incidence of malignant mesothelioma continues to increase in these countries with the peak incidence expected to occur before 2030[1]. In contrast, developing counties such as Brazil, China, India, Indonesia, Kazakhstan, Mexico, Pakistan, Russia, and Thailand increased their use, production, and exportation of asbestos. Therefore, in these countries, asbestos exposure is still on the rise. This ongoing asbestos consumption in these nations will contribute to an additional mesothelioma burden in the future[2]. Currently, the median survival malignant pleural mesothelioma (MPM) is only 9-12 months, so more effective therapeutic agents are evidently needed for patients.

The goals of the studies presented in this thesis were:

- Provide further insight in the interplay between the immune system and mesothelioma tumor cells.
- To study opportunities to exploit the patient's immune system as a therapeutic agent in mesothelioma.

For this, we focused on macrophages and T-cells to analyze if their presence and activity could predict survival and/or local tract outgrowth (LTO) in **chapters 3 and 4**. We demonstrated that tumor associated macrophages (TAMs) are abundantly present in PE of MPM patients and are predominantly of the pro-tumor phenotype in **chapter 5**. Furthermore, in **chapter 7** we expand on our former study in which patients were treated with autologous tumor lysate-loaded dendritic cell (DC) based immunotherapy[3]. In this study, metronomic cyclophosphamide (CTX) was added with the intention to reduce immunosuppressive regulatory T-cells. A sustained tumor response was found and the addition of metronomic CTX resulted in a decrease of Tregs. However, because of the need of fresh tumor material, the number of ineligible patients was high. In order to expand the eligible number of patients that can be included for DC-based immunotherapy, allogeneic tumor lysate could be used instead of autologous tumor lysate. The current study using allogeneic DC-based immunotherapy in patients is outlined in **chapter 8**.

## THE GOOD, THE BAD AND THE UGLY

A tumor does encompass more than only malignant derailed cells. Macrophages, fibroblast, lymphocytes, DCs, natural killer (NK) cells, tumor blood vessels, and tumor stroma are among the cells and structures present in the so-called tumor microenvironment. A complex interplay between cellular components within the tumor microenvironment exists, which can be simplified in a model which subdivides the many players in three categories:

*The Ugly* – The tumor cells; the route that a cell makes to eventually become a malignant cell is long and still not fully understood. Advances in whole-genome sequencing has revealed that malignant cells have undergone a staggering amount of deoxyribonucleic acid (DNA) mutations[4–10] resulting in a cell that has all the capacities that are necessary to become a clinical entity as described by Hanahan and Weinberg[11,12] by Darwinian selection[13].

*The Good* – Anti-tumor immune cells; specific immune cell types play a major role in the tumor environment with part of these immune cells recognizing malignant cells as being out-of-control and acting against the developing and persisting tumor. For example mature DCs, cytotoxic T cells, M1 macrophages, and NK cells.

*The Bad* – Pro-tumor cells; several immune and other cell types are abused by the tumor for pro-tumor activities. This is mainly done by release of cytokines that the cells interpret as a signal to aid in wound healing process, e.g. neovascularization and dampening inflammation. Examples are immature DCs, regulatory T cells (Treg), M2 macrophages, myeloid-derived suppressor cells (MDSC), and fibroblasts.

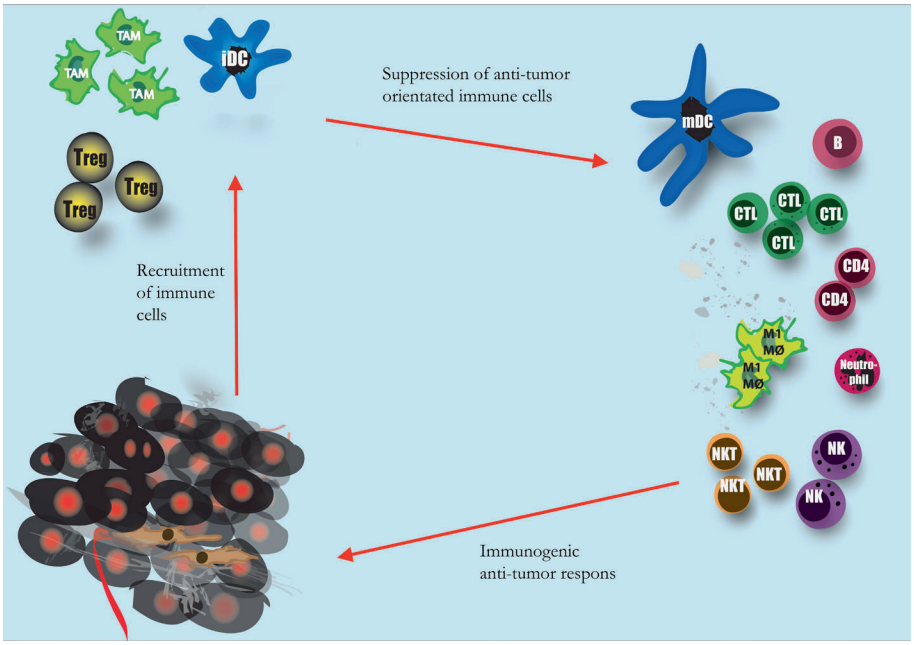


**Figure 1: Mexican standoff between the Good, the Bad and the Ugly.**
Picture showing the main interactions between tumor (depicted as black cells), the anti-tumor immune respons and the immune cells that are abused by the tumor in order to suppress the anti tumor response, among other capabilities.

## TUMOR MICROENVIRONMENT



By analyzing "the Good" and "the Bad" components in "the Ugly" tumor microenvironment, we were able to predict survival in surgically and non-surgical treated mesothelioma patients in **chapter 3**. In addition, by using the ratio of anti-tumor M1 macrophages and pro-tumor M2 macrophages within TAMs, it was possible to predict the occurrence of LTO in MPM patients in **chapter 4**.

The number of M2 TAMs is correlated with the growth potential of tumors and thus negatively associates with survival[14–17]. In addition, macrophages have been described to correlate with the metastatic potential of several tumors[18,19]. This finding is further supported by studies that demonstrate that depletion of the M2 macrophages reduced the incidence of metastasis[20–22]. Mounting evidence is currently present that

M2 macrophages are associated with tumor angiogenesis and lymphangiogenesis. Indeed, M2 macrophages express mediators such as vascular endothelial growth factor (VEGF) A and C, matrix metalloproteinase 9 (MMP-9), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) and several chemokines, such as CXC chemokine ligand 8 (CXCL8) which are directly or indirectly involved in new blood vessel formation and sprouting[23–27]. M2 macrophages exert also an immunosuppressive activity, through the expression of a wide range of molecules, such as arginase-1, indoleamine 2,3-dioxygenase (IDO), TGF-β, inducible nitric oxide synthase (iNOS) and interleukin-10 (IL-10), known for their immunosuppressive role[28–31].

Although our data presented in this thesis shows significant results, these need to be validated in a larger patient cohort. A larger cohort of patient also can give further insight in the relation between M1/M2 macrophages with survival as well as local outgrowth, as this M1/M2 ratio seems to be a predictive for both. Since the patients in the local outgrowth study were matched for survival, no conclusions regarding this relation can currently be drawn from that study.

