Targeting Immune Suppression to Refine Dendritic Cell-based Immunotherapy in Mesothelioma

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Targeting Immune Suppression to Refine Dendritic Cell-based Immunotherapy in Mesothelioma

Beïnvloeding van immuunsuppressie ter verfijning van dendritische cel-immunotherapie in mesothelioom

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“Het logische beeld van de feiten is de gedachte”
Ludwig Wittgenstein

Malignant mesothelioma (MM) is a highly aggressive neoplasm caused by neoplastic transformation of mesothelial cells that line the body's serous cavities and the internal organs. In the majority of patients mesothelioma is localized within the pleural cavity. At this moment, no curative medical procedures are available. With median survival of 9 to 12 month after first signs of illness, the prognosis is poor.
Mesothelioma is strongly associated with the exposure to airborne asbestos particles. Although the relation between exposure to asbestos and the occurrence of disease was already reported by the ancient Greek geographer Strabo and the roman naturist Gaius Plinius Secundus in 23 AD*, the first scientific report was published in the 1960's by Wagner et al. (1). He reported a strong correlation between exposure to asbestos and the occurrence of mesothelioma, among asbestos-mine-workers in South-African's Cape Province. Studies of Stumphius et al. noticed an increased incidence in mesothelioma cases among shipyard-workers in the "Royal Schelde" shipyard (2).
Even though it became more distinct that asbestos exposure was one of the main causes for mesothelioma, the commercial use of asbestos peaked during the seventies. Tons of asbestos-containing materials are still present in buildings, ships and infrastructures. Production and usage of asbestos-containing material maintained in the Netherlands till the interdiction of asbestos-use in 1993. As millions of people were exposed to carcinogenic fibres, with accompanying serious health risks undisputed there is a responsibility for governments to support mesothelioma research. The United Kingdom's Ministry of Justice has recently announced that the U.K. government is obligated to address the medical aspects of mesothelioma **.
It is a misconception that with the ban of asbestos-use, the occurrence of mesothelioma will disappear within the following decades. Many people world wide are incidentally exposed to asbestos fibres. During the collapse of the New York World Trade Centre in 2001 the citizens of New York not only faced the tragedy of a terrorist attack, but as a consequence thousands of people were exposed to a dust-cloud containing 400 tons of asbestos fibres (4% of all fibres within the dust-cloud). Soon after 9/11 the Mesothelioma Applied Research Foundation (MARF) was established to fund research, investigating new treatment strategies to cure mesothelioma.

Figure 1: Demonstration of ships at Alang India by unprotected workers (left), asbestos industry in India and protest against importation of asbestos from Canada (right) (photo's E. Burtynski (left) and Hazards Compaign)

Asbestos is a well known carcinogen to most people in western countries, however this does not apply to people in developing countries. As the western world tries to ban asbestos, the utilisation in Asian countries, especially India, is growing (3). Asbestos is collected in ship breaking yards in Alang India from western ships that contain high amount of asbestos-containing material, making these ships to expensive to dismantle in developed countries (due to high costs of safety measurements). After grinding the asbestos-containing materials, the asbestos fibres are remodelled into asbestos-cement and corrugated roof-plates (Figure 1). Besides the extraction of asbestos from ships, asbestos is also imported on large scale by Asian countries from Canada *** . The Canadian government funds an industry lobby group, the Chrysotile Institute, which promotes the "controlled use" of chrysotile asbestos in developing countries. The distribution of chrysotile asbestos is still possible, since chrysotile asbestos was not mentioned in the Rotterdam Convention in the 1980's. The Rotterdam Convention did reject the use of all other types of asbestos, in their effort to ban toxic agents for health or environmental reasons. At that time is was a misapprehension that chrysotile is less carcinogenic, since chrysotile can be cleared from the lungs more easily. Despite the opposition of the world health organisation (WHO), Canada increased its asbestos mining activities and exported more asbestos last year (4). With the increased production, the total production is similar to the peak period in the sixties. Besides Canada, Russia, China and Kazakhstan are also mining for asbestos.
In the absence of official actions the use of asbestos is increasing in large parts of the world. In 2006, Greenpeace successfully prevented the dumping of warships containing tonnes of asbestos by making the France public aware of these activities by their government. Growing concerns are reported by public health experts and the WHO (3, 5). Although the awareness from western communities is rising, stopping the export of asbestos and asbestos-containing material to third-world countries is still problematic, since there are economical interests involved. It's becoming more distinct that the ban of asbestos did not solve but only transferred the problem to third-world countries. An underestimated number of 90.000 people die of asbestos-related diseases each year and the incidence is still rising.

* R.C. Barbalace, 1995-10-22; Environmentalchemistry.com; "History of Asbestos"

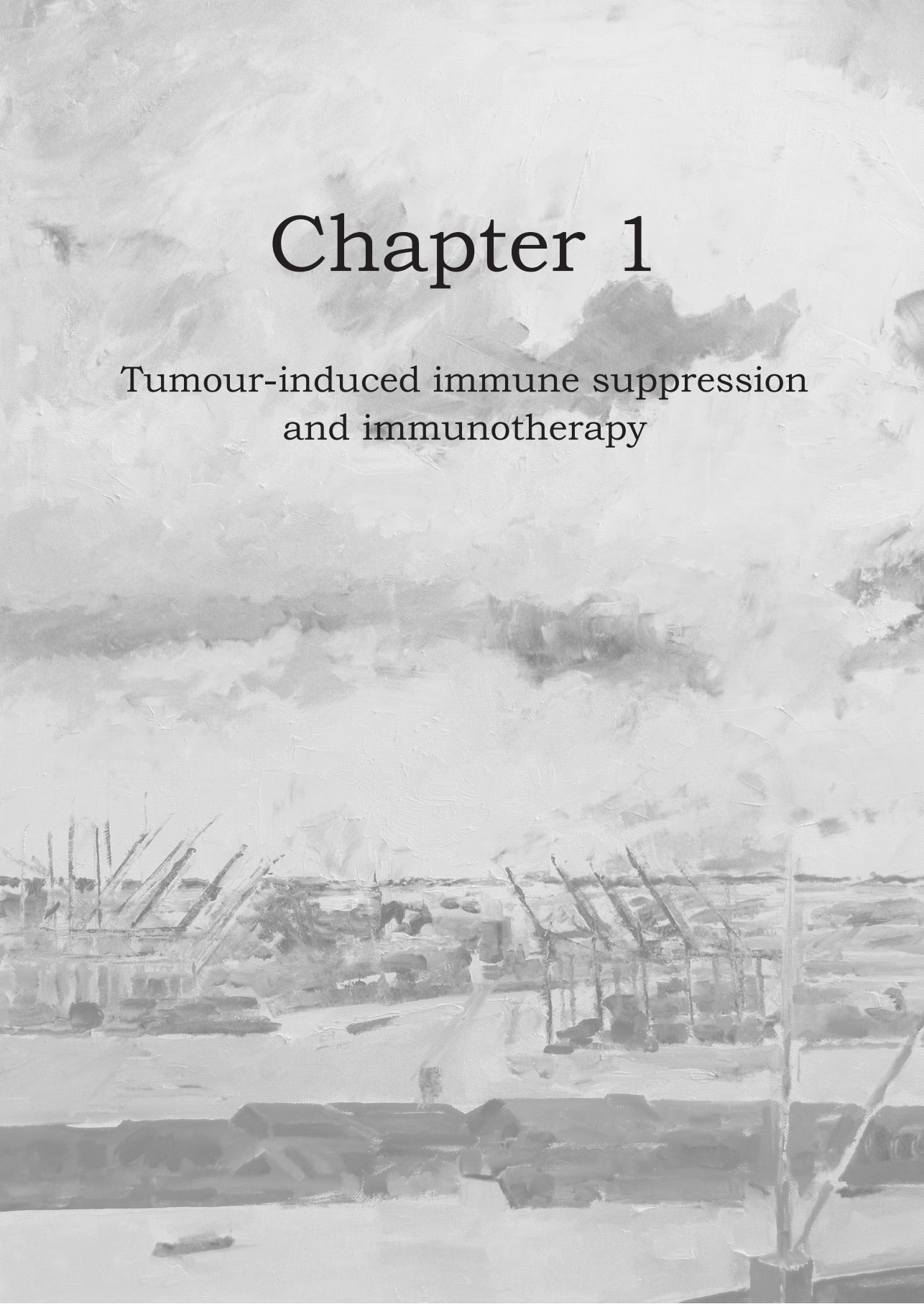
** Ministry of Justice U.K., 27-10-02 ; Government announces meassures on asbestos-related illness

*** D. Allen & L. Kazan-Allen; www.ibasecretariat.org; India's asbestos time bomb

(ref 1-5 are included in the reference list of Chapter 1)

Chapter 1

Tumour-induced immune suppression and immunotherapy



CHAPTER 1: Tumour-induced immune suppression and immunotherapy

Cancer is a major worldwide health problem and one of the most important causes of morbidity and mortality. Cancer is a disease which is characterized by abnormal proliferation of genetically altered cells, which typically invade other tissues and can spread to other parts of the body (metastasize). Defects in regulatory circuits that govern normal cell proliferation and homeostasis can occur in different cell types leading to distinct kinds of cancer. Changes in the genome, causing abnormal proliferation, are influenced by environmental factors and genetic-predispositions.

Progress in cancer treatment using current treatment strategies (e.g. surgery, chemotherapy, radiotherapy, or combinations of these) has been made in the last decades. However, most cancers are still difficult to treat because in many cases the cancer recurrences. Also, these treatments can cause major late-onset morbidity and even mortality for instance due to cardiovascular diseases or an increased rate of secondary malignancies. Therefore, researchers are focussing on new approaches to develop treatments to increase cure rates, and which are more tumour-specific with fewer side effects. One of the approaches, which recently gained a lot of interest, is cancer immunotherapy. It attempts to harness the exquisite power and specificity of the immune system to recognize and to fight tumour cells. As scientific knowledge increases, it is becoming more evident that during tumour progression there is a constant interaction between the immune system and the developing tumour. While some immune cells are trying to bring tumour growth to a hold, other cells of the immune system are favouring tumour progression. This thesis reports on investigations to improve the effectiveness of immunotherapy approaches by targeting tumour escape mechanisms.

1.1. The hallmarks of cancer

In 2000, Hanahan and Weinberg described six essential alterations in normal cell physiology, which together define the prognosis of most human malignancies (1). Although the pathways that cells undertake on their way to transform to malignant cells are highly variable, it is well established that during this process several mutations in onco-genes and tumour suppressor genes take place (2-3). Mutations in these genes are essential in the acquisition or loss of biological capabilities. These mutations can occur at different time points during tumour progression and differ between tumour types. Once formed, all cancer types are composed of a complex mixture of various cell types; including tumour cells, immune cells and stromal cells; that collaborate to create malignant growth. Extensive investigations are elucidating the complexity of these different cell types that exist symbiotically with the tumour cells. Genetic and epigenetic alterations of tumour cells are eventually reflected in biological properties of cancer; that are common in all types of cancer (1, 4). These characteristics in tumour biology led to the description of six essential alterations in cell physiology that collectively dictate malignant growth; including 1: self-sufficiency in growth signals, 2: insensitivity to growth inhibitory (anti-growth) signals, 3: tissue invasion and metastasis, 4: unlimited replicative potential, 5: sustained angiogenesis, and 6: evading programmed cell death (apoptosis). Recently, cancer-related inflammation is recognized as the seventh hallmark [7] (5-6) (Figure 1). These will be briefly discussed in the next part.

A B

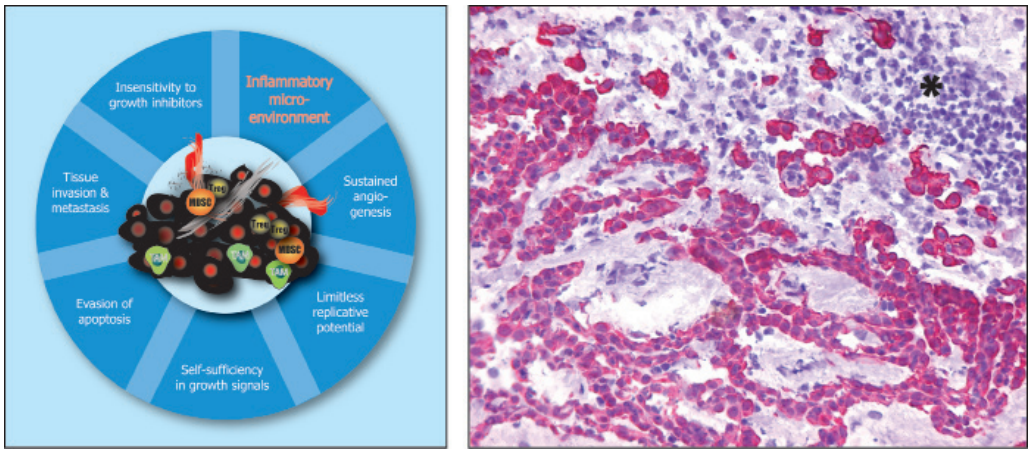


Figure 1 A) In 2000, Hanahan described the six hallmarks of cancer. Recently a seventh hallmark, the inflammatory micro-environment was recognized as a important hallmark of cancer progression. B) Infiltration of inflammatory cells into tumour tissue is frequently observed in several types of cancer: cytokeratine 19 expression on mesothelioma cells (red) with infiltrating inflammatory cells at the rim of the tumour (*).

1. Self-sufficiency in growth signals: Normal cells require growth signals for proliferation. Commonly, these stimuli are produced by one cell type in order to stimulate proliferation of another. However, in cancer cells, signalling molecules are over-expressed and growth-stimulating signals are amplified. Thus tumour cells acquire the ability to synthesize growth factors to which they are responsive, promoting uncontrolled division and cell growth (7).
2. Insensitivity to growth inhibitory signals: In normal tissue, multiple anti-proliferative signals exist to maintain cell quiescence and tissue homeostasis, however cancer cells become insensitive to such anti-growth signals and maintain replicative capacities (6).
3. Tissue invasion and metastasis: Growth of a tumour can lead to invasion of surrounding tissue or to metastasis to other tissues in the body. In order for cancer to spread, cells must acquire mutations that turn on genes which allow them to break free from the primary tumour and travel to other sites in the body. Metastatic growth is the cause of death in 90% of all human cancers (8).
4. Unlimited replicative potential: Replication without limitation requires maintenance of the chromosomal ends, known as telomeres. As cells divide, telomere length is reduced. Tumour cell can overcome this limitation by up regulating expression of telomerase enzymes (9). Thereby cells can keep their telomeres of the chromosomal ends above a critical threshold, making them capable of unlimited multiplication. In addition, fusion of chromosomes ends is frequently observed in cancer cells, which also facilitates multiplication without limitations (10)
5. Sustained angiogenesis: Blood supply is a vital process in the progression of cancer from small, localized neoplasms to larger growing tumors. The expression of growth factors that recruit new vasculature from existing blood vessels is essential to fuel continued growth and division (11).
6. Evading programmed cell death (apoptosis): Normal cells that accumulate excessive DNA damage undergo apoptosis. However, cancer cells are resistant to apoptosis, and thus they continue to grow and divide even as they accumulate mutations (12-15).
7. Cancer-related inflammation: Epidemiological studies have revealed that chronic inflammation predisposes for certain forms of cancer. Increasing evidence indicates

that the "inflammation-cancer" connection is not only restricted to the initiation of cancer process, since all types of cancer appear to have an inflammatory component in their microenvironment. Therefore, inflammation seems a critical component of tumour progression (6, 16-17). Recent reports have shed new light on the molecular and cellular mechanisms linking cancer and inflammation (18-19). Two pathways have been identified. First, the intrinsic pathway leads to activation of different classes of oncogenes driving cells to express inflammation-related mediators that guide the formation of the inflammatory tumour microenvironment. Secondly, in the extrinsic pathway, the inflammatory conditions facilitate tumour growth. To understand these relations, physiological interactions between the immune system and tumour cells need to be discussed.

1.2. Tumour-immunosurveillance

By using murine tumour transplantation-models, Llyod Old, George Klein, and others showed that the immune system of healthy recipient mice was able to detect the transformed malignant cells and differentiate them from normal cells (20-21). The concept that nascent transformed cells could be detected by the immune system was embodied in the cancer immunosurveillance hypothesis by Frank MacFarlane Burnet and Lewis Thomas in 1957; "it is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence" (22). At that time the hypothesis was very controversial, however recently it's becoming more and more evident that the immune system plays a crucial role in host prevention against cancer.

Tumour-associated antigens (TAA) are antigens acquired by a tumour cell in the process of neoplastic transformation that can elicit a specific immune response by the host. Expression of these antigens is caused by mutations leading to synthesis and overexpression of abnormal proteins. The immune system can discriminate between malignant cells and their normal counterparts through recognition of these TAA expressed by tumour cells.

It is known that several immunological cells are involved in the recognition and destruction of tumours during early stages of development. These include cells and factors of the innate and adaptive immune system (23-25). Cells that play a role during the early reaction of the immune system (innate immunity / naive immunity) provide the early lines of defence against pathogens and abnormal cell growth. Macrophages, neutrophils, complement components, $\delta\gamma$ T cells, natural killer (NK) cells, NKT cells and certain cytokines (IL-12, IFN-γ) participate in the innate immune mechanisms protecting against cancer. For example, NK cells can prevent the development of tumours by their ability to produce perforin, leading to the induction of cell death (26-27).

In contrast to the innate immune reaction, cells of the adaptive (specific immunity) phase of the immune reaction have adapted a response to certain antigens. To generate a specific immune response against cancer cells, TAA need to be presented to the cells of the adaptive immune system. Dendritic cells (DCs) play an important role in the activation and modulation of lymphocyte subsets in the adaptive immune system (28-29). DCs are widely acknowledged for their antigen presenting capacity. These antigen presenting cells (APCs) 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. When tumour cells die, TAA are set free. These TAA can be taken up by antigen presenting cells. After taken up of these antigens, the DCs mature and migrate to regional draining lymphoid organs. The captured antigen is processed by major histocompatibility complex (MHC) molecules of DCs and induce antigen-specific activation of lymphocytes. B lymphocytes are capable of producing antibodies, functioning as mediators of the humoral immunity. For example, antibodies can bind tumour cells and assist in their elimination via various mechanisms like enhancing

phagocytosis (opsonisation). Tumour-specific T lymphocytes mediate the cellular immunity. T lymphocytes consist of functionally distinct populations, the best defined of which are helper T cells (Th cells) and cytotoxic T lymphocytes (CTL). In the response to antigen, Th cells stimulate proliferation and differentiation of B and T cells (and others). CTL are capable of killing cells that express foreign antigens.
After activation by dendritic cells in the lymphoid organs, B and T lymphocytes accumulate and enter the circulation. In the elimination of tumours, tumour-specific CTL play a dominant role. CTL can infiltrate and kill tumour cells after recognition of TAA.