In addition to the impact of our findings on prognostic value of the overall survival and predictive value on the development of LTO of patients, the M2 macrophages may also reveal as a potential target for therapeutic intervention. Targeting the total macrophage population would be not ideal, since besides the M2 macrophages the beneficial M1 macrophages would also be decreased. This hypothesis is emphasized by our earlier study in which we showed that depleting the entire macrophage population increased the onset and progression of tumor in a murine model of mesothelioma[32]. There are several proposed strategies to counteract specifically the M2 macrophages, including inhibiting the M2 macrophage recruitment, M2 macrophage depletion and blocking M2 tumor-promoting activity of TAMs[33–35]. However, since M2 macrophages remain the plasticity for polarization[36], re-polarization from M2 to M1-type could be the ideal method to tip the balance between M1 and M2 to an overall more effective anti-tumor microenvironment. One of the proposed strategies is to make use of antibodies against CD40 in order to stimulate the secondary lymph node resident macrophages to migrate into the

tumor tissue with interferon gamma (IFN-γ) to reprogram tumor-induced M2 into M1 macrophages[37]. Currently this is under investigation in our institution. In addition, several other therapeutic strategies are under investigation[38–43].

In **chapter 3 and 4**, in addition to M1 and M2 macrophage ratios on respectively survival and LTO in mesothelioma, $^{CD8+}$tumor infiltrating lymphocytes (TIL) were also analyzed. The $^{CD8+}$TIL count showed a positive trend when correlating with survival. In LTO, the presence of a high amount of $^{CD8+}$TIL negatively correlated with the occurrence of LTO. The $^{CD8+}$TIL count seemed to correlate with CD163/CD68 ratio and therefore the ratio of M2 macrophages on the total macrophage count. These results could be explained by a complex interplay within the tumor microenvironment in which M2 macrophages could directly induce Tregs, which in turn are able to suppress tumor-specific cytotoxic T-cells[44]. Likewise, (M1) macrophages may be directly suppressed by Tregs[45,46], resulting in a similar pro-tumorigenic environment.

The M2/total TAM ratio, if validated in a larger cohort of patients, could prove to be useful in clinic:

- The ratio could be used to select patients for surgery.
- Predicting survival can be done more accurate in both groups of controlled trials testing a therapeutic intervention.
- In LTO, patients that prove to be prone to develop LTO can be included in trials studying the effect of prophylactic radiotherapy, enriching the incidence of LTO in the group to be studied.

The effect of decreasing the M2/total TAM ratio on survival and/or LTO is subject for future research. A stated above; since macrophages remain their plasticity[47] a reorientation from M2 into M1 macrophage could prove to be feasible[48]. A murine model after in vitro testing of agents would be the first step to test this hypothesis.

Pleural effusions of mesothelioma patients are often rich in immune cells and associated cytokines with opposing inflammatory phenotypes. In **chapter 5**, we demonstrated that PE is rich in cytokines associated with an immunosuppressive environment which could be tumor cell derived. The highest levels of the measured

cytokines in PE was found in those cytokines that have been associated earlier with immunosuppression and a worse prognosis in cancer patients (IL-6 and TGF-β)[49,50]. In addition, we demonstrated that TAMs are abundantly present in PE of MPM patients and are predominantly of the M2 phenotype. In addition, we found a negative correlation between these TAMs and T-cells in the pleural effusion of MPM patients. These findings demonstrate that the pleural cavity is an important immunosuppressive compartment in MPM and that TAMs can play a pivotal role in hampering the anti-tumor immune response. This finding could have a profound impact especially for the current trials which apply intrapleural therapeutic strategies.

In conclusion, the tumor microenvironment can give an insight in the complex interplay between the malignant cells and the immune system. By analyzing these immune cells in mesothelioma, outcome and LTO can be predicted.

## IMPROVING DC-BASED IMMUNOTHERAPY IN MESOTHELIOMA PATIENTS

Within immunotherapy in mesothelioma, DC-based immunotherapy is one of the most promising agents and will possibly prove to be a vital option for patients that have no anti-tumor T cell response present, as is discussed in **chapter 6**. However, autologous tumor loaded DC based immunotherapy is only possible in limited number of patients in mesothelioma because of the need of autologous tumor material, which is not easily obtained in patients without pleural fluid. Furthermore, the established T-cell response is diminished by the suppressive tumor microenvironment in place, among which the Tregs are most prominent to suppress cytotoxic T cell responses.
By targeting Tregs using metronomic CTX, we showed that DC-based immunotherapy outcome could be improved in a murine model[51]. CTX is indeed an interesting compound, devoid of significant toxicity at low dosing, and has been extensively studied for its immunomodulatory effects. The precise dose, route, and scheduling for the CTX-based chemo-immunotherapies remained unclear for decades, But current knowledge supports low-dose administration of CTX to have the most immunostimulating effect[52].

A metronomic dose of CTX was administrated to investigate its potency to overcome immunosuppression in MPM patients. We show the safety and feasibility of this combined treatment with DC-based therapy in mesothelioma patients and found sustained tumor regression in **chapter 7**. Although no conclusions can be drawn in regard to survival in a phase I trial, the survival was indeed very promising with 7 out of 10 patients having a survival of ≥ 24 months.

In this trial, we demonstrated that the addition of CTX resulted in a significant decrease of $^{CD4+CD25+FOXP3+}$Treg percentage of the total CD4 cells. However, the obtained results in patients were not comparable to our murine model in all patients. This result further highlights the limitations of the use of murine models, where the use of one tumor cell line in identical mice only presents one phenotype of malignancy to the investigator. In contrast, in the clinical setting many phenotypes are seen. Our clinical study in that regard is exemplary, with only 4 out of 10 patients that have an upregulated Treg status at the start of the study and thus had a comparable Treg status to our laboratory setting. The addition of CTX would theoretically only benefit these four patients. This finding opens up the road to personalized immunotherapy, which will be discussed below.

In addition, the effect of CTX does not seem to be limited to Treg reduction alone. CTX also has effects on B cells, T Helper 1 (TH1) cells, DCs, and TH 17 cells[52]. Ultimately, CTX could prove to be beneficial in a subset of patients in which the phenotype of the microenvironment fits the target of CTX.



We explored a new approach to overcome one of the major limitations that arise in this form of DC therapy: the need for (fresh) autologous tumor material. We have shown in mice that DCs loaded with allogeneic tumor lysates are as immunogenic as DCs loaded with autologous tumor lysate. Therefore, allogeneic tumor lysates or tumor cell lines may serve as an efficient alternative strategy to load DCs in patient's DC-based immunotherapy. A phase I clinical trial is currently in progress, this study is outlined in **chapter 8**.

## PERSONALIZED THERAPY

Non-small cell lung cancer (NSCLC) treatment has been revolutionized in the last decade with the introduction of personalized therapy with targeted agents. Erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKi), was approved in 2005 for second line therapy in all NSCLC patients based on a response rate of 8.9% that was similar to docetaxel[53]. It was not until 2009, however, that the pivotal Iressa Pan-Asia Study (IPASS) study showed impressive response rate of 71,2% and survival of 24 months using gefitinib (also an EGFR-TKi) in a selected population[54]. This study also shows that the beneficial effect of this therapy is most evident in patients harboring a sensitive EGFR mutation.