1.3. Tumour immune escape

Increasing evidence reveals that when tumour progress in time, tumour cells undergo changes to escape immune surveillance. To account for this, the concept of immune surveillance was refined to a process called "cancer immunoediting" (23). The process encompasses three phases: Elimination, Equilibrium, and Escape. During the first phase, immune surveillance takes place. However, tumour cells that are not eliminated by the immune system can enter the equilibrium state, in which there is an equilibrium between tumour growth and tumour killing by cells of the immune system. In this stage, tumours can persist for years without progressing to more severe tumour stages. However, during this period, tumour cells may further accumulate mutations; potentially generating variants that can escape the immune system, by either evading the induction of an immune response or the inhibition of anti-tumour responses via a variety of mechanisms. Some of these mechanisms are related to the tumour cells, whereas others act via the attraction and interaction with infiltrating immune cells.

1. Decreased expression of MHC class I molecules leads to decreased TAA presentation to T cells. Downregulation of MHC molecule expression makes tumour cells less recognizable for cytotoxic T cells.
2. Tumour cells can also lose the expression of TAA that elicit an immune response. If these antigens are not required for growth of the tumour or maintenance of the transformed phenotype, the antigen-negative tumour cells have a growth advantage in the host.
3. Cell surface antigens on tumour cells can also be hidden from the immune system by glycocalyx molecules, such as sialic acid-containing mucopolysaccharides.
4. By the production of immune suppressive substances such as vascular endothelial growth factor (VEGF), interleukin 10 and 23 (IL-10, IL-23) and transforming growth factor beta (TGF-β), tumour cells can directly affect the function of DCs and T cells within the tumour microenvironment. In addition, tumour cells can also exploit mechanisms leading to the abolishment of the induced anti-tumour response. Several tumour types express programmed death ligand-1 (PD-L1) which inhibits T cells through interaction with the negative co-stimulatory receptor, PD-1 (30). Some malignancies may also employ nutrient catabolism to establish tolerance by depriving effector T cells of the necessary nutrients to mount a robust immune response. For example, many tumour tissues have an increased expression of indoleamine 2,3-dioxygenase (IDO), which catalyzes the essential amino acid tryptophan, arresting T cells in the G1 phase, and thereby providing immune escape (31).
5. Tumour cells may also induce specific immunologic tolerance. Tolerance may occur because tumour cells present TAA in a tolerogenic form to lymphocytes.

1.4. Immune suppressive mechanisms

Another important escape mechanism how tumours can resist immune destruction is the induction of an immunesuppressive microenvironment. In the tumour microenvironment, inflammatory cells and molecules influence almost every aspect of the cancer progress. For

example, specialized white blood cells (M2 type macrophages [TAM] and myeloid derived suppressor cells [MDSC], see below) in the tumour environment assist the malignant behaviour of tumour cells, not just by producing cytokines, but also by secreting growth factors and matrix-degrading enzymes. These matrix-degrading enzymes can mobilize the cytokines (e.g. IL-1β, IL-6) and growth factors (e.g. VEGF) from the extracellular matrix which promote tumour progression or enhance metastasis. These cells can also act directly on tumour cells, endothelial cells and on antigen-specific T cells. Furthermore, tumour tissue, peripheral blood and lymph nodes contain significant amounts of regulatory T lymphocytes (Tregs), capable of suppressing the effector arm in anti-tumoural responses. It is evident that immune cells can either protect the host against cancer development or promote the emergence of tumours with reduced immunogenicity leading to a complex interplay of tumour growth and tumour regression mechanisms (17). In the next sections, three types of immune suppressive cells are discussed; myeloid derived suppressor cells (MDSC), tumour-associated macrophages (TAMs) and regulatory T cells (Tregs). These cells can blunt the immune response against cancer and inhibit the effect of therapeutic approaches. Therefore, targeting the immune suppressive microenvironment is proposed as a strategy to improve and refine novel therapies (4, 17) (Figure 2).

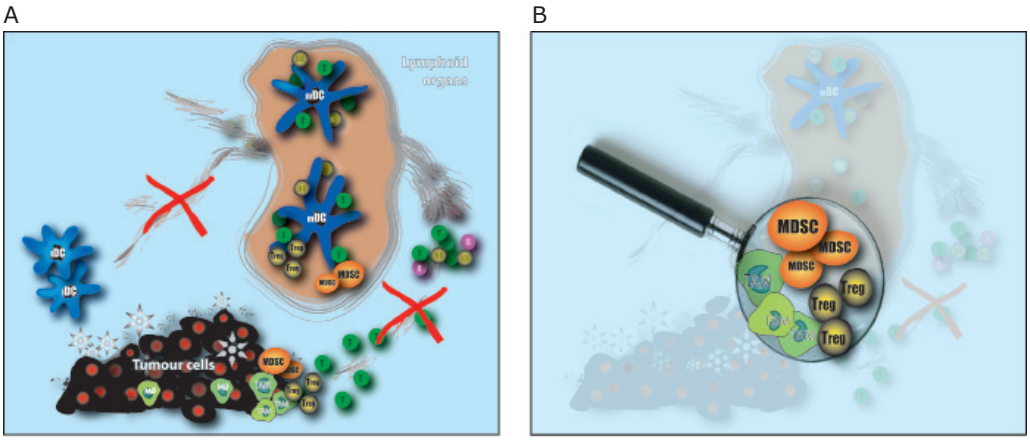


Figure 2 A) Tumours escape immune surveillance by avoiding recognition by the immune system via distinct mechanisms (left part) or by inhibiting anti-tumour responses via the induction of a suppressive microenvironment. B) Within this environment there are three immune cell types abundantly present; myeloid-derived suppressor cells (MDSC), tumour-associated macrophages (TAMs) and regulatory T cells.

1.5. Myeloid-derived suppressor cells

1.5.1. Identification of MDSC

Myeloid-derived suppressor cells (MDSC) were first described by Bronte and Gabrilovich in 2000/2001 (32-33). MDSC are a heterogeneous population of bone marrow derived myeloid cells (thus include both mature and immature/precursor phenotypes), comprising of immature monocytes/macrophages, granulocytes, and DCs at different stages of differentiation and expressing a number of surface markers with immunosuppressive functions (34). To prevent confusion in the field, the previous terms of 'myeloid suppressor cells (MSC)' or 'immature myeloid cells (iMC)' are nowadays replaced by MDSC (34).

Accumulation of these cells has been reported under pathologic conditions, including cancer, various infectious diseases, sepsis, trauma, bone marrow transplantation and some autoimmune disorders. In pathological conditions, a partial block in the differentiation from immature myeloid cells to mature myeloid cells results in the expansion of this

population. Factors released in blood from affected tissue, promote the expansion of MDSC through stimulation of myelopoiesis and inhibition of the differentiation towards mature myeloid cells (35). Although recent publications clearly demonstrate that the expansion of an immunosuppressive MDSC population is frequently observed in many pathological conditions, most of the attention has been focused on the role of these cells in cancer (33-42). Up to a tenfold increase in MDSC numbers were detected in the blood of patients with different types of cancer and in many murine-tumour models, as many as 20–40% of nucleated splenocytes are MDSC (35).

MDSC are characterized by the expression of immature markers and myeloid markers. Most importantly, these cells possess a high potential to suppress immune responses in vitro and in vivo (34). MDSC can upregulate genes related to the metabolism of amino acids (L-arginine), production of reactive oxygen species (ROS) and nitrogen species (NOS), as well as upregulation of immune suppressive surface molecules. How this influences immune responses is discussed in 1.5.3. More recently, subsets of MDSC have been identified within the heterogeneous group of immature myeloid cells. Two major groups of MDSC were recognized based on their morphology, the intensity of surface marker expression and cell metabolism (36-38). Poly-morph nuclear (PMN)-MDSC are phenotypically granulocyte-like, whereas mononuclear (MO)-MDSC are phenotypically monocyte-like. In mice both MDSC types express the markers: CD11b, Gr-1, CD1d, CD16, CD32, CD80 (B7.1), CD115 (c-fms) and CD124 (IL-4Rα), but differ in the expression of F4/80, CD54, CD68 and CCR2 (that are low expressed by MO-MDSC and absent on PMN-MDSC) and CXCR1 (that is only expressed by PMN-MDSC) (34, 39-40). In human these cells express CD11b, CD33, CD66b and CD124. In addition, PMN-MDSC expresses CD15 and VEGF-receptor and MO-MDSC express CD14 and are able to express HLA-DR at low levels (35, 40).

Additionally, a third population has been defined; however this group is rare compared to the PMN-MDSC and MO-MDSC populations. Since most studies focus on the more abundant groups of MDSC, not much is known about this subpopulation. This population consists of Gr-1low expressing myeloid cells, but lack markers of mature myeloid cell types. Based on sideward scatter (SSC) of CD11b+Gr-1low cells, this population can be subdivided into two populations; SSClow and SSChigh cells. SSClow cells harbour immune suppressive capacities, whereas the SSChigh cells are not immune suppressive (36, 39). More research is required to determine how these cells contribute to tumour progression.

1.5.2. Recruitment of MDSC

MDSC are found in blood, the lymphoid organs and in the tumour of tumour-bearing hosts. Recruitment of MDSC is largely depending on tumour-derived factors and factors expressed by stromal cells / inflammatory cells associated with tumour cells. Several chemokines are reported to play a dominant role in the recruitment of MDSC from the bone marrow to the lymphoid organs and the tumour site. Under pathological conditions stimulation of myelopoiesis and inhibition of differentiation of myeloid cells leads to the accumulation of these cells. One of the most important factors during carcinogenesis enhancing the myelopoiesis of MDSC is VEGF. VEGF is best known as a pro-angiogenic growth factor, stimulating tumour vascularisation, however, it is now well established that VEGF is also required for MDSC recruitment (35). Other tumour-derived factors, which are involved in MDSC recruitment, are granulocyte /macrophage colony stimulating factors (GM-CSF, G-CSF and M-CSF). Stromal cells associated with tumours can produce factors (like IL-1β, IL-4, IL-6, IL-10, IL-13, IFN-γ and prostaglandins) that stimulate the recruitment of MDSC (35, 41). In addition, MDSC themselves can also contribute to accumulation and activation via the production of pro-inflammatory mediators (like IL-6 and S100A8/A9) functioning as an autocrine feedback loop (42). The signalling pathways in MDSC that are triggered by most of these factors converge on Janus kinase (JAK) protein family members and signal transducer and activator of transcription (STAT). STAT3 is most likely the main

transcription factor that regulates the expansion of MDSC (43-45).

1.5.3. Immune suppression by MDSC

Activation of MDSC not only requires tumour-derived factors, but also IFN-γ produced by T cells and factors secreted by tumour stromal cells (like IL-1β, IL-4, IL-6, IL-10, IL-13). Most cytokines trigger STAT family of transcription factors. In addition, the arginase metabolism in MDSC is depending on tumour-derived prostaglandin E2 (PGE2). Activation of cytokine receptors on MDSC leads to activation of STAT-signalling pathways, resulting in the production of immune suppressive substances (like TGF-β, ROS and NOS) (35, 41). MDSC contribute to tumour-associated immune dysfunctions through a myriad of mechanisms, including production of nitric oxide, arginine depletion, and production of reactive oxygen species

MDSC are found at different locations in tumour-bearing hosts. These cells can accumulate in tumour tissue, blood, and in lymphoid organs. MO-MDSC are mainly found at the tumour site and can inhibit the anti-tumour response in an antigen non-specific manner by the production of NO. High expression of the enzyme iNOS, leads to the generation of NO. NO can suppress T cell function though various mechanisms involving the inhibition of the cell signalling pathways and inducing DNA-damage to T cells. DNA-damage leads to the induction of T cell apoptosis. High arginase metabolism in MO-MDSC prevents the upregulation of cell cycle regulators in T cells, leading to the inhibition of the proliferation (35, 46). The suppressive activity of MDSC has been associated with the metabolism of L-arginine. L-arginine serves as a substrate for two enzymes: iNOS, which generates NO, and arginase, which converts L-arginine into urea and L-ornithine. MDSC express high levels of both arginase and iNOS, and a direct role for both of these enzymes in the inhibition of T-cell function is well established (46-48). Recent data suggest that there is a close correlation between the availability of arginine and the regulation of T-cell proliferation (49-50). The increased activity of arginase in MDSC leads to enhanced L-arginine catabolism, which depletes this non-essential amino acid from the microenvironment. The shortage of L-arginine inhibits T-cell proliferation through several different mechanisms, like for example preventing their upregulation of the expression of the cell cycle regulators (46).

PMN-MDSC are found in blood, lymphoid organs and at the tumour site where they can suppress T cell activation in an antigen specific manner by the production of reactive oxygen species (ROS). MDSC capable of producing ROS are also capable of producing peroxynitrite, which is responsible for most of the adverse effects on T cells, linked to ROS. Peroxynitrite induces the nitration of several amino acids. Changes caused by nitration of the T cell receptor makes T cells incapable of interacting with the MHC complex on antigen presenting cells, which is necessary to obtain T cell specific stimulation (51-53). Besides, increased arginase 1 metabolism in MDSC enhances L-arginase catabolism, resulting in the downregulation of the CD3z-chain expression. This leads to alteration of the T cell receptor, contributing to the inducing of T cell tolerance by PMN-MDSC (35, 51).

Besides the induction of T cell anergy and T cell apoptosis, MDSC can also inhibit T cell proliferation by producing TGF-β. In addition, MDSC can also inhibit other anti-tumour cells, like NK- and NKT- cells, via TGF-β depending mechanisms. MDSC can bind to the TGF-β receptor on target cells via membrane bound TGF-β, leading to activation of intra cellular pathways resulting in downregulation of NK specific receptors (54).

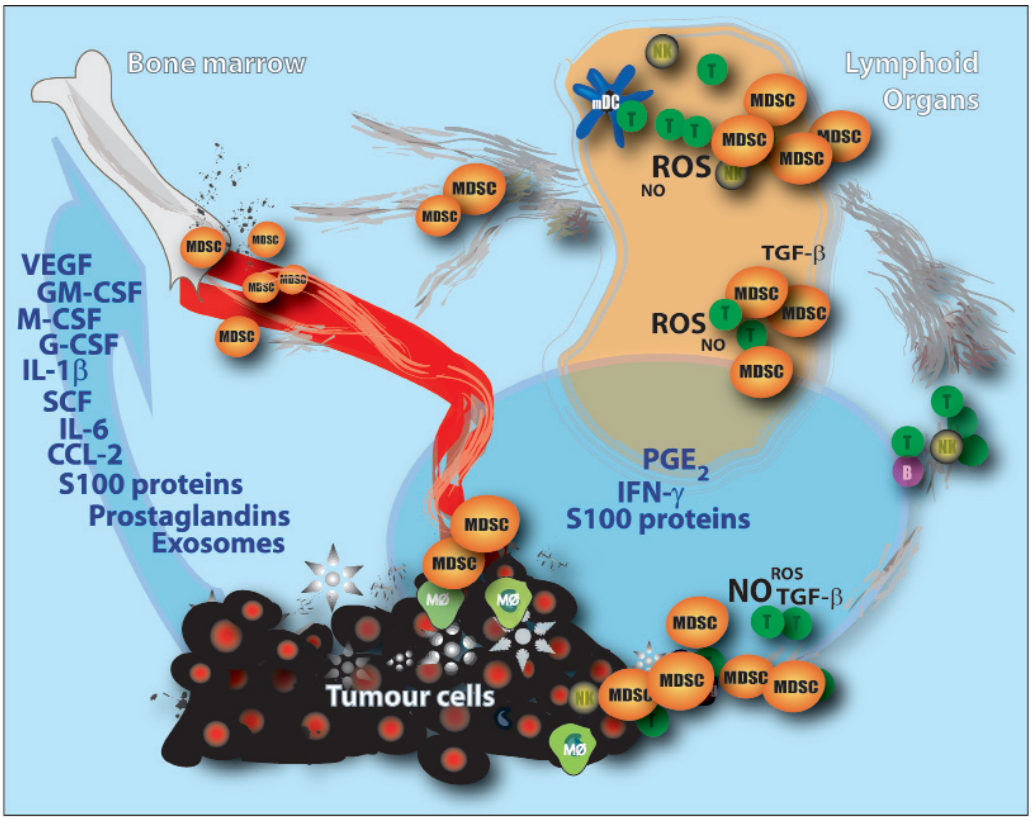


Figure 3 MDSC are accumulation in the lymphoid organs as well as at the tumour site by tumour/stromal cell derived factors, like VEGF. This leads to an influx of MDSC in the lymphoid organs and in tumour tissue. In the lymphoid organs and at the tumour site the anti-tumour response is blocked by products secreted by MDSC. ROS production leads to downregulation of the ζ-chain on T cells. MDSC can inhibit NK and NK-T cells via membrane bound TGF-β, which leads to the downregulation of NK-specific receptors. NO production by MDSC can induce T cell apoptosis and inhibit T cell proliferation. MDSC require tumour-derived PGE2 and T-cell-derived IFN-γ for their function. In addition, the S100 family of inflammatory mediators production by MDSC can function as an autocrine stimulating factor.

1.5.4. Interaction with Tregs

In addition to their direct immune suppressive capacities, MDSC can also indirectly enhance immune suppression via the induction of regulatory T cells. Several studies reported that there is a distinct relation between regulatory T cells and MDSC. Although the exact mechanisms are not completely understood and may differ between tumour types, is has been shown that MDSC can induce Treg-expansion. Moreover, MDSC can also promote suppression of tumour-specific cytotoxic T cells by Tregs by processing and presenting TAA to T cells. TAA presentation in combination with IL-10 and TGF-β stimulation leads to the transcription of FoxP3+ by T cells (59-62).

1.5.5. MDSC as precursor cells

Although MDSC are mainly known for their immune suppressive capacities, they also contribute to tumour progression via several other mechanisms. Since the heterogeneous population of MDSC consists of immature myeloid cells, part of these cells are pluripotent and capable of differentiating into mature cell types. In the presence of appropriate growth factors and cytokines, MDSC can differentiate to dendritic cells, macrophages or endothelial cells (35, 52, 59).
Recent investigations led to the consideration that MO-MDSC can differentiate to TAMs. Since tumour-associated macrophages are the major inflammatory cell type within the tumour microenvironment, it has been proposed that MO-MDSC can function as a source for TAMs (60-61). As MO-MDSC migrate to the tumour site they downregulate ROS production and upregulate their arginase 1 and iNOS expression. Part of this cell population is rapidly differentiated to macrophages. The increased expression of arginase 1 and NO is maintained by TAMs that are already present in the microenvironment (59).
It has been suggested that distinct MDSC populations can function as endothelial progenitor cells (EPC) (62). These cells are characterized by the expression of the endothelial marker CD31 and Tie-2 and can be incorporated into the vascular endothelium. However, it is still unclear under which conditions these MDSC can differentiate to endothelial cells but it has been established that MDSC can contribute to lympho- and angiogenesis (62). PMN-MDSC are capable of producing matrix metallo-proteinase-9 (MMP-9), an enzyme involved in matrix degradation. MMP-9 contributes to the regulation of tumour-vascularisation. It has been suggested that the cleavage of the extracellular matrix contributes to the bioavailability of VEGF within the tumour microenvironment (63). In addition, MDSC themselves can also produce pro-angiogenic factors (like VEGF and prokineticin-2) enhancing blood vessel formation.

1.5.6. Conclusion

Recent studies investigating the role of these cells in cancer have led to the establishment of this cell type as an important immune regulatory cell (35). However, several aspects of this heterogenic cell population require further investigation. For example, the roles of specific MDSC subsets in mediating T-cell suppression, and the molecular mechanisms responsible for inhibition of myeloid-cell differentiation, need to be elucidated. In addition, as many investigations on these cells are performed in murine models, better characterization of human MDSC and whether targeting these cells in patients will be of clinical significance should be the main focus of future research. Nevertheless, it is indisputable that these cells contribute to tumour immune escape by inhibiting anti-tumour responses and therefore these cells are an interesting target to improve novel therapeutic approaches.

1.6. Tumour-associated macrophages (TAMs)

1.6.1. Identification of TAMs

Among the cells present in the tumour microenvironment, TAMs are a major component of the host leucocytic infiltrate (64). Classically activated (M1) macrophages, following exposure to IFN-γ, have tumouricidal activity and elicit tissue destructive reactions. In response to IL-4 or IL-13, macrophages undergo alternative (M2) activation. In general, M2 macrophages are oriented to tissue repair and remodelling, immunoregulation, and tumour promotion. TAMs generally have the phenotype and functions similar to M2 macrophages and display a defective NF-κB activation in response to different pro-inflammatory signals (65-66). They can be characterized on surface expression of general macrophages markers (Murine: F4/80, MHCII, CD11b and CD11c; Human: HLA-DR, CD11b, CD11c) and the expression of mannose-receptor (CD206) and scavenger receptor (CD163). Adjacent to this, macrophage phenotype can be identified based on expression of cytokines, chemokines and growth factors (For example, M1 macrophages are capable of producing IL-12 and IL-6, whereas M2 macrophages can produce IL-10, CCL17 and CCL22) (64, 67).

1.6.2. Recruitment of TAMs

TAM recruitment in tumours is mediated by cytokines belonging to different classes including colony stimulating factor-1, VEGF and chemokines (like CCL2) (68). As described previously (1.5.5.), it has been shown that MO-MDSC are capable of differentiating towards TAMs. Therefore, similar recruitment factors are described that contribute to the infiltration of TAMs and MDSC into tumour tissue (69-70).

In addition, dynamic changes of the tumour microenvironment occur during the transition from early neoplastic events toward advanced tumour stages. These events drive the switch from a M1 macrophage toward the M2 type by profound changes occurring in the tumour microphysiology (e.g. hypoxia, glucose levels, pH). The presence of hypoxic areas is found in most forms of solid tumour. TAMs accumulate in areas where hypoxia occurs. Hypoxia-inducible factor 1 (HIF-1) has been shown to control the cellular response to hypoxia and is essential for myeloid infiltration. As M1 macrophages are attracted to the tumour site during the elimination phase to eradicate tumour cells, changes in tumour microphysiology can occur during the equilibrium phase which leads to a shift in phenotype toward M2. This shift drives macrophages to production of factors (like MMP-9, VEGF and TGF-β) facilitating tumour cell proliferation, tumour cell migration and invasion, angiogenesis and tumour immune escape (68).

1.6.3. Immune suppression by TAMs

TAMs are able to suppress the adoptive immune response through various mechanisms. TAMs acquire a M2 phenotype, which is characterized by the production of immune suppressive cytokines, like IL-10, IL-6, IL-1β, TGF-β, CCL17, CCL18 and CCL22. The inability to produce pro-inflammatory cytokines has been assigned to defective NFκB activation in TAMs. IL-10 in combination with IL-6 can lead to upregulation of molecules in TAMs, which are implicated in suppression of tumour-specific T cell immunity (71). In addition, T cell activation is blocked by the enzyme indoleamine dioxygenase (IDO). IDO catalyzes the catabolism of tryptophan, an essential amino acid acquired for T cell activation (72).

In addition, TAMs also contribute to immune suppression via indirect ways. Secretion of CCL18 leads to recruitment of naïve T cells. Attraction of naïve T cells into the tumour microenvironment is likely to induce T cell anergy (73-74). Besides CCL18, CCL17 and CCL22 are abundantly expressed. These cytokines interact with CCR4 receptor, expressed

by Tregs and induces T-helper 2 polarization (75). Via expression of VEGF, TAMs can block antigen uptake by antigen presenting cells and attract MDSC, which can function as TAM precursors but are also actively suppressing T cell function (as described previously 1.5.3 and 1.5.5). MDSC are depending on PGE2 for their function. PGE2 is secreted by many types of cancer; however TAMs are also capable of producing PGE2 and therefore assist MDSC function (39).

1.6.4. Angiogenesis and tumour invasion

In literature TAMs are particularly reported for their contribution to angiogenesis and promoting tumour invasiveness. In tumour stroma, macrophages can produce enzymes leading to degradation of the extracellular matrix. MMPs are overexpressed in various types of cancer, especially MMP-2 and MMP-9. MMP can be produced by PMN-MDSC, tumour cells and TAMs. Overexpression of MMPs leads to increased cleavage 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 (76). Besides indirect mechanisms, angiogenesis is also directly stimulated by TAMs. TAMs can produce proangiogenic factors like VEGF and platelet

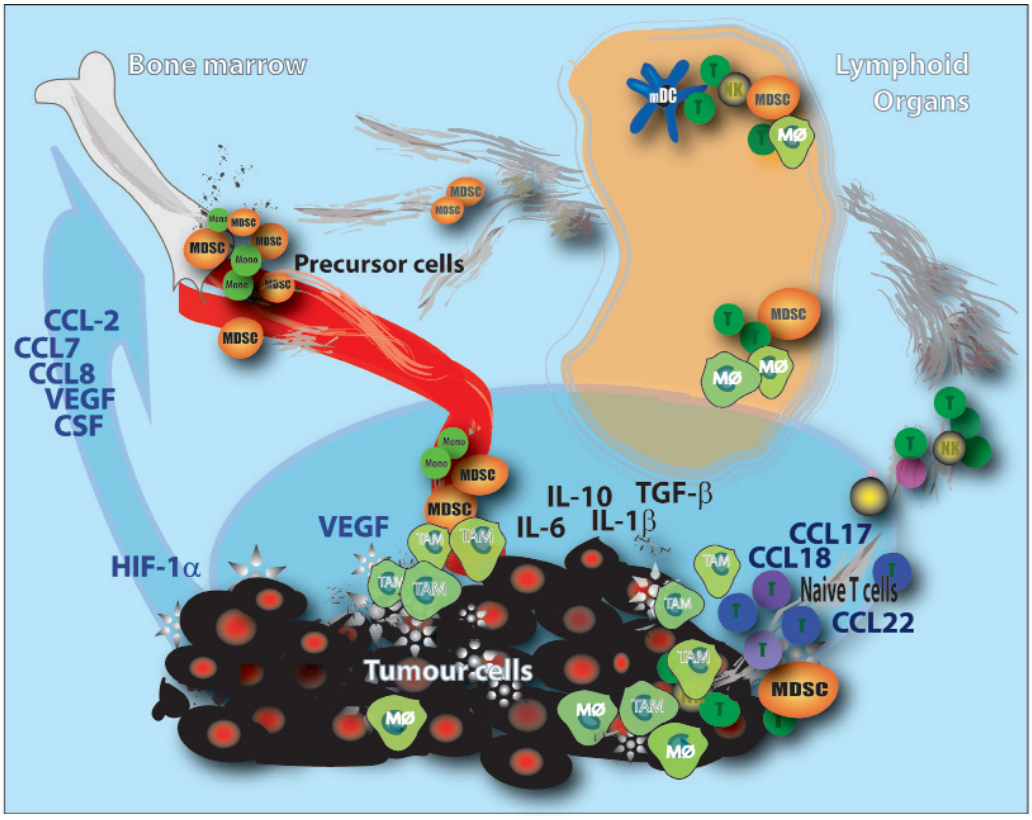


Figure 4 TAMs are recruited by HIF-1α-induced and tumour/stromal cell secreted factors, like CCL2 and VEGF. These factors stimulate the myeloid-haematopoiesis, leading to the accumulation of myeloid precursor cells. These cells are recruited to the tumour site where they rapidly differentiate into macrophages. TAMs are distinctly polarized M2 macrophages, capable of producing immune suppressive cytokines (e.g. TGF-β). In addition they can recruit naïve T cells, which can lead to the induction of T-cell tolerance.

derived growth factors (PDGF) (74, 77). The release of these factors leads to the formation of (lymph)angiogenic structures and subsequent dissemination of metastasis (78).

1.6.5. Conclusion

TAMs are key orchestrators of the cancer-related inflammation present in the tumour microenvironment. TAMs produce a host of growth factors for endothelial cells, as well as inflammatory cytokines and chemokines that contribute to tumour survival, proliferation and invasion. In addition, immunosuppressive mediators released by TAMs affect anti-tumour responses and facilitate tumour immune escape. Therapeutic targeting of macrophages represents a valuable strategy to complement established anticancer treatments.

1.7. Regulatory T cells (Tregs)

1.7.1. Identification of Tregs

Regulatory T cells (Tregs) entail a suppressive 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 (83-85).
Characteristic of Tregs is the expression of CD25 (IL-2 receptor-α chain), forkhead/winged-helix transcription factor box P3 (Foxp3), glucocorticoid-induced TNF-receptor-related-protein (GITR), lymphocyte activation gene-3 (LAG-3), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), however all these markers are not truly Treg-specific (86). Tregs can be divided into natural Tregs and adaptive Tregs. Natural Tregs are important in the suppression of autoreactive T cells that slip through 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 (87). Adaptive Tregs arise from naïve 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) and TGF-β producing Tregs (Th3 Tregs). These cells are characterized by the secretion of immune suppressive cytokines directly inhibiting T cells and converting DCs into suppressive APCs (88-90). Elevated levels of Tregs have been identified in blood of cancer patients compared with normal individuals and their presence predicts for poor survival (91). Tregs were first recognized to infiltrate human cancers and the prevalence of Tregs in tumour-infiltrating lymphocytes is much higher than their proportion in peripheral blood, constituting 20% or more of tumour-infiltrating lymphocytes (92-93).

1.7.2. Recruitment of Tregs

Natural Tregs are derived in the thymus and migrate into the periphery. Although it is generally accepted that the thymic function is largely reduced after adolescence, Tregs persist throughout human lifespan. It has been proposed that Tregs need to be activated and/or expended 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. However, it is poorly understood which factors are involved in trafficking and regulation of Tregs (94).
It has been shown that Tregs play an important role in tumour immunity. Induction of suppressive activity of both, natural and adaptive Tregs, require T cell receptor triggering by antigen or stimulation with TGF-β (95-96). Weak stimulation or the absence of co-stimulatory molecules leads to the induction of long-lasting suppressive activity. Via this mechanism, Tregs can also be directed against TAA and contribute to T cell anergy against

tumours. TAA-specific Tregs accumulate in the peripheral lymphoid organs and at the tumour 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 inducing T cell tolerance before these cells enter the circulation (94). Although exact mechanisms are not fully explored, it has been shown that CCR4+ (receptor for CCL22) Tregs migrate toward tumour microenvironments expressing CCL22 (97). Also CD62L and CCR7 have been described as important homing markers on Tregs (98). 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) (97, 99).

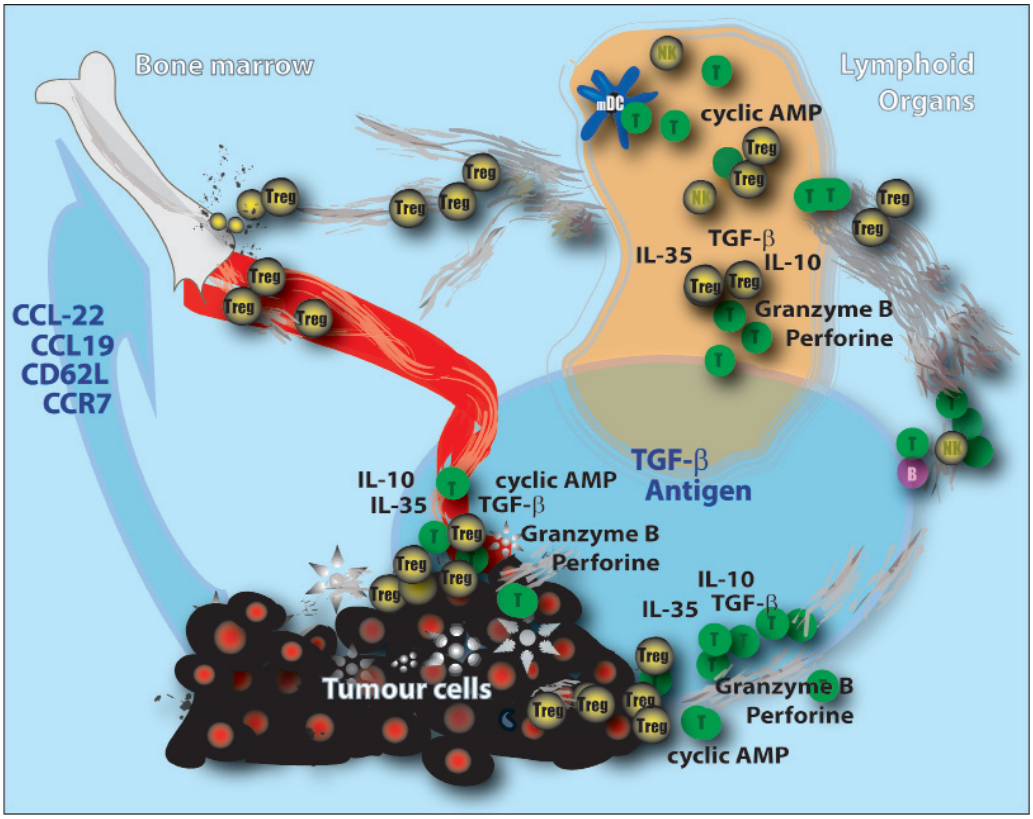


Figure 5 Tregs are attracted to the tumour site and the lymphoid organs by tumour-derived factors, like CCL22, CCL19 and CD62L. Here they can inhibit the anti-tumour response using a variety of mechanisms. They can inhibit effector cells by secretion of immune suppressive cytokines (e.g. IL-10, IL-35 and TGF-β). Besides, Tregs can kill APCs and effector cells by producing granzyme B and perforin. Via upregulation of cyclic AMP Tregs can inhibit proliferation of effector cells.

1.7.3. Immune suppression by Tregs

Tregs inhibit CD4+ and CD8+ effector cells, dendritic cells and natural killer cells and secrete nonspecific, immunosuppressive molecules such as IL-35, IL-10 and TGF-β to blunt the immune response (100). However, recent studies showed that these cytokines are not the main suppressive factor, facilitating Treg-induced immune suppression (101). In contrast, it's becoming more distinct that direct cell-cell interaction between Tregs and target cells play a dominant role in tolerance induction by Tregs (102-105). Like

MDSC, Tregs can inhibit NK and NK T cells via membrane bound TGF-β . 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 downregulation of the NKG2D-receptor on NK and NK T cells (106). Other studies showed that cell-cell binding interacts with mechanisms leading to apoptosis (by activation of programmed cell death-ligands and the release of perforin and granzyme B) and reduced proliferation (by upregulation of intracellular cyclic AMP) (101).
Besides the effects on target cells, Tregs disturb the induction of the adaptive immune response by preventing proper antigen presentation. Tregs forming aggregates around DCs prevent contact between DCs and T cells [94]. In addition, Treg-aggregation leads to decreased upregulation of CD80 and CD86 on immature DCs and downregulate the expression of CD80 and CD86 on mature DCs. These phenomena are antigen specific and dependent on lymphocyte function-associated antigen 1 (LFA-1) and CTL-associated protein 4 (CTLA-4) (107-108). Though, the exact role of CTLA-4 in Treg-mediated suppression is still under extensive investigation. Additionally, activated Tregs, which express high-affinity IL-2R, may absorb IL-2 from the microenvironment (109). More recently, it has been reported that Tregs can prevent the induction of an anti-tumour response by killing DCs in tumour-draining lymph nodes via a perforin-dependent manner (110).