Therefore, selection for this type of therapy proved to be crucial. Currently, a plethora of mutations and translocations are being selectively studied and targeted in patients in clinical or research setting. Among the most studied targets are anaplastic lymphoma kinase (ALK)[55–64], c-ros oncogene 1 (ROS-1)[65–72], v-Raf murine sarcoma viral oncogene homolog B1 (BRAF)[73–81], rearranged during transfection (RET) gene[82–87], met proto-oncogene (MET)[88–95], mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)[96–98], phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) [81,99–105], and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)[106–109], although especially KRAS has proven to be a difficult mutation to target[110]. In all these studies, patients are selected on the basis of DNA mutation or chromosome translocation for these therapies. Due to the low incidence of these mutations, this selection is mandatory; a beneficial response in a patient group with low mutation prevalence would not prove to be significant in a randomized trial in an unselected population.

## PERSONALIZED IMMUNOTHERAPY

Immunotherapy, aiming at harnessing or restoring the natural ability of the immune system to recognize and attack tumor cells, has made great progress over the last decade. Currently, immunotherapy can be divided into four categories:

- Biological response modifiers are compounds, which can (non-)specifically enhance the immune response such as components that trigger inflammation.
- In vitro generated antibodies to enhance the immune response or target components on tumor cells.
- Peptide or complex preparations of tumor antigens that are designed to boost T cell responses or innate immune cell responses.
- Cellular immunotherapies include the adoptive transfer of autologous or allogeneic activated immune cells into patients who can be partly immunoablated.

While immunotherapy has finally made its first steps into the clinical setting in cancer treatment, currently the response rates are modest and an analogy can be made to the first entry of targeted therapy in NSCLC, in which selection of patients is a requirement to increase the response rates for the therapeutic strategies that currently are under development. In contrast to targeted therapies in NSCLC, tumor mutation status most likely cannot be used as a predictive marker since most immune therapies do not directly target the tumor. Until now, immunotherapeutic therapies are applied to all patients, for example our phase I study of the efficacy of DC therapy in MPM and the large international multi-center randomized phase II trial of the cytotoxic T-lymphocyte-associated protein 4 (CTLA 4) antibody tremelimumab as second- or third line therapy in MPM patients[111]. Predictive markers that are able to select patients that benefit most from each therapeutic strategy could enhance the outcome of such trials.



The knowledge of the immune-tumor interactions could prove to be the basis of personalized immunotherapy, in which the immunological composition of the patient dictates the choice of therapy. This selection should be based on tumor specimens of the patient involved. However, in most patients no resection material of the entire tumor is available, but only biopsy material. Biopsy material could be inadequate to analyze the immunological composition of the whole tumor microenvironment since it is known that immune cells tend to reside at specific locations. For example M2

macrophages reside mainly in those areas of the tumor with hypoxic, acidic and low nutrients[112], while regulatory T cells are located at the rim of the tumor[113–115]. Multiple biopsies could be used to minimize the problem of sample-bias. For evaluation of the immunotherapy and selection of new immunotherapy when the tumor shows progression, repeat biopsies are indicated. This will pose a burden for patients, for repeat biopsies may involve painful procedures with the risk of complications. Blood analysis could reduce this burden. However, in blood, it is unclear if tumor-induced alterations in peripheral blood mononuclear cells (PBMC) represent the changes in immune cells within the tumor tissue. Furthermore, it is unclear if the process of metastasizing results in alteration of immunological cells in the blood. In addition, the interaction between the primary tumor and its metastasis is a field of research in development[116]; its effect on the blood immune cells is unknown. Future study on immune cells in blood possibly will reveal its use as surrogate marker for the immune system and tumor interplay.

Using this immunological composition of the tumor, an analysis can be made which component in the anti-tumor response in the patient is suppressed or blocked by the tumor to counteract this effect. For example; when no cytotoxic T-cells are found, patients should first be treated with therapies that stimulate the immune response in order to boost the amount of cytotoxic T cells that target the tumor, for example transferred T-cell therapy or DC therapy. Likewise, programmed death (PD) receptor oriented immunotherapy seems more effective when it's ligand, programmed death ligand 1 or 2(PD-L1/PD-L2) are expressed on the primary tumor[117]. It has to be noted, however, that the possibility exists that these ligands are up- and down regulated over the course of tumor development and therefore could false negatively correlate for absence of response. Anti-CTLA 4 therapy seems only effective if the tumor is invaded by cytotoxic T cells that can be stimulated by this form of therapy, this should be taken into account and T cell induction could be accomplished by methods stated above[118]. When Tregs are the dominant immunosuppressive cell type, CTX could prove to be necessary to reduce their immunosuppressive effects[52]. Likewise, zoledronic acid may be indicated if M2 macrophages seem responsible for the immunosuppressive effects[119].

This could result in the identification of tumors that use several mechanisms to evade or suppress the immune system. In this situation, multiple immunotherapeutic agents may prove to be necessary. Indeed, the simultaneous use of immunotherapeutic substances already has resulted in promising results, even in spite of not being personalized yet; for example, when anti-PD-1 therapy is combined with anti-CTLA 4 therapy, and therefore removing the inhibition of two immune checkpoints instead of one, the response rates appear to be better than single agent therapy[120]. In this trial, immunotherapy was not selected on immune system-tumor interaction biology. Therefore, it is unknown if some patients would have had the same response using only a single agent. The suggested additional effect could be explained by an increased number of patients responding to each individual agent or the actual combination of the two agents.

Eventually an effective anti-tumor immunotherapy could face acquired resistance or Darwinian selection from the tumor. Therefore, if a patient that initially did not show sufficient cytotoxic T cells in the initial biopsy and is started on DC-based immunotherapy shows signs of progression, re-biopsy is indicated to make a re-assessment of the immunological composition of the tumor to select a new therapeutic strategy. For example, by the administration of PD1 blocking antibodies when tumors upregulate PD-L1 in response to cytotoxic T cells[121,122].