1.7.4. Conclusion

Increased Treg numbers have been reported in the blood, lymphoid organs and tumour tissue of patients with distinct types of cancer. Elevated levels of Tregs have also been identified in blood of cancer patients and predicts for poor survival (91). In addition, Tregs in tumour-infiltrating lymphocytes is much higher than their proportion in peripheral blood, constituting 20% or more of tumour-infiltrating lymphocytes (92-93). Since these cells can inhibit the anti-tumour response in various ways, it leaves no doubt that Tregs play a critical role in tumour immune surveillance. Targeting these cells may therefore enhance the efficacy of novel therapeutic approaches.

1.8. Re-programming of the immune system

For many years, all efforts to treat cancer have concentrated on the destruction/inhibition of tumour cells. New insights have led to the establishment that immunosuppressive cell types in tumour environments play an important role in cancer progression. Therefore, it has been proposed that targeting MDSC, TAMs and/or Tregs is promising in refining innovative therapeutic strategies. Strategies to modulate the host microenvironment offer a complementary perspective. However, targeting of immune suppressive cells may lead to prolongation of survival by slowing down carcinogenesis; however in combination with activation of the immune system these strategies can unfold therapeutic potential. Therefore, the interest in the development of cancer vaccines has increased tremendously over the last few decades.

1.9. Immunotherapy

Cancer immunotherapy attempts to make use of the ability of the immune system to generate an antigen specific response against tumour cells. Numerous approaches for immunotherapy have been developed. They can be divided into two strategies: passive and active therapy.

Passive therapies rely on the administration of ex vivo expanded/produced immunologic effectors that are capable of directly targeting tumour cells.

1. Monoclonal antibody therapy: Humanized monoclonal antibodies are effective in several human malignancies and during the last decade. Monoclonal antibodies can target cells in various ways. They can target cells directly (like for example Rituximab (anti-CD20) in the treatment of non-Hodgkin lymphomas (107) and Trastuzumab (anti-HER-2/Neu) in breast cancer) or by inducing tumour cell apoptosis. Other monoclonal antibodies can also target angiogenenic factors like VEGF (VEGF-inhibitor Bevacizumab) or be used as immunomodulators to inhibit immune suppressive molecules/cells or activate immune stimulatory molecules. Efficacy of this approach can be enhanced by linking these antibodies to a toxin (e.g. radionucleotides and anticancer drugs).
2. Cell transfer of effector cells: Adaptive transfer of antigen specific effector cells (like T cells and NK cells) can be expanded and/or activated ex vivo and subsequently administered back into the patient to attack the tumour. 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 (108). This led to the concept that antigen specific T cell transfer can be used as an anti-tumour therapy to eradicate established tumours. The effectiveness of adaptive T cell transfer to eradicate malignancies is challenging (109).

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

1. Cytokines: Several cytokines are capable of activating and recruiting specific immune cells that can enhance anti-tumour immunity (e.g. IL-2, IL-12, IL-15, TNF-α, GM-CSF). These cytokines can be used as an approach as single treatment or in combination with other immunotherapy strategies (110)
2. Peptides & proteins: Defined TAA epitopes have been used to vaccinate cancer patients; however this approach is limited by the relatively low number of identified epitopes and by the requirement of MHC typing. Nevertheless, some authors have reported the applicability of this approach. The need of peptide identification can be circumvented by using the whole TAA protein for immunization. These proteins can be taken up by APC and endogenously processed into epitopes for presentation to T cells. Adjuvants need to be added to induce APC activation and avoid tolerance induction (111-113).
3. DNA: In this approach DNA sequences coding for specific TAA are directly injected into the skin. DNA then needs to be taken up, transcribed into mRNA, translated into a protein and processed into peptides by APC. An important restriction is the relatively inefficient delivery into APC (111-113).
4. Recombination viral vectors: Viruses engineered to express TAA can be injected directly into the patient. The virus then transduces the host cell, leading to cell death and presentation of antigenic isotopes to the immune system. A wide variety of viral vectors are available. However there are concerns regarding the immuno-dominance of viral antigens over TAA, resulting in a strong antivirus response leading to virus eradication and attenuation of the anti-tumour immune response (111-112).
5. Whole tumour cells: The ideal source of TAA is the tumour itself, since it expresses all the TAA that need to be targeted. Tumour cell-lines are often used as source for this approach. Tumour cell-lines can be genetically modified to co-express cytokines or co-stimulatory molecules to enhance their immunologic capacity. However, in general, tumour cells display a rather weak antigen presentation capacity and because of the need for ex vivo tumour cell culture, this approach is rather expensive, time consuming and labour intensive (111-112).
6. Dendritic cells: DCs have emerged as the most powerful initiators of immune responses. In the natural activation of the adaptive immune system against tumour cells, DCs play a crucial role since they are capable to engulf tumour antigens and activate lymphocytes in an antigen specific manner. Therefore, the application of dendritic cells to therapeutic cancer vaccines has been prompted (114-115). DCs

can be generated in large amounts ex-vivo, and can be pulsed with tumour antigens in optimal conditions. Subsequently, the injection of matured tumour antigen-pulsed DCs leads to the induction of an anti-tumour response in murine models as well as in patients (116-119). Moreover, DC activation also induces the formation of antibodies against tumour components (117). Therefore, DC-immunotherapy can potentially induce long lasting immune protection.

Over the last decades numerous groups have investigated the safety and applicability of DC-based vaccines in the treatment of cancer in preclinal / clinical studies. DC-based vaccines are studied both in solid tumours and non-solid tumours. Most of the clinical trails have been performed in patients with advanced stages of disease. In the next chapter we introduce DC-based immunotherapy for the adjuvant treatment of mesothelioma.

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Chapter 2

Aims and outline of the thesis

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2.1. Malignant mesothelioma

Malignant mesothelioma (MM) is caused by a neoplastic transformation of mesothelial cells and occurs predominantly in the pleura and less frequently in the peritoneum, pericard or tunica vaginalis testis. Exposure to airborne asbestos fibers is the major causative agent. For the Netherlands, the peak of the epidemic is predicted around the year 2017. The most plausible scenario predicts an increase in pleural mesothelioma mortality up to 950 cases per year in men, with a total death toll close to 18.000 cases during 2000-2028. Currently, nearly 700 new cases are diagnosed each year with MM in the Netherlands. Rotterdam has the highest risk of MM at the national level and is responsible for 1% of overall mortality in men in this area.

With median survival durations of 9-12 months from onset of clinical symptoms, the prognosis is poor (1). Because of the limited success of conventional treatments, novel therapeutic regimens are urgently needed. Currently the combination of pemetrexed (ALIMTA, registered trademark of Eli Lilly and Company) and cisplatin is considered standard of care for patients with mesothelioma, as an increase in median survival of 3 months was noticed (2-5). This combination chemotherapy can reduce tumour burden, however this therapy is used as palliative treatment. A systemic review of the literature provides very limited evidence for the role of surgical cytoreduction in treating mesothelioma (6). It is extremely difficult, painful and not without serious complications (7). Therefore, there is a great need for new therapeutic approaches in these patients. One of the most challenging approaches is DC-based immunotherapy.

2.2. Dendritic cell-based immunotherapy in malignant pleural mesothelioma

Pulsing of DCs can be done in various ways in-vivo and ex-vivo. In-vivo activation of DCs has been attempted by subcutaneous or intratumoural injection of GM-CSF with and without tumour lysate. However, since DC function is suppressed in-vivo by immune suppressive cells (MDSC and Tregs) and tumour derived soluble factors (e.g. VEGF) this strategy leads to difficulties. Therefore, expending the number of DCs ex-vivo, in the absence of immune suppressive components holds more potential. When specific tumour antigens are identified, single peptides can be used to load DCs (8). However, it has become increasingly clear that this "single target" approach is problematic. Since antigen expression by tumour cells is often very heterogeneous and not all tumour cells express similar antigens. Therefore, the use of single peptides results in an immune response limited to the epitopes used for immunization that might be insufficient to rapidly eliminate all tumours. As described previously, tumour cells can lose TAA expression to elicit an immune response. If these antigens are not required for growth of the tumour or maintenance of the transformed phenotype, the antigen-negative tumour cells have a growth advantage in the host. Eventually these will lead to outgrowth of those cells that do not express these TAA and thereby these tumour cells escape detection by T cells that are triggered to target that single TAA. Besides this, the efficacy of vaccination against a single or a few TAA is limited by peptide restriction to a given HLA type and the induction of CTL without CD4+ T helper cell activation.

Therefore, polyvalent therapeutic strategies, aimed at targeting many antigens at once, may overcome these problems. One such strategy is to load DC with tumour cell lysates, either from autologous or allogeneic background, without further defining the antigens. Lysate can be generated after collection of tumour material. Tumour cells can be UVB irradiated leading to apoptotic tumour lysate or subjected to freeze-thawing cycles giving rise to necrotic lysate. There is considerable controversy as to whether necrotic or apoptotic lysate is the superior source of tumour antigens to pulse DC for immunotherapeutic

applications (9). Ex-vivo generated DCs can encounter these fragments and take up different antigens, providing them with a large scale of tumour-associated and tumour-specific antigens. Therefore, tumour lysates might be advantageous in providing the full antigenic repertoire of the tumour and, particularly, unique tumour antigens, which will theoretically decrease the ability of tumours to evade the immune response by down regulation of a single antigen (10). It greatly diminishes the chance of tumour escape compared to using single epitope vaccines. Besides the role as antigen source, tumour lysate also serves a role in protein supplementation in DC culture (11). Furthermore, the use of tumour cell lysate theoretically eliminates the need to define, test and select for immunodominant epitopes. An example of a cancer which needs to rely on lysate pulsed DC-immunotherapy is malignant mesothelioma.

Many antigens that were originally identified as tumour specific are now known to be expressed in many other tissues. Mesothelioma-associated tumour antigens are limited to date and no mesothelioma specific tumour antigens are known. Mesothelin, calretinin, SV-40, Wilms tumour 1 (WT-1), and telomerase have been described as TAA but these proteins are not expressed on the membranes of all MM tumours. Although research is performed to identify "better" TAA for MM, much work remains to be done. This may be useful in the early detection of mesothelioma for diagnostic purposes. However, immunotherapy based on a polyvalent therapeutic strategy is directly accessible for all types of cancer and diminishes the chances of rapid immune escape by lose of single TAA on tumour cells. In 2005 Hegmans et al. showed that is was feasible to use lysate pulsed dendritic cells to induce anti-tumour responses against MM in a murine model (12). To investigate if this strategy was also applicable in MM patients, a clinical trail, using dendritic cells loaded with autologous tumour-lysate, was adapted in the Erasmus MC (**CHAPTER 3**). Although we were able to show that an immune response could be generated and an anti-tumour response could be induced in part of the patients, refining this approach was recommended. One of the main reasons for reduced efficacy of immunotherapy in cancer patients is the presence of tumour-induced immune suppression (**CHAPTER 1**).

The aim of the work addressed in this thesis was to refine immunotherapy by targeting immune suppressive cells with the intention to enhance the affectivity of the induced anti-tumour response.

To investigate approaches to refine immunotherapy, we aimed to target immune suppressive cells. In the immunesuppressive environment MDSC, TAMs and Treg play a dominant role (**CHAPTER 1**). We aimed to determine the capacity of agents to target these cells in a murine model for mesothelioma. Simplifying the nature of cancer by analyzing only the cancerous cells either in tissues or after propagating in culture will lose important information on the biological reality of tumours in vivo. In our need to make investigations translational to the clinic, we analyzed three clinical applicable agents with are on the market, but used for the treatment of other diseases then cancer.

We evaluate a strategy to overcome immune suppression by MDSC. MDSC are a relatively new cell type and were first described by Bronte and Gabrilovich in 2000/2001 (13-14). These cells can affect antitumour-immunity via an antigen-specific and non-specific manner. However, both suppressive mechanisms are largely depending on tumour-derived PGE2. COX-2 is essential in the formation of PGE2. Therefore we tested a selective COX-2 inhibitor (Celecoxib) to reduce PGE2 production by tumour cells. This was tested in an animal model for mesothelioma. Results of this refinement are reported in **CHAPTER 4**.

The role of TAMs during tumour pathogenesis and tumour progression has been studied extensively. However, targeting TAMs by using clinical applicable agents seems difficult. One of the agents which seem to hold promise in targeting TAMs are bisphosphonates. Recently, is has been discussed that Zoledronic acid (ZA) may have anti-tumour potentials, since Gnant et al. showed that ZA prolongs disease-free survival in patients with breast

cancer (15). The mechanism via which ZA prevents disease progression is still unclear. It has been suggested that ZA modulates the immune response and may influence macrophage phenotype (16-18). In **CHAPTER 5** we determined the effect of ZA on TAMs in a murine model for mesothelioma.

In **CHAPTER 6** we focussed on the role of regulatory T cells in mesothelioma. Hegmans et al. showed that Tregs can be reduced with a depleting antibody PC61, however the specificity of this antibody is not sufficient in the combination with immunotherapy since PC61 also depletes activated T and B cells (19). Recent clinical studies have shown that low-dose cyclophosphamide (CTX) induces beneficial immunomodulatory effects in the context of active or adoptive immunotherapy (20-30). CTX is widely used to treat various types of malignancies and some autoimmune disorders. It displays either immunosuppressive or immunopotentiating effects, depending on the dosage and the timing of drug administration (31). CTX was given to mice in a metronomic low-dose treatment protocol in a mesothelioma mouse model. The results of this treatment strategy have eventually led to the initiation of a new clinical trail. **CHAPTER 7** reports the study design of the newly initiated trial.

In **CHAPTER 8** we postulate the hypothesis that dendritic cell-based immunotherapy can be refined by simulteneously targeting tumour-induced immune suppression. Furthermore, the contribution of this thesis to the therapeutic application of immunotherapy and the treatment of cancer patients in general are discussed.

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Chapter 3

Consolidative dendritic cell-based immunotherapy elicits cytotoxicity against malignant mesothelioma

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Consolidative Dendritic Cell-based Immunotherapy Elicits Cytotoxicity against Malignant Mesothelioma

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Rationale: We previously demonstrated that dendritic cell-based immunotherapy induced protective antitumor immunity with a prolonged survival rate in mice. However, the clinical relevance is still in question. To examine this, we designed a clinical trial using chemotherapy followed by antigen-pulsed dendritic cell vaccination in mesothelioma patients.

Objectives: The aim of this study was to assess the safety and immunological response induced by the administration of tumor lysate-pulsed dendritic cells in patients with mesothelioma.

Methods: Ten patients with malignant pleural mesothelioma received three vaccinations of clinical-grade autologous dendritic cells intradermally and intravenously at 2-week intervals after chemotherapy. Each vaccine was composed of 50×10^6 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.

Measurements and Main Results: Administration of dendritic cells pulsed with autologous tumor lysate in patients with mesothelioma was safe with moderate fever as the only side effect. There were no grade 3 or 4 toxicities associated with the vaccines or any evidence of autoimmunity. Local accumulations of infiltrating T cells were found at the site of vaccination. The vaccinations induced distinct immunological responses to KLH, both *in vitro* and *in vivo*. Importantly, after three vaccinations, cytotoxic activity against autologous tumor cells was detected in a subgroup of patients.

Conclusions: This study demonstrated that autologous tumor lysate-pulsed dendritic cell–based therapy is feasible, well-tolerated, and capable of inducing immunological response to tumor cells in mesothelioma patients.

Clinical trial registered with www.clinicaltrials.gov (NCT00280982).

Keywords: antitumor; clinical trial; vaccinations; tumor lysate-pulsed

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

In an earlier study, we described the use of dendritic cell (DC)-based immunotherapy in a mouse model for mesothelioma. The potency of antigen-pulsed DCs was demonstrated by the increase in antitumor immunity leading to a prolonged survival.

What This Study Adds to the Field

We show for the first time the safety and feasibility of tumor lysate–pulsed DCs as therapeutic adjuvants in patients with mesothelioma and found distinct immune responses and antitumor responses in these patients.

Malignant pleural mesothelioma is a fatal disease with median survival time from first signs of illness to death of less than 12 months (1, 2). The incidence of mesothelioma is closely associated with exposure to airborne asbestos fibers (3). Although the application of asbestos is prohibited in most developed countries, incidences of mesothelioma are increasing due to the incubation period of 20 to 50 years from initial exposure to asbestos to the onset of disease. For The Netherlands and most other European countries, deaths related to this disease are expected to continue rising until the year 2020 (4). However, in numerous eastern and developing countries, asbestos is still used, and as consequence, incidences of mesothelioma worldwide will continue to rise (5). This anticipated increase in the incidence of mesothelioma has spurred considerable interest in the development of better treatments for mesothelioma.

Chemotherapy consisting of a combination of pemetrexed and cisplatin is regarded as the standard of care for selected patients with mesothelioma (6). The survival benefit is limited (~3 mo) and new or additional treatment options like anti-angiogenesis drugs (bevacizumab), photodynamic therapy, gene therapy, and a variety of immunotherapy approaches are currently being tested.

Immunotherapy is a promising approach in the treatment of cancer. It tries to harness the potency and specificity of the immune system to attack cancer cells, aiming for a nontoxic treatment with minor side-effects and a long-lasting immunological memory. One approach of immunotherapy uses dendritic cells (DC) to present tumor-associated antigens (TAA) and thereby generate tumor-specific immunity (7, 8). DC are extremely potent antigen-presenting cells specialized for inducing activation and proliferation of $CD8^+$ cytotoxic T lymphocytes (CTL) and helper $CD4^+$ lymphocytes. We previously investigated the effect of DC-based immunotherapy on the outgrowth of mesothelioma in a murine model (9). Whereas the TAA are not known for mesothelioma, we used tumor cell lysate as antigen source to pulse DC. We established that DC-based immunotherapy induced strong tumor-specific CTL responses leading to prolonged survival in mice (9). The efficacy of immunotherapy was dependent on the tumor load. The most beneficial effects were established at early stages of tumor development. This is in agreement with our current

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knowledge of the effect of immunotherapy in other tumor types (10).

On the basis of these preclinical animal studies, we have now completed a clinical trial in which autologous tumor lysate–pulsed DC were administered intradermally and intravenously in mesothelioma patients after cytoreductive therapy with chemotherapy. Patients received 50×10^6 mature DC pulsed with tumor lysate and keyhole limpet hemocyanin (KLH) every 2 weeks for a total of three injections. The safety, feasibility, and immunological effectiveness of this approach are reported.

METHODS

See the online supplement for more details regarding the preparation of the tumor lysate, flow cytometric analysis of clinical-grade DC, delayed-type hypersensitivity skin testing, immune response assessment against KLH, flow cytometric assays for interferon-γ and granzyme B expression, and the cytotoxicity assays.

Patient Population

The study was approved by the institutional Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands. Procedures followed were in accordance with the ethical standards of this committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000. Ten patients, recently diagnosed with malignant pleural mesothelioma of the epithelial subtype, were enrolled after informed consent. Patient characteristics are summarized in Table 1. 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.

A further description is detailed in the online supplement. From all participants, blood and serum samples were taken at regular intervals. Blood was tested for immunological responses and liver and renal functioning. In addition, serum samples were screened for the development of auto-immunity.

Preparation of Tumor Lysate

A detailed description of the preparation of the lysate can be found in the online supplement. Single cell suspensions of tumor tissue (cases 2, 4, 5, and 10) and/or pleural effusions (cases 1, 3, 4, 6, 7, 8 and 9) were counted and resuspended at a concentration of 50×10^6/ml in phosphate buffered saline (PBS). 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 ($200 \times g$ for 5 min), 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 a previously described method to generate clinical-grade mature dendritic cells in conformity with Good Manufacturing Practice guidelines (11). Detailed information is outlined in the online supplement. In brief, concentrated leukocyte fractions were generated through peripheral blood leukapheresis. Peripheral blood mononuclear cells were then enriched using counterflow centrifugal elutriation (Elutra; Gambro BCT, Zaventem, Belgium) as described by Berger and colleagues (12). Fractions were further purified by Ficoll-Paque Premium density gradient centrifugation when percentage of polymorphonuclear cells exceeded 10% (all cases except for patient case 7 and 9; Table 2). Monocytes were resuspended in XVIVO-15 supplemented with 2% pooled human AB serum (DC-culture medium). The next day, half of the medium was replaced by the same volume of DC culture medium supplemented with IL-4 and granulocyte macrophage-colony stimulating factor (GM-CSF; CellGenix, Freiburg, Germany). After 6 days of culture, cells were harvested using a pipet and then seeded in fresh DC culture medium supplemented with tumor cell lysate, IL-4, GM-CSF, and KLH (Calbiochem, La Jolla, CA). The next day the maturation cocktail was added (prostaglandin E2 [Pharmacia & Upjohn, Puurs, Belgium], tumor necrosis factor-α [TNF-α], IL-1β, and IL-6 [all CellGenix]). Cells were harvested at Day 9 and 50×10^6 cells were used for immediate vaccination; remaining cells were cryopreserved in dimethyl sulfoxide for later vaccinations and for the delayed type hypersensitivity (DTH) skin testing.

Dendritic Cell Vaccination

Patients received three immunizations with mature DC, loaded with autologous tumor lysate and KLH, in 2-week intervals (Figure 1). Each immunization, consisting of 50×10^6 cells, was administered intradermally and intravenously. Dosage was divided 1/3 intradermally in the forearm and 2/3 though the intravenous route by mixing the components in 100 ml of normal saline drip. Constant monitoring was done for 4-hours after the administration of vaccine therapy. The vaccine was routinely analyzed for DC purity and tested for infectious agents before administration to patients.

RESULTS

Ten patients who met the inclusion/exclusion criteria were enrolled after informed consent; all Caucasian men ranged in age from 56 to 78 years (median 65 yr) and newly diagnosed with malignant pleural mesothelioma of the epithelial subtype. Six patients had more than 150×10^6 cells in their pleural fluid, which was sufficient for pulsing DC; from the others tumor material was obtained by thoracoscopy (>0.2 g wet weight). Nine patients received four cycles of chemotherapy consisting of 500 mg/m^2 of body surface area (BSA) premetrexed (Alimta, Eli Lilly and Co., Fegersheim, France) and 75 mg/m^2 BSA cisplatin every 4 weeks according to the treatment schedule described. One patient received 80 mg/m^2 BSA carboplatin instead of cisplatin because of a hearing implant (case 4). Dietary supplementation with low-dose folic acid and vitamin B_{12} before and during the treatment was given to limit toxicity. During the four cycles of chemotherapy, none of the patients

TABLE 1. PATIENT CHARACTERISTICS AT THE TIME OF DIAGNOSIS

No.	ECOG Performance Status	Chest Pain	Weight Loss (>5%)	PLT (x10^9/L)	LDH (U/L)	HGB (mM)	WBC (x10^9/L)	Age (yr)
1	1	No	Yes	326	298	9.1	9.8	68
2	0	No	Yes	438 H	231	6.5 L	11.0 H	64
3	1	Yes	No	124 L	470 H	7.2	4.0 L	78
4	0	No	No	175	154	10.2	5.6	60
5	0	No	No	481 H	168	7.8 L	7.0	71
6	1	No	Yes	315	368	9.8	7.7	57
7	1	No	Yes	223	354	8.6	7.0	77
8	0	No	Yes	206	268	9.1	5.6	56
9	0	Yes	No	484 H	162	8.8	9.8	66
10	0	Yes	Yes	231	471 H	7.9	4.4	59

Definition of abbreviations: ECOG – Eastern Cooperative Oncology Group; H – above upper limits of reference range of individuals; HGB – hemoglobin level; L – below lower limits of the reference range of individuals; LDH – lactate dehydrogenase enzyme level; PLT – platelet count; WBC – white blood cell count.
H and L values deviate from the normal distribution.

TABLE 2. DENDRITIC CELL CULTURING PROCESS

No.	Material Used for DC Loading	Monoctes at Day 0 (x10^6 Cells)	Additional Purification after Elutra (% Polymorphonuclear Cells Before/After Ficoll)	DC at D10 (x10^6 Cells)*
1	PF	2250	Yes (22/8)	295
2	TT	3000	Yes (75/7)	175
3	PF	2000	Yes (50/5)	160
4	TT/PF	1850	Yes (16/9)	375
5	TT	2400	Yes (47/4)	240
6	PF	2100	Yes (13/6)	230
7	PF	2150	No (7/–)	365
8	PF	3100	Yes (75/7)	276
9	PF	3500	No (9/–)	190
10	TT	1600	Yes (18/8)	255

Definition of abbreviations: DC – dendritic cell; PF – pleural fluid; TT – tumor tissue.
* The initial cell number of the immature DC used for loading was 420 $\times$ 10^6 cells.

experienced any serious adverse events. Four patients had a stable disease and six patients showed a partial response after their last chemotherapy and returned at that stage to their usual activities (World Health Organization/Eastern Cooperative Oncology Group performance status of 0 or 1). Patient characteristics are depicted in Table 1.

All ten patients reacted to subcutaneous injected tetanus toxoid in a DTH skin testing 8 to 12 weeks after the last chemotherapy cure indicating that the chemotherapy drugs did not (or no longer) exert their influence on the patients' immune system. Patients underwent a single leukapheresis with a mean total volume processed of 9.0 $\pm$ 0.5 L. None of the patients experienced any toxicity during the procedure except for mild citrate reactions that were compensated by calcium administration. The leukapheresis procedure was well tolerated. The products (135 $\pm$ 20 ml) were enriched for monocytes using Elutra counterflow elutriation system. Fractions V of most cases (except cases 7 and 9, Table 2) were further purified by Ficoll-Paque Premium density gradient centrifugation because percentages of polymorphonuclear cells exceeded 10% that might otherwise alter DC quality. Blood monocytes were cultured with GM-CSF and IL-4 for 10 days to allow DC differentiation. From all patients, sufficient clinical-grade dendritic cells could be generated for three vaccinations (160×10^6 – 375×10^6 cells). Although cell numbers showed patient-to-patient variability, the phenotype of the cells harvested at Day 10 of culture were large cells with indistinct and veiled morphology, and more than 95% were negative for CD14 and positive for $CD40^+$, $CD80^{2+}$, $CD83^{2+}$, $CD86^{+++}$, expressing additional high levels of human leucocyte antigen DR (HLA-DR) molecules, which were compatible with the characteristic features of mature DC. Routine sterility testing did not detect microbial contamination in any of the vaccines.

Toxicity

Overall, the vaccination regimen with loaded dendritic cells was well-tolerated in all patients. No dose adjustments or dose discontinuations were necessary. 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. In one patient (case 1), 3-millimeter skin punch biopsies were taken at Day 2 and Day 14 after the last injection of KLH and lysate-pulsed DC. In addition, adjacent normal, noninjected skin was also biopsied from the same individual. Microscopical examination of the immunohistochemical stainings showed a prominent thickening of the epidermis after 14 days (Figure 2). Compared with normal skin, a mild lymphocytic infiltrate and a more intense interstitial mononuclear cell infiltrate in the mid and deep dermis was demonstrated in the vaccinated site. The infiltrates consisted mainly of HLA-DR, DQ, and DP alleles, macrophages (CD68), and T lymphocytes (CD3, CD4, and CD8) (Figure 2). With regard to the dendritic cells, these were present at increased levels at Day 2 in both epidermis and dermis and probably originated from the vaccine, as demonstrated in other studies (13).

Eight patients developed mild to severe flu-like symptoms after the vaccination, particularly fever, muscle aches, chills, and tiredness. Two patients showed these symptoms after the first vaccination; the others after the second and/or third injection. In seven patients these symptoms normalized after one day. Most patients took paracetamol (acetaminophen) for 1 d as analgesic and antipyretic agent. One patient (case 6), in whom the reaction started after the first injection, the symptoms were more severe and paracetamol treatment was continued for 3 days. This patient did get chills after second and third vaccination. In none of the patients was grade 3 or 4 toxicities observed. No clinical signs and laboratory data of any autoimmune diseases (antinuclear auto-antibodies, extractable nuclear antigens, rheumatoid factor immunoglobulin M, and anticyclic citrullinated peptide [anti-CCP]) were observed in all patients until the final follow-up. There were no substantial changes in the results of routine blood tests.

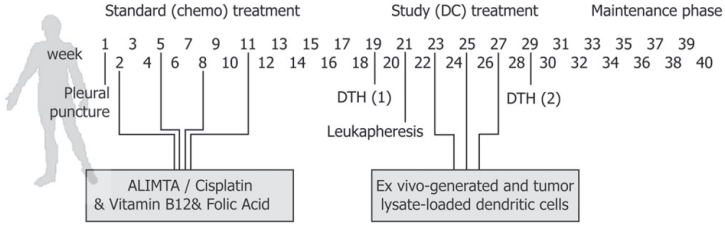

Figure 1. Synopsis of the study consisting of a combined treatment with chemotherapy followed by active immunotherapy using autologous tumor lysate–loaded dendritic cells (DCs). DTH = delayed type hypersensitivity.

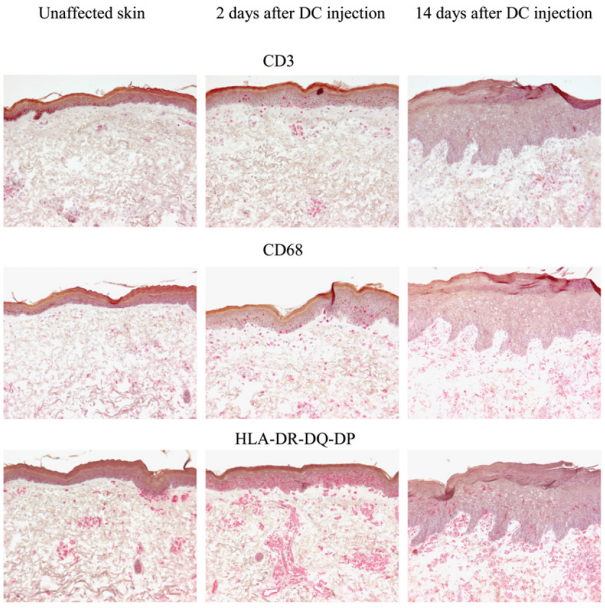

Figure 2. Immunohistochemical stainings for CD3, CD68 and HLA-DR, DQ, and DP alleles of skin biopsies taken from unaffected skin 2 days and 14 days after the second vaccination from the injection site (patient 1; Table 3). DC = dendritic cell.

Efficacy

The clinical responses were evaluated before and after immunotherapy by computed tomography (CT) scans and chest X-rays and analyzed according to the modified Response Evaluation Criteria in Solid Tumors (modified RECIST). Three patients showed partial responses after DC-immunotherapy, one showed stable disease, and six had no response after vaccinations (Table 3 and 4). The CT scans of patient nine, before and after the vaccinations, is shown in Figure 3. Two weeks after the DC vaccinations, a second DTH skin test was performed. Cell lysates of autologous tumors, KLH, DC loaded with tumor lysate and KLH, DC loaded with tumor lysate, and an appropriate positive (tetanus toxoid) and negative (saline) control, were injected intradermally and read 48 hours later. DTH skin testing revealed a response to dendritic cells loaded with tumor lysate and KLH, and to KLH alone in all patients (Table 3). Five patients responded to tumor lysate–loaded dendritic cells without KLH (Table 4). No DTH reaction was observed with tumor lysate in any of the patients. Reactions varied from induration and erythema to only slight erythema.

Serum samples from all patients showed a significant increase of pre-vaccine versus post-vaccine antibodies reactive to KLH, both of the IgG and IgM isotype (Table 4). No or very low amounts of antibodies against KLH 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 KLH gradually increased with the number of vaccinations (Figure 4) suggesting that several vaccinations were necessary to induce a more potent humoral response. Antibodies against KLH in serum, diluted up to 100,000 times, could easily be detectable by ELISA in all patients after three vaccinations (Figure 4). The response remained at the same level for several months after the last DC injection and gradually decreased after 6 to 12 months (data not shown).

Chromium release assays were performed in 6 of 10 patients from whom pleural fluid was obtained (cases 1, 3, 6, 7, 8, and 9, Table 4). Only these samples were suitable because viable single-cell suspensions are needed for labeling with radioactive chromium for the cytotoxicity assay, and therefore, patients from whom tumor tissue was obtained (cases 2, 4, 5, and 10) were excluded for this cytotoxicity assay. Almost no lysis of autologous tumor cells was observed before DC-treatment in these 6 patients. In 4 patients (cases 6, 7, 8, and 9) clear inductions of cytoxicity against autologous tumor cells were measurable. The cytotoxicity levels of patient number 9 increased after every vaccination; for the three other patients (case 6, 7, and 8) three vaccinations were necessary to induce cytotoxicity (Figure 5). Another assay that was used to assess the T cell capacity for cell lysis was the flow cytometric detection of $CD3^+CD8^+$ T cells expressing granzyme B. Nine patients (1 remained at the same level) showed a significantly increased percentage of granzyme B^+ $CD8^+$ T cells after vaccination ($P = 0.023$; paired t test was used for comparing within-subject changes (Figure 6), and the granzyme B expression per CD8 cell increased in most patients (Table 4).

DISCUSSION

To our knowledge, this is the first study assessing autologous monocyte-derived dendritic cells loaded with autologous tumor cell lysate in patients with mesothelioma. The primary objectives of this study were to determine the toxicity and feasibility of clinical grade DC and to investigate if mesothelioma might be susceptible to immunotherapy treatment. Ten patients fulfilled the inclusion and exclusion criteria and were enrolled in the study. Feasibility was defined as producing three doses of 50×10^6 autologous tumor-lysate–loaded DC, each for intravenous (two-thirds) and intradermal (one-third) administration. In all apheresed subjects, sufficient cells were obtained

from a single leukapheresis product, processed *in vitro*, injected (first vaccination), and cryopreserved for later administration (second and third vaccination). These results indicate that our method for producing large amounts of clinical-grade DC is feasible in patients with malignant mesothelioma.

The possibility to harness the potency and specificity of the immune system underlies the growing interest in cancer immunotherapy. One such approach uses the patient's own DC to present tumor-associated antigens and thereby generate tumor-specific immunity (7, 8). We generated DC in large amounts *ex vivo*, in the absence of the suppressing tumor milieu, and subsequently loaded them with a preparation of autologous tumor antigens. To prevent antigens from being presented by immature DCs, which might tolerate tumor antigens and potentially enhance tumor growth (14, 15), cells were matured using a standard cytokine cocktail. Mature DCs are injected both intraveneously (distribution to the liver, spleen and bone marrow) and intradermally where they then migrate to the regional lymphatics. In this way, they can maximally stimulate cytotoxic T cells, B cells, T cells, NK cells and NKT cells that are essential for killing tumors at different immunological organs.

We used a generally known and widely accepted method for the preparation and maturation of clinical grade dendritic cells (11, 12). Autologous tumor lysate was used as the source of tumor antigen to load onto DC because in mesothelioma, specific tumor-associated antigens (TAAs) are undefined to date. Although mesothelin, calretinin, SV-40, Wilms tumor 1 (WT-1), and telomerase have been described as TAA in mesothelioma, these proteins are not expressed on the membranes of all mesothelioma tumors. Besides this, the efficacy of vaccination against a single or a few TAAs is limited by peptide restriction to a given HLA type and the induction of CTL without Th1 response. Therefore, we investigated in an earlier animal study whole tumor lysate as an antigen source with satisfying results (9). We demonstrated that DC immunotherapy was effective in controlling this aggressive cancer in which TAA remain undefined. Other investigators have also shown that human DC pulsed with apoptotic mesothelioma cells were able to induce a CTL response *in vitro* directed against the tumor, illustrating that malignant pleural mesothelioma cells contain unknown TAA that can lead to an antitumoral immune response (16). Autologous 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 (17). On the other hand, sufficient amounts of tumor lysate must be available for DC pulsing and this often limits the applicability of tumor-lysate pulsed DC immunotherapy. Forty-three biopsies and pleural fluids were collected from patients with mesothelioma of which 10 patients had sufficient tumor material (pleural fluid >150 × 106 tumor cells or >0.2 g tumor tissue) to pulse the dendritic cells, although most patients had a high tumor burden on the computerized tomography (CT) scan. Tumor material was obtained from patients for dyspnea relief or diagnostic purposes. For ethical reasons, no tumor material was collected from patients without any medical need for removal. In this study, none of the patients developed any clinical or laboratory signs of autoimmune disease, indicating that the fear expressed by some researchers, that such highly stimulatory DC pulsed with undefined tumor lysates might induce autoimmune disease, is unfounded.