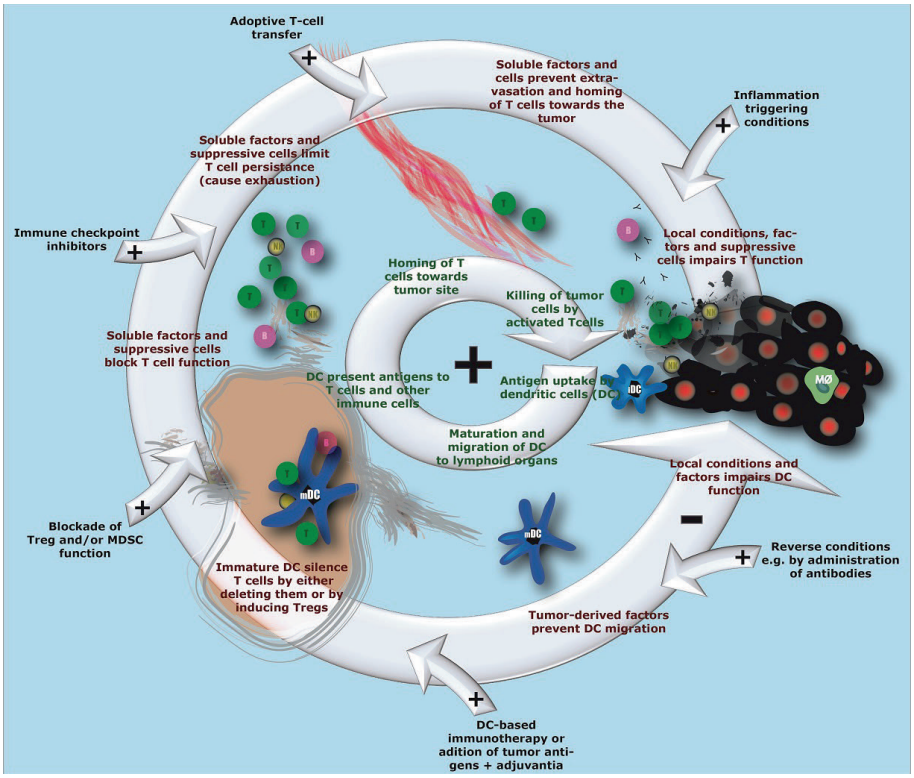


**Figure 2:** This figure shows the natural induction of immune responses by dendritic cells (DC) to tumors in the inner circle. Immature DC (iDC) take up and process tumor antigens, mature en route, and migrate to regional lymphoid organs where they stimulate antigen-specific cytotoxic T lymphocytes (T), B cells (B), natural killer (T) cells (NK(T)), essential for tumor killing. The outer circle demonstrates a variety of the tumor-induced mechanisms that interfere with the development of antitumor responses. The smaller arrows (+) show some therapeutic approaches that intervene with this down-regulation of anti-tumor immunity.

## COMBINING IMMUNOTHERAPY WITH OTHER ANTI-CANCER THERAPIES IN MESOTHELIOMA

Immunotherapy could also be combined with the existing anti-cancer therapies; i.e. surgery, radiotherapy, chemotherapy, and targeted therapy. In addition, epigenetic therapy might prove to be a very interesting partner for immunotherapy.

*Surgery*

At first sight, immunotherapy adjuvant to surgery seems an unlikely combination; surgery is targeted at the complete removal of the tumor, thus after successful removal of the tumor there would be no target left for immunotherapy. However, it has been shown that partial, but not complete, tumor surgery seems to improve outcome combined with chemotherapy and adjuvant immunotherapy[123]. This remarkable outcome could be because debulking surgery could lead to antigen exposure to the immune system, which could boost the effect of immunotherapy. Furthermore, it has been shown that immunotherapy seems most effective in patients with a limited tumor burden[124–130], since the level of tumor-induced immunosuppression is the result of the total burden of the tumor[131]. Indeed, certainly in mesothelioma, surgery could prove to be the ideal tool to reduce tumor load as an induction for immunotherapy; the majority of patients present with a large tumor burden in and the surgical options do not lead to microscopic complete resection.

*Radiotherapy*

Tumor cells can upregulate expression of immune target molecules such as Fas and major histocompatibility complex I following irradiation[132–135]. These mechanisms, along with radiation induced cell death, result in an increased antigen presentation of antigen presenting cells to cytotoxic T cells, resulting in an anti-tumor immune response. This is called immunogenic cell death (ICD) and seems to be particularly induced by ablative radiotherapy, where irradiation is applied in high single doses of 10 Gy or more[136]. Even in clinic, evidence for these effects can be seen; Irradiation can also reduce tumor growth outside the treatment field, often referred to as the abscopal effect[137]. Also, preclinical data suggest a biologic interaction between radiation therapy and immunotherapy[138]. Several clinical studies corroborate these findings[139].

The extent of MPM, covering the entire hemithorax in most patients, results in dose-limitations in the application of radiotherapy. However, ICD could be induced by applying ablative radiotherapy to only a fraction of the tumor by which an anti-tumor immunoresponse could be induced. Currently, trials are commencing using this novel strategy.

However, radiotherapy can also negatively alter the tumor microenvironment; in a study on glioblastoma multiforme, radiation induced recruitment of vasculogenic bone marrow-derived cells through stromal cell-derived factor-1 (SDF-1), which restored vasculature allowing tumor recurrence[140]. Also, thoracic radiotherapy can also induce radiation pneumonitis and/or organizing pneumonia[141], while immunotherapy is also able to induce similar lung toxicity[117,120,142]. The safety of combining thoracic radiotherapy and immunotherapy in lung cancer and mesothelioma therefore has to be evaluated thoroughly.

*Chemotherapy*

Although immunotherapy and chemotherapy have historically been considered antagonistic due to the occurrence of bone marrow suppression of chemotherapeutic agents, this concept has been challenged over the past decade with experimental evidence that chemotherapy may in fact potentiate the efficacy of immunotherapy[143,144]. Various chemotherapeutic agents can affect the tumor microenvironment in multiple ways. Gemcitabine, oxaliplatin and paclitaxel can reduce the amount of Tregs and/or MDSCs infiltrating tumors, thereby reducing their immunosuppressive effects[145–150]. In addition, oxaliplatin can induce immunogenic cell death in a proportion of tumor cells, which can lead to the release of tumor associated antigens for uptake and processing by antigen presenting cells (APC)[151]. Anthracyclines can recruit APCs and enhance their differentiation to an activated phenotype, better able to present antigen to lymphocytes[152]. Pemetrexed, in addition to its already proven positive effect on mesothelioma patient when combined with cisplatinum[153], also seems to harbor an synergistic effect when combined with immunotherapy in a murine model[154].

In mesothelioma, gemcitabine has been studied for years as monotherapy or as part of a combination therapy. However, the response rate, certainly as monotherapy, is limited[155,156]. The addition of immunotherapy to gemcitabine for its capacities stated above may prove to be effective. In addition, a recently published study demonstrated that while DC cross-presentation within the tumor microenvironment is defective, this effect can be reversed by the addition of gemcitabine[157]. The immunological effects of pemetrexed, as well as other chemotherapeutic compounds, warrant further study.

*Targeted therapy*

Targeted therapy aims to inhibit certain molecular pathways that are crucial for tumor growth and maintenance. In addition, targeted therapies have been shown to promote effective DC maturation, T cell priming, activation and differentiation into memory T cells, as well as effector T cell function[158]. For example temsirolimus enhances CD8+ T cell activation and IFN-γ production and bortezomib sensitizes tumor cells to cytotoxic T cell mediated lysis[159,160]. Also targeted therapies can aid in overcoming local immunosuppression; bevacizumab, dasatinib, vemurafenib, and sunitinib seem to possess inhibitory activities on Tregs and/or MDSCs[161,162]. In addition, targeted therapies may sensitize tumor cells to immune-mediated killing by increasing the expression of death receptors or 'distress' ligands while simultaneously diminishing the expression of pro-survival signals[158]. In mesothelioma, targeted therapy has not shown to be beneficial to this date[163], but it is possible that the combination of targeted and immunotherapeutic agent could provide a synergistic effect.