From animal studies and reports of other tumor types it is generally accepted that DC-based immunotherapy is most effective in cases of relatively small tumor loads. To decrease

TABLE 3. OVERVIEW OF THE RESPONSES FOUND IN THIS STUDY

Clinical Response	No.	%
Response to prior chemotherapy		
Complete response	0	0
Partial response	6	60
Stable disease	4	40
No response	0	0
Response to immunotherapy		
Complete response	0	0
Partial response	3	30
Stable disease	1	10
No response	6	60
Immunological response		
^{51}Cr release	4 of 6*	66
KLH response in serum	10	100
Skin-test reaction	10	100
KLH	0	0
Tumor lysate	10	100
DC + tumor lysate + KLH DC + tumor lysate – KLH	5	50
Survival in months after diagnosis		
Median (range)	19 (11–34)	90
DOD	9	10
AWD	1	

Definition of abbreviations: DC – dendritic cell; DOD – died of disease; AWD – alive with disease; KLH – keyhole limpet hemocyanin.

* ^{51}Cr release assay was performed in six patients, from remaining patients no viable tumor cells were obtained.

TABLE 4. CLINICAL AND IMMUNE RESPONSE EVALUATION OF PATIENTS WITH MESOTHELIOMA PARTICIPATING IN THE TRIAL

No.	Response on Chemotherapy	IgG anti- KLH*	DTH skin test (loaded DC)	Cyto-toxicity	Difference in Granzyme B Expression(%/MFI)	Radiological Findings Before and After DC Immunotherapy	Interval between Diagnosis and Death (Months)	Follow-up
1	PR	+++	+	—	+2.8/+16.34	PR	23	DOD
2	PR	++	+	ND	0.0/+7.03	PD	34	AWD
3	SD	+	—	—	+4.6/+34.91	PD	15	DOD
4	PR	++	—	ND	+10.0/+6.78	PD	15	DOD
5	SD	++	—	ND	+8.8/+38.82	SD	15	DOD
6	PR	+++	—	+	+21.5/−3.58	PD	13	DOD
7	SD	+	+	+	+1.2/−2.04	PD	11	DOD
8	SD	++	—	+	+4.1/+ 5.86	PR	19	DOD
9	PR	++	+	+	+3.1/+6.78	PR	30	DOD
10	PR	+	+	ND	+35.4/−19.29	PD	15	DOD

Definition of abbreviations: AWD – alive with disease; DC – dendritic cell; DTH – delayed type hypersensitivity; DOD – died of disease; KLH – keyhole limpet hemocyanin; MFI – mean fluorescence intensity; ND – not done; SD – stable disease; PR – partial response; PD – progressive disease.

* IgG antibodies detected against KLH in serum at 1,000 times (+), 10.000 times (++) or at 100.000 times (+++) diluted serum.

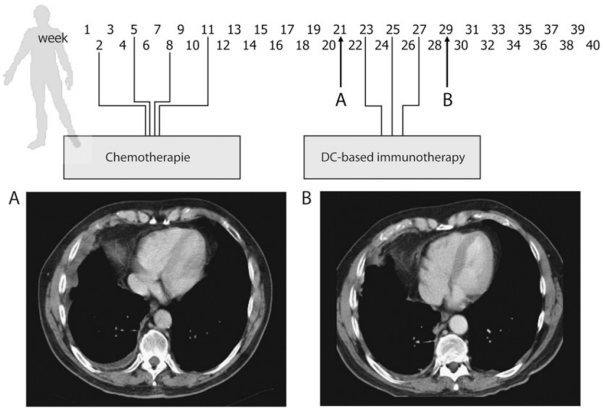

Figure 3. (*A*) CT scans 2 weeks before the first dendritic cell (DC) vaccination and (*B*) two weeks after the third vaccination (indicated by *arrows* on the time scale) revealed a decline in pleural fluid and a regression of the tumor (case 9). Of the 10 participating patients, 6 had progressive disease; 1 had a stable disease, and 3 showed a partial response in this 8-week time period.

a patient's tumor load a combination of pemetrexed and cisplatin (one patient with a hearing implant [cochlear] received pemetrexed and carboplatin as alternative) was given because it is considered the clinical standard of care in mesothelioma. This combination chemotherapy is the only treatment with activity proven in phase III trials and is approved by the U.S. Food and Drug Administration. Patients could only participate in the trial when no progressive disease was present after chemotherapy (exclusion criteria). The downside of these drugs is the high toxicity level (e.g., inducing lymphopaenia), and therefore, a recovery period of 8 to 12 weeks was formulated to achieve immunological recovery from the chemotherapeutic treatment. Preclinical studies have shown certain chemotherapy drugs can synergize with immunotherapy directly (18). Currently several groups are investigating different drugs that can induce optimal immunogenic mesothelioma cell death and can thus be combined with immunotherapy directly leading to an enhanced anticancer immune response (19, 20). A recent report in the form of an abstract has shown that, despite the decrease in lymphocyte numbers, cisplatin/pemetrexed modulates the immune system and provides a rationale for combining cisplatin/pemetrexed with immunotherapy at the same time (21).

Imaging techniques (CT scans) were performed before and after immunotherapy in all participating patients. However, tumor extension in patients with mesothelioma is rather difficult to access due to the widespread tumor over the large surface of the pleura (22). Six patients had progressive disease; one patient had a stable disease, and three showed partial responses after DC-immunotherapy. CT scans 2 weeks before the first DC vaccination and 2 weeks after the third vaccination revealed a regression of the tumor in three patients during this 8-week period. However, these tumor regressions seen on CT scans cannot be attributed solely to the DC treatment but might also be caused by a delayed reaction of the chemotherapy. Imaging scans of a proper control group, patients with high tumor burden in their pleural effusion and not receiving DC vaccinations at corresponding intervals are necessary to draw conclusions on tumor regressions. The evaluation of the overall survival is also difficult to interpret in the absence of a randomized trial and, therefore, these results should be interpreted with caution. As shown in Table 4, median survival was 19 months (range 11–34 mo). Nine patients died of disease; one patient is alive with disease (currently at 48 mo after diagnosis). These survival data are difficult to compare with historical controls as patients are selected. Although the number of patients in this study is limited, these data indicate that a selected group of patients may benefit from DC immunotherapy without major adverse effects.

The immunogenic protein keyhole limpet hemocyanin (KLH) was used in this study as helper antigen and as tracer molecule, allowing *in vivo* and *in vitro* monitoring of immunological responses induced by the vaccinations (23, 24). In the serum samples of all patients, antibodies against KLH induced by DC-therapy were detected, both of the IgG and IgM isotype. Also, all patients revealed strong responses to DCs loaded with tumor lysate and KLH and to KLH alone 48 hours after DTH skin testing. In five patients (50%), DC pulsed with tumor lysate (without KLH) caused induration, which supports the idea that

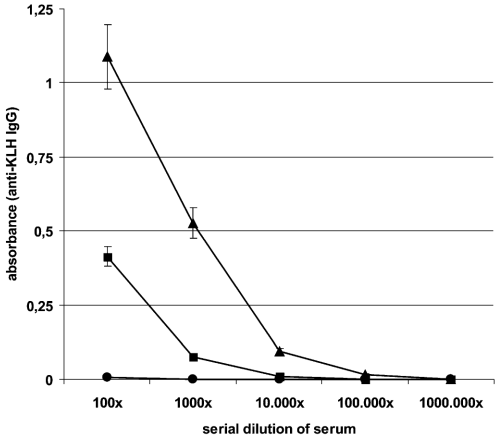

Figure 4. Anti–keyhole limpet hemocyanin (KLH) immunoglobulin responses increase after dendritic cell (DC) injections. Kinetics of antibody responses against KLH was measured in serially diluted serum of representative patient (case 4) at the first (*circles*), second (*squares*), and two weeks after the third (*triangles*) injection of tumor lysate–pulsed DC.

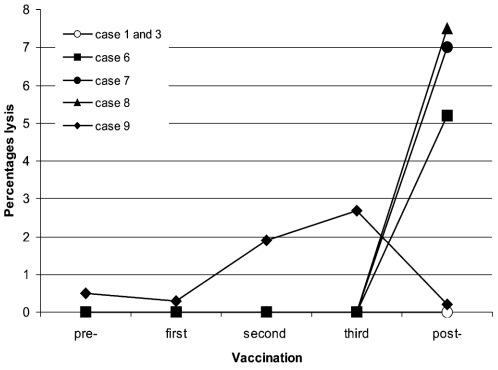

Figure 5. Cytotoxicity for ^{51}chromium-labeled (^{51}Cr) autologous tumor cells by peripheral blood mononuclear cells during dendritic cell (DC) vaccination. Of the six patients that were evaluable, four patients showed increase in ^{51}Cr release caused by lysing of autologous tumor cells. The percentage of lysis was calculated using the formula: corrected % lysis = 100 × (experimental release − spontaneous release[target cells incubated in medium alone])/(maximum release[2% Triton X-100 as lysing agent] − spontaneous release).

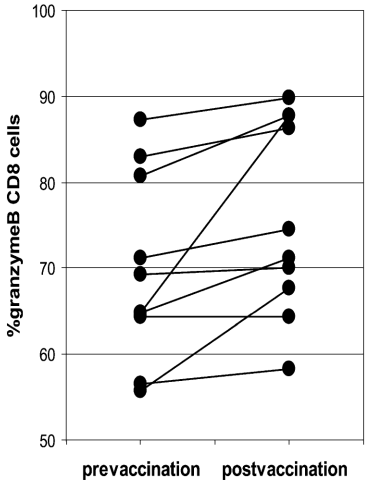

Figure 6. Granzyme B positive CD3$^+$ CD8$^+$ T lymphocytes were measured in blood samples of patients before and after dendritic cell (DC) vaccination using flow cytometry. Significant increases in granzyme B$^+$ T lymphocytes were detected after three vaccinations in all patients (P = 0.023; paired t test).

specific immune responses are induced. This was confirmed by the increase of HLA-DR, DQ and DP$^+$ cells, macrophages and T-lymphocytes in skin biopsies taken from the temporary local skin reactions after DC vaccination. Blood samples revealed an increase in antitumor T cell activity in four of the six patients after DC vaccination. Most studies in other cancer types could not establish these findings directly in peripheral blood and needed more sophisticated techniques to demonstrate T cell activity (25–27). Therefore, our results clearly support the fact that this form of immunotherapy induced specific immune responses in mesothelioma.

Own research and that of others have shown that mesothelioma cells, like other tumor cells, produce many immunosuppressive factors that can affect DC, effector T cells, macrophages, NK and NKT cells. We were the first to demonstrate that human mesothelioma tissue contains significant amounts of Foxp3$^+$ regulatory T cells (28). Depletion of these cells led to increased survival in a transplantable mouse model for mesothelioma. Other studies have revealed that myeloid-derived suppressor cells and M2 macrophages within the tumor promote growth and metastasis by directly acting on tumor cells, endothelial cells, and on antigen-specific T cells (29–32). We cannot exclude that the up-regulation of antitumor activity in our study will be negatively influenced by an immunosuppressive environment. Manipulation of these suppressive factors might therefore be used in combination with DC immunotherapy to improve the outcome of mesothelioma. Recently we started a study with dendritic cell immunotherapy in combination with a low-dose of cyclophosphamide (Endoxan) in mesothelioma patients to inhibit T-regulatory function to increase the success rate of tumor eradication.

The administration of DCs loaded with autologous tumor cell lysate to patients was safe. Some patients developed self-limited fever a few hours after the vaccinations. Local skin reactions were seen at the site of the intradermal injection suggesting that some form of immunity was induced. There was no clinical or radiological evidence of any autoimmunity. Distinct immunological responses to the surrogate marker KLH were induced by the vaccinations, both *in vitro* as *in vivo*. Importantly, antitumor cytotoxicity activity against autologous tumor cells was measured in the blood of patients. An increase in systemic CTL activity was seen in a subset of treated patients (4 out of 6). Multiple vaccinations were necessary as the increase in CTL activity was seen only after 3 vaccinations for most of the patients in this assay. Another immune monitoring assay that was used, the expression of granzyme B in CD8$^+$ T cells, increased significantly in all patients by the vaccination protocol. No correlations between vaccine responders (CTL activity, increase of granzyme B expression, KLH antibodies) and clinical outcomes could be detected.

Although this trial is relatively small with 10 patients, it includes a homogeneous group of patients with regard to histology, prior treatments, performance status, and study design and execution. It is the first human study on DC-based immunotherapy in patients with mesothelioma. In conclusion, DC loaded with autologous tumor cell lysate administrated to patients was safe and feasible and no adverse effects were observed. Antitumor immune responses were detected in a few patients with mesothelioma after DC-immunotherapy. Whether this has a beneficial effect in improving survival will be the subject in successive studies. Influencing the immunosuppressive cells (Tregs, M2 macrophages, and MDSC), cells abundantly present in the tumor environment, and their subsequent effect on DC-mediated anti-tumor responses, seems of critical importance for future clinical trials. Also other sources of antigens to pulse DC must be investigated to make DC immunotherapy more accessible for larger numbers of patients to perform comparative studies.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Chapter 4

COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived suppressor cell mesothelioma

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COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived suppressor cells in mesothelioma. Celecoxib influences MDSC function

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Abstract

Background: Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature cells that accumulates in tumour-bearing hosts. These cells are induced by tumour-derived factors (e.g. prostaglandins) and have a critical role in immune suppression. MDSC suppress T and NK cell function via increased expression of arginase I and production of reactive oxygen species (ROS) and nitric oxide (NO). Immune suppression by MDSC was found to be one of the main factors for immunotherapy insufficiency. Here we investigate if the *in vivo* immunoregulatory function of MDSC can be reversed by inhibiting prostaglandin synthesis by specific COX-2 inhibition focussing on ROS production by MDSC subtypes. In addition, we determined if dietary celecoxib treatment leads to refinement of immunotherapeutic strategies.

Methods: MDSC numbers and function were analysed during tumour progression in a murine model for mesothelioma. Mice were inoculated with mesothelioma tumour cells and treated with cyclooxygenase-2 (COX-2) inhibitor celecoxib, either as single agent or in combination with dendritic cell-based immunotherapy.

Results: We found that large numbers of infiltrating MDSC co-localise with COX-2 expression in those areas where tumour growth takes place. Celecoxib reduced prostaglandin E2 levels *in vitro* and *in vivo*. Treatment of tumour-bearing mice with dietary celecoxib prevented the local and systemic expansion of all MDSC subtypes. The function of MDSC was impaired as was noticed by reduced levels of ROS and NO and reversal of T cell tolerance; resulting in refinement of immunotherapy.

Conclusions: We conclude that celecoxib is a powerful tool to improve dendritic cell-based immunotherapy and is associated with a reduction in the numbers and suppressive function of MDSC. These data suggest that immunotherapy approaches benefit from simultaneously blocking cyclooxygenase-2 activity.

Background

MDSC are a heterogeneous population of immature myeloid cells. These cells can inhibit anti-tumour responses in an antigen-specific and in a non-specific way [1,2]. Antigen-specific suppression takes place in lymphoid organs and depends on reactive oxygen species (ROS) production, leading to T cell tolerance. Antigen-non-specific suppression takes place at the tumour site and is mainly dependent on nitric oxide (NO) secretion, causing T cell specific apoptosis [2-4]. More recently, it has been shown that the heterogeneous group of MDSC can be subdivided into three major groups. Polymorph nuclei MDSC (PMN-MDSC), mononuclear MDSC (MO-MDSC) and Gr-1^{low} MDSC [5-7]. Greifenberg *et. al.* showed that the MO-MDSC and a subpopulation of Gr-1^{low} MDSC can inhibit T cell proliferation by the production of NO, in contrast to the PMN-MDSC that did not show this suppressive capacity [5]. However, the exact mechanisms that play a role in

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the induction of T cell tolerance by MDSC subtypes need to be further explored. As ROS production by MDSC also contributes to the induction of tolerance we determined the ROS producing capacity of these subpopulations of MDSC. In addition, we investigated if ROS production by these different MDSC populations can be influenced.

The production of ROS by MDSC is highly depending upon cyclooxygenase-2 (COX-2) enzyme activity [8,9]. The inducible COX-2 enzyme is essential in the biosynthesis of prostaglandins. Over-expression of COX-2 has been described as an important factor in tumour development. Therefore, high expression of COX-2 has been correlated with poor prognosis in cancer [10-12]. In addition, several studies showed the relevance of COX-2 inhibition in cancer progression [13-15]. Although the relation between COX-2 over-expression and prostaglandin E2 (PGE_2) synthesis in cancer has been studied extensively, the impact on the tumour microenvironment is still under investigation [16-18].

Increased evidence indicates that immune suppressive cells, recruited by tumour-derived factors, are the main cause of failure of novel anti-cancer therapies, including immunotherapy [4,19-24]. We investigated dendritic cell (DC)-based immunotherapy in a murine model of mesothelioma. It was found that the efficacy of this treatment is hampered by the highly immunosuppressive environment in mesothelioma [25]. Adjacent to this, it was found that COX-2 over-expression in mesothelioma correlates with poor prognosis [26]. Combining the current knowledge on MDSC, COX-2 over-expression and immune suppression, we aimed to determine the effect of selective COX-2 inhibition on MDSC populations in the optimization of DC-based immunotherapy. We studied the effect of celecoxib on different MDSC populations in a murine model for mesothelioma. Since ROS is one of the principal factors leading to induction of T cell tolerance, we focused on the effects on ROS production by different MDSC populations during celecoxib-treatment.

Methods

Animals and cell lines

BALB/c mice (specific pathogen free [SPF], female, 6-8 weeks old) were purchased from Harlan (Zeist, The Netherlands) and were housed under pathogen-free conditions at the animal care facility of Erasmus MC. All experiments were approved by the local ethical committee for animal welfare (Erasmus University Committee of Animal Experts, Rotterdam, the Netherlands) and complied with the guidelines for the welfare of animals in experimental neoplasia by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR), and by the Code of Practice of the Dutch Veterinarian Inspection. The mesothelioma AB1 cell line was kindly provided by Professor B.W.S. Robinson (School of Medicine and Pharmacology, Sir Charles Gairdner Hospital Unit, The University of Western Australia, Perth, Australia).