*Epigenetic therapy*

Chromatin is the macromolecular complex of DNA and histone proteins, which provides the scaffold for the packaging of our entire genome. Deep sequencing technologies aimed at mapping chromatin modifications have accelerated the insight in epigenetic abnormalities in cancer. Analysis for histone modifications and the binding of chromatin regulators have raised intriguing correlations between cancer-associated DNA hypermethylation and genes marked with ''bivalent'' histone modifications in multipotent cells[164,165]. These bivalent genes are marked by active and repressive histone modifications[166] and appear to identify genes that are integral to development and lineage commitment. Interestingly, many of these genes are targeted for DNA methylation in cancer. Equally intriguing are recent comparisons between malignant and normal tissues from the same individuals. These data demonstrate broad domains within the malignant cells that contain significant alterations in DNA methylation[167].

Epigenetic therapy refers to the use of agents with hypomethylating and histone-deacetylase inhibitory(HDACi) activity. Importantly, HDACis have been shown to

enhance the immunogenicity of cancer cells; Several groups have reported the upregulation of natural killer cell activating ligands, major histocompatibility complex (MHC) class I and II molecules, components of the machinery for antigen presentation, and co-stimulatory molecules on the surface of cancer cells exposed to HDACis[168–170]. In addition, the pre-treatment of malignant cells with HDACis has been employed to generate an effective anticancer vaccine for therapeutic use, and HDACi-treated malignant cells exhibit an increased propensity to be taken by DCs[168,171]. Vice versa, the immune system even seems to be a critical component of the antitumor effects of HDACis[172].

## FUTURE STRATEGIES IN MESOTHELIOMA

The optimal strategy for combining each form of therapy is subject for extensive further research. It is likely the selection, order, or combination of therapeutic approaches will have to be personalized, too. For example, in theory, a mesothelioma patient with an extensive tumor load may benefit from debulking surgery to reduce tumor load followed by personalized immunotherapy. In addition, chemotherapy and/or targeted therapy could be added as neoadjuvant therapy or concurrent with immunotherapy. In contrast, in limited disease, the role of debulking surgery could prove to be of limited additional benefit and radiotherapy might be used instead as initial therapy to increase tumor-associated antigens that in turn could induce an anti-tumor immune response.

However, the overwhelming amount of different options and possible combinations of therapies, most of which should be “targeted” to patients’ immune status and reconsidered at tumor progression poses a major problem for research in mesothelioma; the possibility to prove the efficacy in a large phase III randomized controlled trial. While this problem is not easily solved, consideration should be given if this method of proving therapeutic efficacy is feasible in the future.

## CONCLUSION

Personalized immunotherapy, probably combined with established anti-tumor therapies, could lead to a "clinical cure" in metastasized cancer that was incurable until date[173]. However, when this scenario is reached, clinicians will have to analyze each move of the tumor using repeat biopsies or blood sampling to counteract on it, quite similar to a game of chess[174]. However, at presentation of the malignancy and at each point of progressive disease the situation is more serious than a game of chess; The Good, the Bad and the Ugly stare each other down at gunpoint in a Mexican standoff[175]. The Ugly and the Bad have the advantage as dictated by Darwinian theory. Clinicians are now given tools to unload the gun of the Ugly and shooting the Bad or possibly to persuade the Bad to the side of the Good, resulting in a possible victory over the Ugly. However, it remains to be seen how many spare guns the Ugly holds up in its sleeve. Undoubtedly, due to the further unraveling of the interplay between immune system and tumor, additional mechanisms will be found in the coming years that will add to the current knowledge and reveal new therapeutic options. An interesting time lies ahead of us, with immunotherapy gradually moving to the forefront of cancer treatment[173,176].

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## NEDERLANDSE SAMENVATTING

Asbest is een natuurlijk mineraal welke opgebouwd is uit microscopisch kleine vezels. Wanneer deze vezels worden ingeademd kunnen ze uiteindelijk op het borstvlies en/of buikvlies terechtkomen. Dit borstvlies (pleura) bekleedt de binnenkant van de ribben, het hart en het middenrif. Het buikvlies (peritoneum) bekleedt alle organen in de buikholte. Asbestvezels zijn biologisch niet afbreekbaar, daarnaast zijn ze te lang om door macrofagen opgenomen te worden; dit zijn cellen van het afweersysteem die in staat zijn om onder andere vreemde stoffen op te ruimen. Op de voorkant van dit proefschrift is een dergelijke macrofaag te zien met een asbestvezel die te lang is voor deze macrofaag om volledig te omvatten. Dat asbest kankerverwekkend is werd reeds aangetoond in 1960; er werd bij mensen met een blootstelling aan asbest een duidelijk hoger percentage van het zogenaamd mesothelioom aangetroffen. Mesothelioom is een kanker van het borst-, long- en/of buikvlies en wordt vaak ook asbestkanker of longvlieskanker genoemd, in **hoofdstuk 1** wordt deze ziekte verder besproken. Een genezende behandeling bestaat (nog) niet en de enige bewezen effectieve palliatieve behandeling is chemotherapie; de behaalde verlenging van het leven is hiermee gemiddeld 3 maanden, maar de variatie is groot.

Er vindt veel onderzoek plaats naar nieuwe middelen om deze ziekte beter te behandelen, maar tot nu toe zijn veel onderzochte behandelmethodes niet effectief gebleken. Immuuntherapie maakt gebruik van de 'natuurlijke' eigenschappen van afweercellen om de tumorcellen aan te vallen. Dit kan bijvoorbeeld worden bereikt door het toedienen van antistoffen tegen de tumor of een onderdeel hiervan. Er kunnen ook cellen van de afweer worden toegediend, met name als deze tevoren zijn bewerkt en zich specifiek kunnen richten tegen tumorcellen. Een andere mogelijkheid is om buiten het lichaam cellen te kweken die verantwoordelijk zijn van het tonen van doelwitten aan andere immuuncellen, de zogenaamde antigeen presenterende cellen. Buiten het lichaam worden deze cellen als het ware opgeladen met kapotgemaakte tumorcellen. Door deze cellen vervolgens terug te brengen in het lichaam kunnen deze cellen door ervoor zorgen dat de tumor wordt aangevallen. Dit doen ze door middel van presentatie van celonderdelen aan een ander deel van

het immuunsysteem. In **hoofdstuk 2** wordt op de mogelijkheden en de vooruitgang binnen de immuuntherapie wordt verder ingegaan.

Het afweersysteem van het menselijk lichaam is opgebouwd uit verschillende celtypen, ieder met eigen taken. De functie van deze afweercellen kan zijn om indringers te doden, bijvoorbeeld bacteriën in het geval van een longontsteking. Ook speelt het afweersysteem een rol in wondherstel, waar het helpt bij de vorming van bloedvaten en het geven van groeisignalen. Op het moment dat een cel ontspoort (bijvoorbeeld door schade aan zijn DNA) en zich kwaadaardig gaat gedragen behoort de afweer te reageren zoals bij de longontsteking. De gemuteerde cel moet kapot gemaakt (gelyseerd) worden waardoor kleine onderdelen (antigenen) vrij komen. Deze antigenen van zo'n tumorcel worden door zogenaamde dendritische cellen opgepakt en getransporteerd naar de lymfeklieren. In de lymfeklier vindt antigeenpresentatie door deze dendritische cellen aan verschillende andere cellen van het immuunsysteem plaats, waaronder T en B cellen. Deze T en B cellen leren op die manier hoe ze de tumor moeten herkennen. Vervolgens moeten de T cellen migreren naar de tumor om daar de tumorcellen aan te vallen en te doden. De B cellen maken op hun beurt antistoffen tegen de tumor.

Op het moment dat er in het bovenbeschreven proces van het immuunsysteem een hapering optreedt, zullen ontspoorde cellen niet adequaat worden opgeruimd en krijgen zij de kans zich te vermenigvuldigen, zo kan een tumor groeien en spreken we van kanker. Aangezien dit proces uit meerdere stappen bestaat kan op elk niveau van deze stappen een probleem aanwezig zijn. Een opmerkelijk fenomeen is dat de hapering kan optreden onder invloed van de eigen immuuncellen die door de tumor worden misbruikt, waardoor de tumor zijn eigen pro-tumor omgeving creëert. Door middel van mediatoren die de tumor uitscheidt (onder andere cytokines) "denken" deze immuuncellen mee te doen aan wondgenezing en gaan op deze manier zorgen voor bloedvatvoorziening nabij de tumor, geven groeisignalen af en onderdrukken afweercellen die gericht zijn op het vernietigen van de tumor cellen. Dit hele proces zorgt ervoor dat de tumor kan groeien en beschermd wordt tegen immuuncellen die tegen de tumor gericht zijn. Cellen die hierin een rol spelen zijn onder andere regulatoire T cellen, macrofagen van het M2 type en immature dendritische cellen.

Het geheel van tumorcellen met omliggende cellen wordt de tumor micro-omgeving genoemd. Naast de genoemde tumor en afweercellen spelen onder andere bloedvaten en fibroblasten hierin een rol. De tumor micro-omgeving is een strijdtoneelplek waar aan de ene kant afweercellen proberen de tumor te doden en aan de andere kant afweercellen aanwezig zijn die dit proberen tegen te houden. Zo ontstaat er een balans welke meer anti-tumor gericht kan zijn, maar ook meer pro-tumor gericht. Deze balans was onderwerp voor onderzoek in **hoofdstukken 3 en 4**. In deze onderzoeken werd de verhouding van de anti-tumor gerichte macrofagen (M1 macrofagen) en pro-tumor gerichte macrofagen (M2 macrofagen) onderzocht in de tumor micro-omgeving van patiënten. Het bleek dat als deze balans werd overheerst door M2 macrofagen, dat de overleving van patiënten korter was en dat er vaker sprake bleek te zijn van lokale doorgroei van het mesothelioom. Deze bevindingen kunnen leiden tot een betere inschatting van prognose van patiënten, daarnaast kan beter worden voorspeld of patiënten risico lopen op lokale uitgroei van het mesothelioom. Dit opent de weg voor onderzoeken naar maatregelen om lokale uitgroei te voorkomen, maar nog interessanter zou zijn om te onderzoeken of het aanpassen van deze M1-M2 macrofaagbalans in de richting van overheersend M1 macrofagen een invloed zou kunnen uitoefenen op overleving en/of lokale uitgroei.

Bij patiënten met een pleuraal mesothelioom ontstaat er vaak vocht tussen het borst en longvlies; dit wordt pleuravocht genoemd. Hierbij kunnen patiënten kortademig worden en moet het vocht worden weggehaald. Dit vocht was het onderwerp voor onderzoek in **hoofdstuk 5**. In dit onderzoek hebben we pleuravocht van mesothelioompatiënten gebruikt om buiten het lichaam macrofagen te kweken. Het bleek dat macrofagen die zich ontwikkelden in dit pleuravocht vanzelf veranderen in M2 macrofagen, dus pro-tumor gerichte macrofagen. Ook komen de cytokines in het pleuravocht overeen met pro-tumor gerichte cytokines. De conclusie van dit onderzoek is dat pleuravocht bij patiënten met een mesothelioom in staat om een pro-tumor gerichte balans van immuuncellen te creëren. Deze bevinding kan een grote rol spelen bij behandelingen welke lokaal in de borstkas gegeven worden. Op dit moment worden er meerdere experimenten uitgevoerd waarbij zo'n lokale behandeling in de borstkas wordt toegepast. Het feit dat in het pleuravocht

bij patiënten een pro-tumorgerichte omgeving heerst kan deze experimenten negatief beïnvloeden en zou aanleiding kunnen geven tot het toepassen van extra medicamenten in deze onderzoeken om de balans van het pleuravocht meer in een anti-tumor gerichte omgeving te laten veranderen.

De meest potente antigeen presenterende cellen zijn dendritische cellen, derhalve wordt veel onderzoek gedaan om juist dit celtype te gebruiken als therapie tegen kanker. Voordelen van een effectief medicijn met dendritische cellen zijn talrijk. Er wordt op een relatief normale wijze gebruik gemaakt van het eigen immuunsysteem, de afweerreactie wordt breed ingezet, dus niet alleen specifieke T cellen of antistoffen gericht tegen een enkel antigeen. Daarnaast zijn de bijwerkingen over het algemeen mild. Een overzicht van het gebruik van dendritische cellen als immuuntherapie wordt gegeven in **hoofdstuk 6**.

In **hoofdstuk 7** wordt ons onderzoek beschreven waarin 10 patiënten zijn behandeld met dendritische celtherapie. Deze dendritische cellen werden gekweekt uit een subtype van witte bloedcellen, genaamd monocyten, uit het bloed van de patiënt. Vervolgens werden ze in het laboratorium “getraind” met behulp van cytokines en opgeladen met kapot gemaakte lichaamseigen tumor cellen om zich te kunnen richten tegen de eigen tumorcellen van de patiënt. De getrainde dendritische cellen werden vervolgens weer aan de patiënt toegediend. Om ervoor te zorgen dat pro-tumorgerichte regulatoire T cellen deze antitumor gerichte reactie niet zouden afremmen werd cyclofosfamide aan de patiënten gegeven. Dit middel remt de werking van deze regulatoire T cellen. Het aantal regulatoire T cellen in het bloed van de patiënten bleek ook duidelijk af te nemen onder het gebruik van cyclofosfamide. Daarnaast waren de bijwerkingen mild en bleek deze vorm van therapie ook haalbaar na het verrichten van een chirurgische verwijdering van de longvliezen. Alhoewel dit onderzoek niet was bedoeld om overlevingswinst aan te tonen werd er bij meerdere patiënten een opmerkelijk lange overleving gezien. Van de 10 deelnemende patiënten hebben er 7 langer dan 2 jaar na diagnose geleefd, dit terwijl de gemiddelde overleving van patiënten met een mesothelioom slechts 9-12 maanden is.