Tumour growth of murine mesothelioma in BALB/c mice

BALB/c mice were divided into 4 groups. Each group consisted of 6 mice. On day 0, all mice were injected with a lethal dose of 0.5×10^6 AB1 tumour cells. From day 0 onwards, group 1 and 3 received a control diet while group 2 and 4 received celecoxib diet (500 mg celecoxib/kg [Celebrex®; Pfizer, New York, NY, USA]). Both were starch-based diets manufactured by Harlan Teklad (Madison, WI, USA) as described by T. Hahn *et al.* [27]. Mice in group 3 and 4 received DC-based immunotherapy consisting of 1×10^6 tumour-lysate loaded dendritic cells at day 10.

Dendritic cells were culture from bone marrow using RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 5% heat inactivated fetal bovine serum (FBS [Hyclone, Waltham, MA, USA]), 20 ng/ml GM-CSF (kindly provided by Kris Thielemans, VU Brussels, Belgium), and 50 μM β-mercaptoethanol (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) for 9 days. At day 8, cells were pulsed with tumour-lysate (to the equivalent of three AB1 cells per DC) and overnight matured with 100 ng/ml LPS E. Coli 026:136 (Sigma-Aldrich BV). On the day of vaccination, DCs were harvested and purified by Lympholyte-Mammal (Cedarlane, Hornby, ON, Canada) density gradient centrifugation, and washed three times in phosphate-buffered saline (PBS [Gibco]). Cells were resuspended at a concentration of 1×10^6 viable cells in 500 μl PBS and intraperitoneally injected.

The occurrence of tumour growth, body weight, physical well-being and survival were measured for 2 months, as described previously [28].

Immunohistochemistry on tumour cells and biopsies

Tumour cells were cultured in RPMI supplemented with 5% FBS on two chambers Falcon culture slides (BD biosciences, Erebodegem, Belgium) starting with 5×10^4 AB1 cells per well. Celecoxib was added to the culture when cells reached a confluence of 60% for 24 hours in a concentration of 10 μg/ml and 100 μg/ml. Resected tumour material was obtained from the peritoneal cavity of AB1 inoculated BALB/c mice at day 15. Tumour biopsies were embedded in Tissue-Tek II optimum cutting temperature (OCT) medium (Miles, Naperville, IL, USA), snap-frozen in liquid nitrogen and stored at -80°C. Tissue sections (6 μm) were cut on a HM-560 cryostat (Microm, Heidelberg, Germany).

For the COX-2 expression a rabbit affinity-purified IgG against murine COX-2 was used (Cayman chemical,

Montigny-le-Bretonneux, France) (this antibody shows no cross-reactivity with COX-1). For the COX-1 expression a rabbit affinity-purified IgG against murine COX-1 was used. These primary antibodies were incubated for 1 hour at room temperature. Binding of antibodies was detected using the goat anti-rabbit alkaline phosphatase (AP) (Sigma-Aldrich). Naphtol-AS-MX-phosphate (0.30 mg/ml [Sigma-Aldrich]) and new fuchsine (160 mg/ml in 2 M HCl [Chroma-Gesellschaft, Köngen, Germany]) were used as substrate. Hematoxylene was used as counterstaining. The specificity of the antibodies was checked using a protein concentration-matched non-relevant monoclonal antibody and PBS.

Mouse mesothelioma biopsies were double stained for Gr-1-FITC (clone RB6.8C5) and COX-2. As secondary antibodies horseradish peroxidase (HRP) conjugated goat anti-FITC (Rockland, Gilbertsville, PA, USA) and AP-conjugated goat anti-rabbit (Sigma-Aldrich) were used. Naphtol-AS-MX-phosphate and 1 mM Fast Blue (Sigma-Aldrich) were used as substrate for AP and NovaRed was used as substrate for HRP, according to the manufacturer's instructions (Vector, Burlingame, CA, USA).

Flow cytometry

Spleens were aseptically removed, and mechanically dispersed in cold HBSS (Invitrogen). Cell suspensions were filtered through a 100 μm nylon cell strainer (BD Biosciences), depleted of erythrocytes by osmotic lysis, washed twice in RPMI medium containing 5% FBS, and adjusted to a concentration of 1×10^6 cells/ml in FACs-buffer.

Splenocytes were stained with the following optimally diluted mAbs: Ly6c (FITC conjugated), MHCII (PE conjugated), CD11b (PercP-Cy5.5 conjugated) (all BD bioscience), CD8 (FITC conjugated), F4/80 (FITC conjugated), CD4 (PE conjugated), CD31 (PE-Cy7 conjugated), B220 (Alexa fluor 700 conjugated), Ly6g (APC-Cy7 conjugated) (all eBioscience), CD11c (PE-Texas red conjugated [Caltag, Burlingame, CA, USA]), and a live/dead marker (DAPI [Invitrogen]). Splenocytes were restimulated in the presence of GolgiStop (BD biosciences) for 4 hours using anti-CD3 and intracellular stained for Granzyme B (PE conjugated [Caltag]) and IFN-γ (APC conjugated [BD Biosciences]). Viability was determined by live dead aqua (Invitrogen). Acquisition of eight to nine colour samples was performed on a FACs LSR II cytometer (BD Biosciences). The analysis and graphical output were performed using FlowJo software (Tree Star Inc., Costa Mesa, CA, USA).

ELISA

PGE_2 levels in the peritoneal washes were determined using a specific ELISA assay for PGE_2 (R&D systems, Abingdon, UK). Manufacturer's recommended protocols were followed. Serum was diluted appropriately to ensure that readings were within the limits of accurate detection.

Nitric oxide and its reactive products (NO)

Equal volumes of peritoneal wash (150 μl) were mixed with Greiss reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in double-distilled water). After 30 min incubation at room temperature, the absorbance was measured at 548 nm using a microplate reader (Bio-Rad). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of a stock solution of sodium nitrite.

Reactive oxygen species (ROS)

The oxidation-sensitive dye dichlorodihydrofluorescein diacetate (DCFDA, Sigma-Aldrich) was used for the measurement of ROS production by splenocytes. Cells were incubated at 37°C in DMEM (Invitrogen) in the presence of 1 μM DCFDA for 60 min, and washed twice with cold PBS. Cells were stained with Abs directed against Gr-1 (PE conjugated [BD biosciences]) and CD11b (PercP-Cy5.5 conjugated [BD biosciences]) as previously described. Cells were washed with cold PBS after 20 min incubation. Acquisition of the samples was performed on a flowcytometer.

Tumour-specific lysis assay

Spleens were aseptically removed, and mechanically dispersed in cold PBS. Cell suspensions were filtered through a 100 μm nylon cell strainer (BD Biosciences), depleted of erythrocytes by osmotic lysis, washed twice in RPMI, and adjusted to a concentration of 4×10^6 cells/ml in RPMI medium supplemented with 5% FBS. After 48 h of culture, spleen cells were washed extensively. Mouse AB1 cells were incubated with 100 μCi of $Na_2\,{}^{51}CrO_4$ (ICN Biomedicals) for 2 hours at 37°C, washed three times, resuspended in culture medium at a concentration of 5×10^4 cells/ml. Splenocytes (150.000 cells per well) from either naïve mice, untreated mice, or mice treated with DC-based immunotherapy were mixed with 5×10^3 radiolabeled AB1 target cells and added to wells of a 96-well round-bottom microtiter plate (0.2 ml final volume) to achieve the desired effector:target (E:T) ratios. To determine the suppressive capacity of splenocytes from mice treated with control diet or celecoxib diet, 150.000 splenocytes from DC treated mice were mixed with 150.000 splenocytes from mice treated with either control diet or celecoxib diet. After 24 hour incubation, cells were mixed with radiolabeled AB1 target cells and added to wells of a 96-well

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round-bottom microtiter plate (0.2 ml final volume) to achieve the desired effector:target (E:T) ratios.

Plates were incubated for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2, and cell-free supernatants were collected from each well. The amount of ^{51}Cr released from lysed AB1 target cells was determined by γ scintillation counting. Percent lysis was calculated using the formula: corrected % lysis = 100×(experimental release - spontaneous release [target cells incubated in medium alone])/(maximum release[2% Triton X-100 as lysing agent]-spontaneous release).

Statistical analysis

Data are expressed as mean ± SD. Comparisons between groups were made using the t-test. A two-tailed p-value < 0.05 was considered significant. Data presented as a percentage of tumour-free animals were analysed with Kaplan-Meier survival-curves, using the log-rank test to determine significance.

Results

Identification of myeloid-derived suppressor cell subsets

Splenocytes from mice that were inoculated with a lethal dose of AB1 tumour cells and placed on a control or celecoxib diet were stained for flowcytometric analysis.

Similar to the most recent published data, three populations of Gr-1$^+$CD11b$^+$ cells were identified [5-7]. The Gr-1^{low} MDSC could be further subdivided in two populations based on size and inner complexity of the cells (forward [FSC]/side scatter [SSC]). The physical characteristics combined with Ly6c expression was used to visualize other intrinsic differences between the different groups. We found that Ly6c expression was lower on the Gr-1^{low} MDSC with a high SSC (subset 1) in contrast to the Gr-1^{low} MDSC with low SSC (subset 2). Ly6c expression on PMN-MDSC was lower then Ly6c expression on MO-MDSC (Figure 1A).

MDSC can induce T cell tolerance by producing ROS. ROS production was highest in PMN-MDSC followed by MO-MDSC. Subset 2 of the Gr-1^{low} MDSC had the capability to produce ROS, whereas the majority of the cells in subset 1 were not able to produce ROS (Figure 1A).

Tumour biopsies were obtained and stained for the presence of Gr-1$^+$CD11b$^+$ cells. Large areas with infiltrating positive cells were found at the rim of the tumour. Since COX-2 is an essential enzyme for catalyzing the biosynthesis of tumour-derived prostaglandin E2 (PGE_2), a molecule that induces MDSC [7], biopsies were stained for COX-2 expression. COX-2 expressing cells were found at the border of the tumour closely located near areas with Gr-1$^+$CD11b$^+$ infiltrating cells (Figure 1B).

Next we investigated if the enzyme activity of tumour-derived COX-2 could be inhibited by celecoxib *in vitro*. Therefore, cultured AB1 tumour cells were incubated with different dosages of celecoxib. After 24 hours supernatant was removed and cells were stained for the expression of COX-2 and COX-1 as a control. COX-1 expression was not effected by the selective COX-2 inhibitor celecoxib. COX-2 expression was reduced in a doses-depended manner (Figure 1C).

In conclusion, these data show that MDSC in the spleens of tumour-bearing mice can be subdivided into three groups. Additionally, we demonstrate that PMN-MDSC and MO-MDSC produce high ROS. The group of Gr-1^{low} MDSC could be further subdivided into two subsets based on FSC/SSC, Ly6c and ROS production. Large areas with infiltrating Gr-1$^+$CD11b$^+$ cells were found at the rim of the tumour in close contact to COX-2 expressing cells. COX-2 expression by AB1 tumour cells was decreased by celecoxib *in vitro* in a dose-depended manner.

Reduction of MDSC by dietary administration of celecoxib

We investigated the effect of celecoxib treatment on the four MDSC subsets that were identified in the spleen of tumour-bearing mice. Splenocytes from mice that were inoculated with AB1 tumour cells and received celecoxib diet or control diet were analyzed for the presence of the MDSC subsets.

Ten days after tumour injection, the absolute number of MDSC was significantly lower in mice receiving celecoxib diet compared with mice receiving control diet. This difference was more pronounced at day 22 after tumour injection (Figure 2A). When MDSC subsets (MO-MDSC PMN-MDSC and the two subpopulations of Gr-1^{low} MDSC as described in Figure 1A), a significant decrease in the percentage of PMN-MDSC was found. In addition, a shift between subset 1 and subset 2 within the Gr-1^{low} MDSC was observed leading to an increase of subpopulation 1. The total numbers of Gr-1^{low} MDSC did not differ between celecoxib treated and untreated animals. More recently, it has been shown that macrophages can be derived from MDSC [6,29]. Therefore this cell population was also analyzed. Macrophages were characterized by FACS based on their FSC/SSC, in combination with the expression of CD11b, F4/80 and MHCII on their membrane. A significant reduction in macrophages was observed in celecoxib treated mice compared to untreated mice (Figure 2B).

To summarize, a significant decrease in the number of MDSC in the spleen was found at day 10 after tumour inoculation and became more diverged when tumour progressed in celecoxib treated mice compared with untreated animals. Furthermore, we found that the reduction in MDSC after dietary celecoxib treatment

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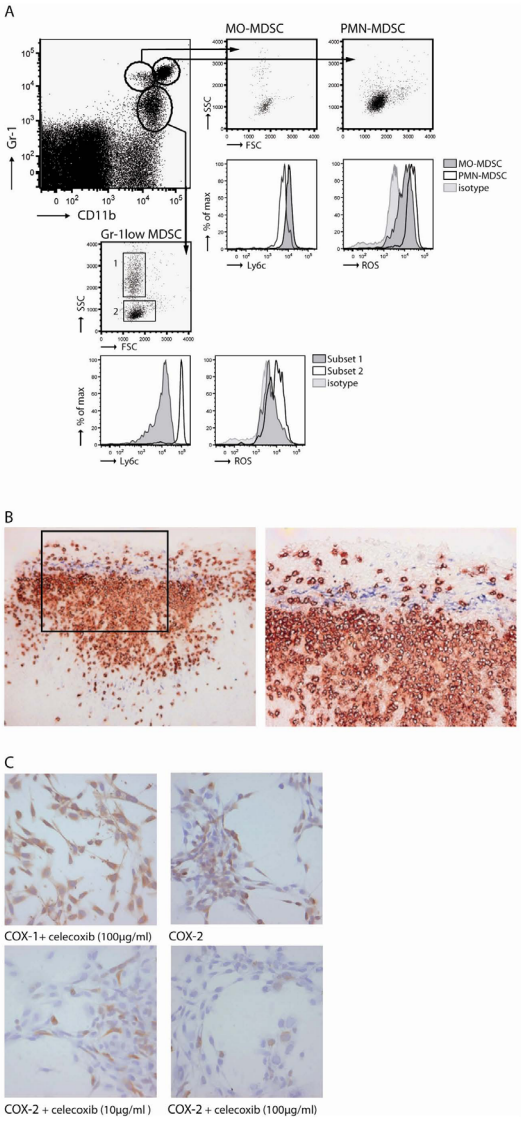

Figure 1 Identification of MDSC and myeloid cell types in tumour-bearing mice. A) PMN-MDSC expressed high levels of Gr-1, CD11b. MO-MDSC have a slightly higher expression of Ly6c. A third population was identified showing a low expression of Gr-1. Based on FSC/SSC this population was further subdivided into two subsets; SSC^{low} expressing high Ly6c and SSC^{high} expressing low Ly6c. ROS production was measured for each subtype. B) Histology on tumour sections showed $Gr\text{-}1^{+}$ (red) at the rim of the tumour in close contact with COX-2 expressing cells (blue). [Magnification: 200× (left) and 400× (right)]. C) Inhibition of COX-2 activity was tested *in vitro* by adding celecoxib to AB1 cell cultures. AB1 cells were cultured for 24 hours with medium, 10 µg/ml, or 100 µg/ml celecoxib and stained with anti-COX-2 and anti-COX-1 (as control) antibodies.

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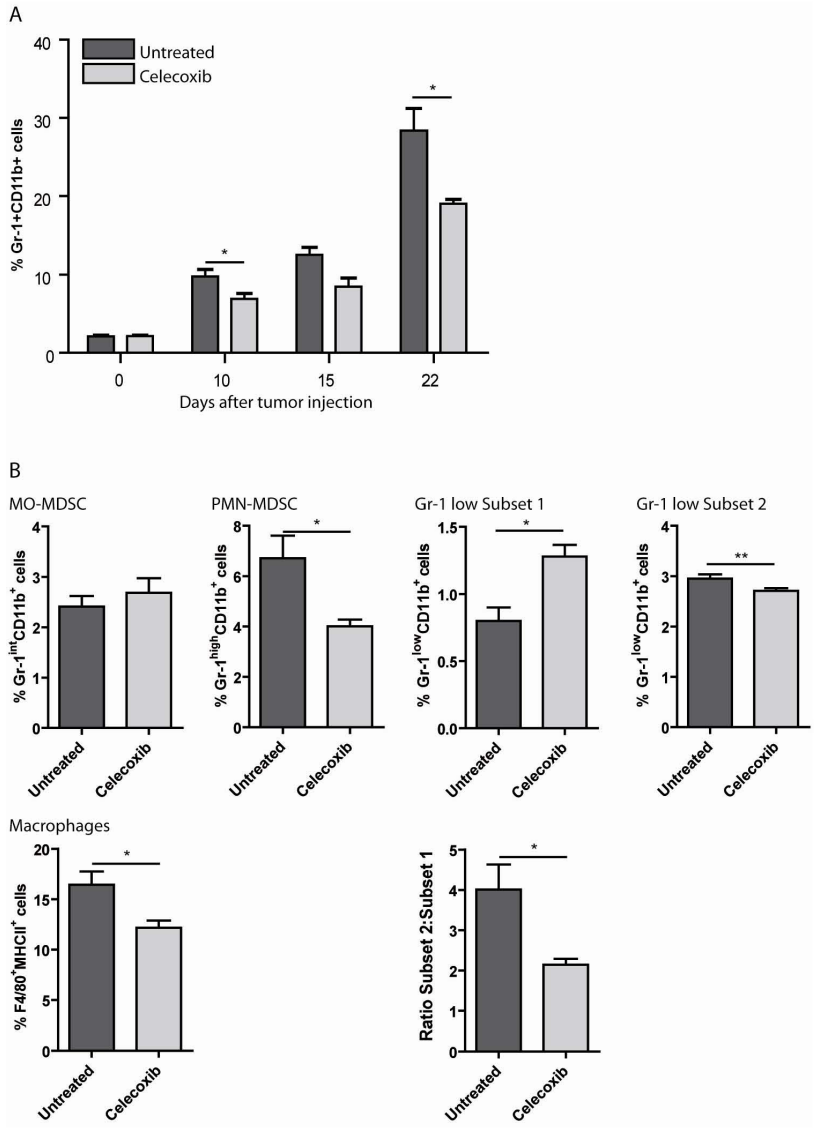

Figure 2 Reduction of MDSC in tumour-bearing mice after celecoxib-treatment. A) Accumulation of MDSC (characterized by the expression of Gr-1 and CD11b) in tumour-bearing mice receiving control diet or celecoxib diet: day 0 n = 4/4, day 10 n = 5/5, day 15 n = 3/2, day 22 n = 3/3). A significant difference was found at day 10 and day 22. * p = 0.034, ** p = 0.031. B) Splenocytes from mice receiving control diet or celecoxib diet were analyzed 10 days after tumour injection (n = 5/5). Subtypes were identified as described in Figure 1. A significant reduction was found in PMN-MDSC (p = 0.021) and in subset 2 from the Gr-1^{low} MDSC population (p = 0.041) in mice receiving celecoxib diet. A significant increase was found in subset 1 from the Gr-1^{low} MDSC population (p = 0.007), leading to a shift in ratio (p = 0.020) in celecoxib treated mice. The percentage of F4/80^{+}MHCII^{+} cells was significantly reduced in mice receiving celecoxib diet (p = 0.022).

was mainly caused by a reduction of PMN-MDSC and a shift in subsets of the Gr-1^{low} MDSC.