Dendritische celtherapie met tumoreigen materiaal heeft als nadeel dat er beschikking moet zijn over een aanzienlijk aantal verse tumorcellen. Bij patiënten bij wie reeds een diagnose was gesteld was er veelal geen indicatie meer om nogmaals tumormateriaal af te nemen. In de onderzoeken welke uitgevoerd zijn in het Erasmus MC bleek derhalve dat slechts 10% van de patiënten deel kon nemen aan deze onderzoeken. Daarnaast is deze vorm van immuuntherapie arbeidsintensief en duur. Dendritische cellen trainen kan ook met behulp van niet-lichaamseigen (allogene) antigenen, echter een uniform antigeen van mesothelioom is niet bekend. Alhoewel er een aantal antigenen bekend zijn, zijn deze niet aanwezig op alle tumorcellen en een behandeling gericht tegen één antigeen kan aanleiding geven tot groei van tumorcellen die dit antigeen niet op hun cel hebben. In muizen hebben wij therapie met dendritische cellen die opgeladen zijn met een combinatie van gelyseerde mesothelioom cellijnen onderzocht. Het bleek dat deze therapie even effectief is als dendritische celtherapie waarbij de dendritische cellen opgeladen zijn met gelyseerde lichaamseigen tumor cellen. **Hoofdstuk 8** beschrijft het onderzoek waarbij deze patiënt-eigen dendritische cellen beladen worden met een mengsel van vijf verschillende mesothelioom tumor cellijn lysaten. Het primaire doel van dit onderzoek is om bewijs te leveren dat deze vorm van behandeling veilig is voor patiënten. Later zal ook gekeken worden naar effectiviteit. Dit onderzoek is op dit moment actief in het Erasmus MC en de eerste patiënten zijn reeds behandeld.

Immuuntherapie is door het wetenschappelijk tijdschrift “Science” uitgeroepen als doorbraak van het jaar 2013. Dit terwijl er slechts enkele therapieën op dit gebied beschikbaar zijn voor een beperkte patiëntengroep, zoals beschreven in **hoofdstuk 9**. Het onderzoek in dit veld is echter immens en de vooruitgang indrukwekkend. In een rap tempo wordt het zeer ingewikkelde immuunsysteem ontrafeld en worden er behandelingen getest. De relatie tussen tumor en immuunsysteem verschilt echter van patiënt tot patiënt. Dit houdt ook in dat de behandeling op maat gemaakt zal moeten worden. Bijvoorbeeld als een patiënt geen T cellen in de tumor heeft die de tumor probeert aan te vallen dan kunnen deze worden toegediend of gestimuleerd. Zijn deze T cellen echter al wel aanwezig dan zal deze therapie nauwelijks meerwaarde bieden en zal het onderzoek zich moeten richten op de vraag waarom

deze T cellen niet effectief hun werk kunnen doen. Immuuntherapie op maat; “personalized immunotherapy” is de sleutel voor een effectieve tumorgerichte therapie welke gebruikt maakt van anti-tumor gerichte immuuncellen (The Good), pro-tumor gerichte cellen tegengaat (The Bad) om zodoende de tumor (The Ugly) uit te schakelen.

## DANKWOORD

Bovenal gaat mijn dank uit naar alle patiënten die hebben meegewerkt aan de beschreven onderzoeken of eraan hadden willen deelnemen. De te verwachten winst voor de patiënt zelf is vaak afwezig of klein en deelname aan een studie dient voornamelijk de toekomstige patiënt. Desondanks was u bereid aan de onderzoeken mee te doen.

**Prof.dr. J.G.J.V. Aerts**: Beste Joachim, ik ken je al vanaf het moment dat ik als AGNIO werkzaam was in het Sint Franciscus Gasthuis. Mede door jou heb ik besloten dat ik longarts wilde worden en mede dankzij jou ben ik het ook geworden. Toen de mogelijkheid zich voordeed om in jouw groep onderzoek te doen aarzelde ik geen moment en heb er ook geen moment spijt van gehad. Je bent een gedreven persoon die ondanks een veel te volle agenda altijd tijd kan vrijmaken voor een discussie over een onderwerp. De beklimming van de Tafelberg is onvergetelijk.

**Dr. J.P.J.J. Hegmans**: Beste Joost, je bent een basale onderzoeker in hart en nieren, maar jij draagt de patiënt een bijzonder warm hart toe. Het feit dat de overleving van de patiënten uit de studie hoopgevend is betekent voor jou niet een studie-uitkomst voor het artikel, jij bent blij voor die mensen die een goede overleving kennen. Ik kon met jou altijd goed discussiëren over onderzoeksprojecten, maar ook over de zin van het leven bijvoorbeeld in een McDonalds op het vliegveld in Boston.

**Prof.dr. H.C. Hoogsteden**: Beste Henk, mijn eerste ontmoeting met jou was tijdens mijn sollicitatiegesprek naar een opleidingsplek longziekten en tuberculose. Er ontvouwde zich al snel een buitengewoon kleurrijk gesprek over onder andere kindergeneeskunde en psychiatrie. Tijdens mijn opleiding ontstond de mogelijkheid om tot jouw staf toe te treden en dit drukwerk is een gevolg daarvan. Je komt op voor je afdeling, gaat problemen niet uit de weg en zorgt voor de opleidingsassistenten alsof het jouw kinderen zijn.

**Prof.dr. R.W. Hendriks**: Beste Rudi, dank voor jouw immunologische kijk op de experimenten en de medewerking aan de artikelen. Het complexe systeem van de immunologie is mij hierdoor duidelijker geworden. Wel bleven er, zoals verwacht, aan het eind van elke bespreking meer vragen over dan antwoorden.

**Prof.dr. P. Baas, Prof.dr. A.J.J.C. Bogers en Prof.dr. J. van Meerbeeck**: Dank voor het plaatsnemen in de kleine en grote commissie en het kritisch doornemen van dit proefschrift. **Dr. M. Gregoire**, merci de prendre un siège dans la grande commission et lecture critique de cette thèse.

Beste **Sanne**, jij was mijn maatje bij een deel van dit proefschrift. Jij op het lab, ik in de kliniek. De hoeveelheden gigabytes die over en weer zijn gegaan zijn onvoorstelbaar, van de Verenigde Staten tot Zuid-Afrika, data werd verwerkt, grafieken gemaakt en de artikelen geschreven. Ik vond het altijd fijn om samen te werken. Voor jou breekt binnenkort het moederschap aan, als dat je even goed afgaat als je onderzoek doet dan mag je kindje daar erg blij mee zijn.

**Cynthia, Femke, Floris, Joris, Koen, Lysanne, Margaretha, Marlies, Niken en Pauline:** Waar er iemand is die met de patiënten contact heeft, zo zijn er ook mensen die enkel een deel van de patiënt zien door een microscoop of nog indirecter via een de FACS. Maar basaal en translationeel onderzoek is en blijft de basis voor klinisch onderzoek. Dank voor de buitengewoon prettige samenwerking en jullie inzet.

Beste **Lex Maat**, chirurgie bij patiënten met een mesothelioom is inspannend en uitdagend, desondanks heb jij je dit als geen ander eigen weten te maken. De complete respons die wij longartsen graag zien werd op deze manier bereikt. Dank voor de prettige samenwerking.

Beste **Jan-Lukas Robertus** en **Lisette de Vogel**, de afdeling pathologie is van groot belang geweest voor het maken, kleuren en beoordelen van de coupes die in dit proefschrift beschreven zijn. Juist wanneer de tijd drong kon er zeer snel worden gewerkt.

**Collega longartsen in het Erasmus MC**: Beste Bernt, Cor, Eva, Henk (Otten), Jelle, Karin, Kim, Leon, Marleen, Marlies, Menno, Robert, Rogier en Thomas, jullie zijn een fantastisch team van collega's waardoor werken een hobby wordt. Een team waarin meerdere subspecialisaties aanwezig zijn die door elkaar op een dergelijke manier gerespecteerd worden is in de huidige medische situatie een noodzaak, maar niet vanzelfsprekend.

**Collega's in het Erasmus MC**: Collega's van de interne geneeskunde, nucleaire geneeskunde, pathologie, radiologie, radiotherapie en thoraxchirurgie, longoncologie vergt bij uitstek een multidisciplinaire benadering. Ons team is daar zeer in bedreven en ik vind deze samenwerking zeer plezierig, dank hiervoor.

**Collega's buiten het Erasmus MC**: De beschreven patiënten in dit proefschrift zijn voor het overgrote deel patiënten waarbij u de diagnose heeft gesteld en die via u zijn verwezen voor een experimentele behandeling. Zonder deze verwijzingen komt de wetenschap niet verder, dus buitengewoon veel dank daarvoor.

Beste **oncologie/research verpleegkundigen** (Annemarie, Arianne, Els, Janneke, Louise, Marian, Marjolijn en Titia) en de **afdelingsverpleegkundigen**, de longoncologie is in de afgelopen jaren sterk veranderd, waarbij onderzoek een prominente rol is gaan spelen. Dit heeft aanpassing gevergd en zonder jullie zou dit niet mogelijk zijn geweest.

Beste **onderzoeksverpleegkundigen**, dank voor de vele malen dat ik van jullie het pleuravocht van de patiënten heb mogen ontvangen en alle keren dat ik (ruim.....) na het reguliere programma toch nog patiënten mocht plannen.

Beste **dames van de polikliniek longziekten**, ook jullie dank voor alle keren dat er bloed van patiënten is afgenomen voor de verschillende studies en dat deze patiënten vaak tussendoor konden komen ondanks plaatsgebrek op de poli.

Beste **Barbara**, dank voor het helpen met de logistiek voor dit proefschrift!

Beste **Ewout**, ik ken je inmiddels zo ongeveer mijn halve leven en met onze gezamenlijke hobby's voorzie ik dat onze vriendschap permanent zal zijn. Jij bent de enige persoon aan wie ik kan vragen om mee te gaan naar een concert die niet zal vragen waar dat dan wel niet is (Oslo……). Dank dat jij mijn paranimf wil zijn.

Beste **Arthur**, ook onze gezamenlijke geschiedenis, samen met Esther, gaat al jaren terug naar mijn afstudeerproject op de afdeling nucleaire geneeskunde. Onze liefde voor films heeft ons inmiddels al honderden keren met z'n vieren samengebracht en ook al viel de film tegen, het gezelschap niet! Ook jij bedankt dat jij mijn paranimf bent.

Beste **Roos**, het combineren van het leven van twee artsen met een steeds groter wordend gezin brengt uitdagingen met zich mee. Jij zorgt voor de oplossing, dank daarvoor!

Lieve **Pa en Ma**, jullie staan aan de basis van dit proefschrift. Ik mocht de keuzes maken die ik wilde en mocht naar de grote stad voor de middelbare school. Die vrijheid heeft mij gebracht waar ik nu ben en daar ben ik jullie voor altijd dankbaar voor.

Lieve **Thijs, Lotte en Anouk**, bij jullie alle drie heb ik continu het gevoel "Hè, hoe kan het nou dat je dat al kunt? Je bent toch nog maar net geboren!". Wat worden jullie ontzettend snel groot. Mama en ik genieten iedere dag van jullie.

Lieve **Inge**, wij kennen elkaar nu al vele jaren, eerst verliefd, nu getrouwd. Ik weet dat ik altijd op je kan bouwen en ik kijk terug op de dingen die wij hebben gedaan en uit naar de vele dingen die wij samen en als gezin nog gaan ondernemen. Te beginnen met de komst van ons 4$^{e}$ kindje.

## CURRICULUM VITAE

The author of this thesis was born on November the 3th, 1976 in Rotterdam. In 1995, he graduated from the Erasmiaans Gymnasium and started the study of medicine in the same year at the Erasmus University in Rotterdam. After obtaining his medical degree, he started working in the Sint Franciscus Gasthuis in Rotterdam at the department of internal medicine, the last years as introduction to the training of pulmonologist under supervision of Dr. H.S.L.M. Tjen and Dr. A.P. Rietveld. In 2006, he started his training to become a pulmonologist in the Erasmus Medical Center in Rotterdam under the supervision of Prof.dr. H.C. Hoogsteden. The last 18 months of this training were completed in the field of oncology. After becoming a pulmonologist in 2010, he started to work in the same hospital with oncology, endosonography and interventional pulmonology as current fields of expertise. Almost simultaneously, in 2011, this Ph.D. project was started under supervision of Prof.dr. J.G.J.V. Aerts and Dr. J.P.J.J. Hegmans.

The author lives in Bergschenhoek, a small town just next to Rotterdam, with his wife Ingeborg and three children; Thijs, Lotte and Anouk. A fourth child is expected in June.

## LIST OF PUBLICATIONS

Bronchiolitis obliterans organizing pneumonia (BOOP) after thoracic radiotherapy for breast carcinoma. **Cornelissen R**, Senan S, Antonisse IE, Liem Y, Tan YKY, Rudolphus A and Aerts JGJV. *Radiation Oncology* 2007, 2:2

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Dendritic cell-based immunotherapy in mesothelioma. **Cornelissen R**, Lievense LA, Heuvers ME, Maat APWM, Hendriks RW, Hoogsteden HC, Hegmans JPJJ, Aerts JGJV. *Immunotherapy (2012) 4(10), 1011–1022*

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Ratio of Intratumoral Macrophage Phenotypes Is a Prognostic Factor in Epithelioid Malignant Pleural Mesothelioma. **Cornelissen R**, Lievense LA, Maat AP, Hendriks RW, Hoogsteden HC, Bogers AJ, Hegmans JP, Aerts JG. *PLoS ONE 2014; 9: e106742*.

A tumor is more than just tumor cells; it contains for example stromal cells, endothelial cells and is often invaded by numerous cells that belong to the natural defense system of the body (immune system). The latter can have a dual role in tumor genesis. At one hand, these immune cells are present at the tumor site to eliminate tumor cells. On the other hand, immune cells are being tricked by the tumor for their tissue repair mechanisms, by which they advance tumor growth by releasing growth factors and inducing angiogenesis. In this thesis, these immunological cells were the focus of my research for their ability to act as predictive or prognostic marker for tumor aggressiveness. Furthermore, modulation of the immune system was tested as therapeutic option for malignant pleural mesothelioma, a disease with limited treatment options.