Reduced ROS production by MDSC subsets

We next investigated if dietary celecoxib treatment not only influenced the number of MDSC in the spleen but also affects the function of the MDSC subsets. It has been shown that MDSC can down regulated the ζ-chain on T cells by ROS production in the lymphoid organs and thereby induce T cell tolerance [19,30]. We analyzed splenocytes of dietary celecoxib treated and untreated mice for their capability to produce ROS.

Celecoxib treatment reduced ROS production in all MDSC subsets, especially in the Gr-1^{low} MDSC subset 2 and MO-MDSC. There was a trend toward a reduction of ROS production by macrophages, though this reduction was not significant ($p = 0.14$). Analyzing ROS production by all myeloid cells revealed that ROS production was decreased in celecoxib treated mice compared to untreated animals (Figure 3).

In summary, ROS production was most pronounced in PMN-MDSC. Dietary celecoxib treatment reduced ROS production in all MDSC subtypes but is most effective in the MO-MDSC and Gr-1^{low} MDSC subset 2 both in percentage as well as the median fluorescence intensity (MFI).

Reduction of COX-2 expression in tumour tissue after dietary celecoxib treatment

After we observed that celecoxib treatment reduced the number and function of MDSC in the spleen of tumour-bearing mice we investigated if dietary treatment of mice with celecoxib also affects MDSC in the tumour environment. Therefore tumour biopsies were analyzed for the expression of COX-2 enzyme and the presence of infiltrating MDSC. In addition, the peritoneal cavity of tumour-bearing mice was washed with PBS and analyzed for tumour-derived PGE_2 and NO production.

Tumour was infiltrated with massive amounts of Gr-1$^+$CD11b$^+$ cells, mainly at the border of the tumour. These cells were in close contact to COX-2 expressing tumour cells. Treatment with celecoxib showed that the Gr-1$^+$CD11b$^+$ cells were more restricted and that COX-2 expression at the rim of the tumour was lost (Figure 4A).

Additionally, tumour-derived PGE_2 was decreased in the peritoneal wash of mice receiving celecoxib diet compared to animals on control diet. Also a reduction in NO concentrations was found (Figure 4B).

These data show that treating mice with celecoxib decreased the expression of COX-2 and thereby reduced MDSC both systemically as well as in the local

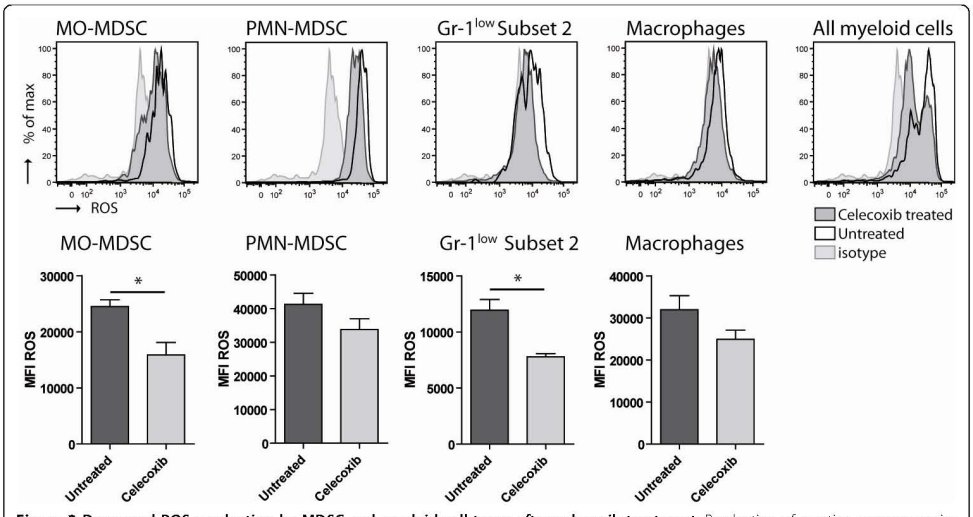

Figure 3 Decreased ROS production by MDSC and myeloid cell types after celecoxib-treatment. Production of reactive oxygen species (ROS) by (immature) myeloid subtypes from the spleen of tumour-bearing mice was measured. Splenocytes were obtained from tumour-bearing mice receiving either control diet or celecoxib diet for 10 days after tumour injection. Subtypes were identified based on characteristics as described in Figure 1. ROS production was reduced by MDSC in tumour-bearing mice treated with celecoxib diet. Mean fluorescent intensity (MFI) of ROS was significantly lower in MO-MDSC ($p = 0.015$) and Gr-1^{low} Subset 2 MDSC ($p = 0.017$) after celecoxib-treatment. The MFI of ROS on macrophages did not significantly change ($p = 0.14$).

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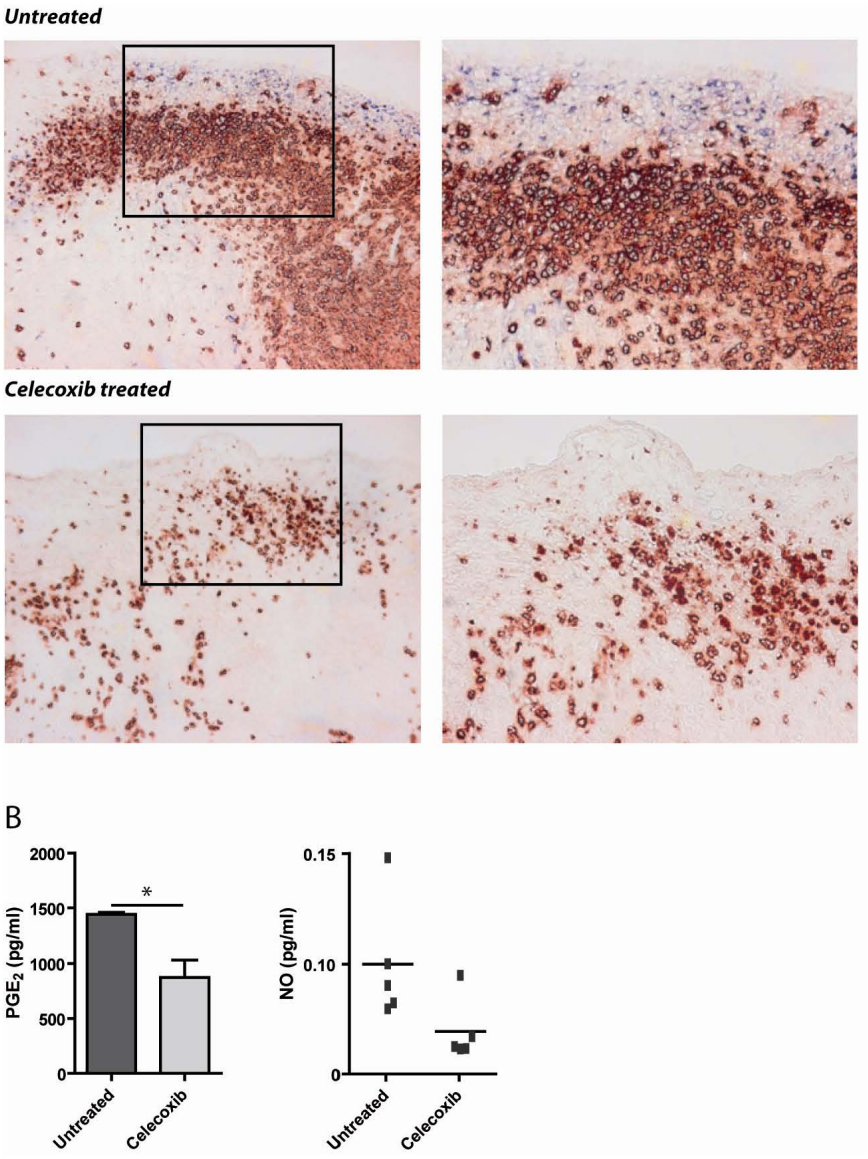

Figure 4 Reduction of COX-2 expression after celecoxib-treatment. A) Tumour sections of untreated (n = 7) and celecoxib treated mice (n = 8) were immunohistochemically double-stained for the presence of Gr-1 (red) and COX-2 expression (blue). No expression of COX-2 was found in tumour sections from mice with dietary celecoxib. Gr-1$^+$ cells were reduced in tumour sections of mice treated with celecoxib [magnification 200× left and 400× right]. B) PGE_2 levels were measured in peritoneal washings of untreated (n = 7) versus celecoxib treated mice (n = 8) (p = 0.0061). NO was detected using Greiss reagent.

microenvironment of the tumour. This reduced COX-2 expression is accompanied by with a reduction in PGE_2 and NO levels. Moreover, the infiltration of Gr-1 sup>+CD11b$^+$ cells in tumour areas was reduced by celecoxib-treatment.

Reduction of immune suppression after celecoxib treatment

In our previous study we have shown that DC-based immunotherapy in this murine model leads to the induction of a strong anti-tumour response [28]. However, the effectiveness of the anti-tumour response was negatively influenced when tumours sizes increased [28]. It has been addressed that one of the main reasons for immunotherapy failure is the induction of T cell tolerance by immune suppressive cells. To determine if treatment with celecoxib also intercepts the induction of T cell tolerance the following experiments were performed. A chromium release assay was performed to investigate the effects on tumour specific lysis in different conditions.

There was no specific lysis of tumour cells when splenocytes of naïve mice or tumour-bearing mice were co-cultured with radioactive labelled tumour cells. When splenocytes from mice treated with DC-based immunotherapy were co-cultured with tumour cells a massive induction of tumour lysis was observed, demonstrating that DC-based immunotherapy induces tumour specific recognition by immune cells. To examine the suppressive effect of MDSC in the spleen, splenocytes from DC-treated mice were co-cultured with splenocytes of tumour-bearing mice treated with either control diet or celecoxib diet. After 4 hour incubation at 37°C, the splenocyte mixtures were co-cultured with the radioactive labelled tumour cells. Although the total amounts of splenocytes were equal in all conditions, a significant reduction in anti-tumour activity was found when splenocytes of control diet animals were added. In contrast, the addition of splenocytes from tumour-bearing mice treated with dietary celecoxib did not reduce the lytic capacity of splenocytes from mice treated with DC-immunotherapy (Figure 5A).

IFN-γ and granzyme B production by CD8$^+$ T cells showed similar results. CD8$^+$ T cells from the spleen of naïve and tumour-bearing mice were not capable of IFN-γ and granzyme B production. Production of IFN-γ and granzyme B was significantly increased after DC-treatment. Co-culture of splenocytes from DC-treated mice with splenocytes from tumour-bearing mice treated with control diet or celecoxib diet, showed that CD8$^+$ cells were affected by splenocytes of mice that had received the control diet while splenocytes of celecoxib treated mice did not affect the capability of CD8$^+$ cells to produce IFN-γ and granzyme B (Figure 5B).

In conclusion, these data show that anti-tumour responses induced by DC-treatment, are affected by suppressive cells in the spleen of tumour-bearing mice. However, the anti-tumour activity as indicated by AB1 lysis and IFN-γ/granzyme B production by CD8$^+$ T cells was no longer influenced when co-cultured with Splenocytes of mice receiving celecoxib diet, indicating that COX-2 inhibition leads to a reduction in suppressive immune cells.

Dietary celecoxib improves DC-based immunotherapy

Next, we wanted to know if combining dietary celecoxib and DC-based immunotherapy would lead to an increased survival benefit. Previous studies showed that mice treated with DC-immunotherapy one day after tumour injection leads to 100% survival in DC-treated mice by inducing anti-tumour T cell activity. However, the efficacy of DC-treatment decreases with increasing tumour burden. To study the possible synergistic effect of celecoxib and immunotherapy a *suboptimal* DC-treatment protocol was used. Therefore in this protocol mice received tumour lysate-loaded DC 10 days after tumour injection. Mice receiving AB1 tumour cells developed signs of terminal illness after 12 days.

Treatment with celecoxib alone prolonged survival to some extent; however this prolongation was not significant. No side effects were observed in mice receiving the celecoxib diet. The combination of dietary celecoxib and DC-immunotherapy led to a significant improvement of the immunotherapy ($p = 0.038$). In addition, combined treatment compared to no treatment significantly improved survival ($p = 0.027$) were single treatment with celecoxib or suboptimal DC-treatment did not improve survival ($p = 0.305$ and $p = 0.455$) (Figure 6).

This experiment showed that the combination of dietary celecoxib and immunotherapy is superior to single treatment in mice with high tumour burden.

Discussion

We show that dietary administration of celecoxib leads to a significant decrease in the number and suppressive function of immature myeloid cells in the spleens and tumours of tumour-bearing mice. Subdivision of the heterogeneous group of immature myeloid cells revealed that three types could be identified in tumour-bearing mice, reflecting findings done by others [5,7]. The PMN-MDSC population was most frequently present in the spleen of tumour-bearing mice. This population was also most capable of ROS production. Previous studies showed that ROS production by MDSC is the main factor for inducing T cell tolerance by down regulation of the ζ-chain on activated T cells [19,30]. Celecoxib-treatment especially influenced this PMN-MDSC population

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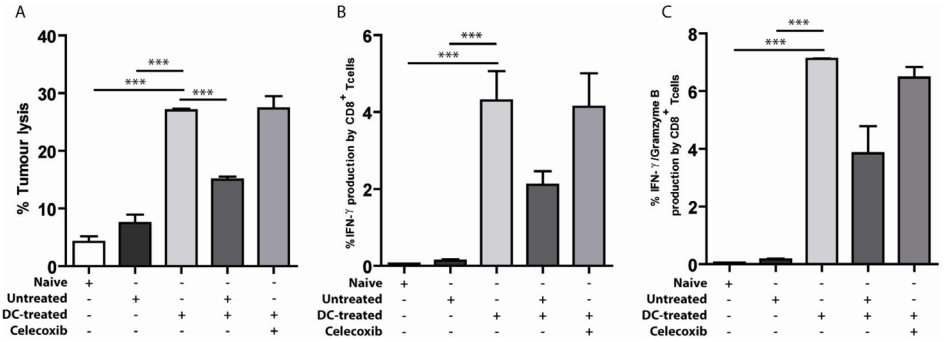

Figure 5 Improved T cell function after celecoxib-treatment. A) The amount of ^{51}Cr released from lysed radiolabeled AB1 target cells was determined to compare cytolytic activity of splenocytes isolated from naïve mice (n = 4) tumour-bearing mice (n = 5) and DC-treated mice (n = 4). To determine the inhibitory effect of MDSC in the spleen, splenocytes from mice receiving control diet or celecoxib diet were co-cultured with splenocytes of DC-treated mice (the number of DC-treated splenocytes was equal in all conditions 150.000 cells/well). Percentage of lysis was calculated using the formula: corrected % lysis = 100 × (experimental release - spontaneous release [target cell incubated in medium alone])/(maximum release - spontaneous release). Splenocytes from DC-treated mice were significantly better capable of lysing tumour cells compared to naïve ($p < 0.0001$) and tumour-bearing mice ($p < 0.0001$). The addition of splenocytes from a tumour-bearing mice treated with control diet significantly reduced the lytic capacity of splenocytes from DC-treated mice ($p = 0.0002$) whereas the addition of splenocytes from tumour-bearing mice treated with celecoxib diet did not affect the lytic capacity of splenocytes from DC-treated mice ($p = 0.887$). B) Percentages of IFN-γ^+ $CD8^+$ cells within the spleen were determined by intracellular FACs staining. DC-treatment significantly improved IFN-γ production by $CD8^+$ cells compared to naïve mice ($p < 0.0001$) and tumour-bearing mice ($p = 0.001$). Conditions as described above. C) IFN-γ/ Granzyme B production by $CD8^+$ cells was measured by intracellular FACs staining revealing a significant difference between naïve ($p < 0.0001$) and tumour-bearing mice($p < 0.0001$) compared to $CD8^+$ cells in the spleen of DC-treated mice.

compared to other MDSC subtypes in the spleen of tumour-bearing mice. Greifenberg *et. al.* recently showed that this population was not capable of NO production and therefore showed no suppressive effect on T cell proliferation in a T cell mixed leukocyte reaction (MLR) [5]. However, our data provides evidence that PMN-MDSC do play a significant role in tumour-induced immune suppression, since PMN-MDSC are most capable of producing ROS and thereby contribute to the induction of tolerance. Induction of tolerance can influence novel treatment strategies like DC-based immunotherapy negatively. Others have shown that immune suppression in the spleen in mainly dependent on ROS production whereas immune suppression in the tumour area is predominately depending on NO production by myeloid cells indicating that the increased PMN-MDSC population suppresses the anti-tumour T cell responses by producing ROS.

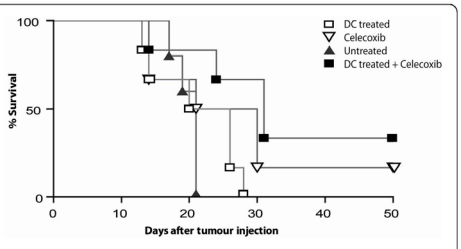

Figure 6 Dietary celecoxib improves DC-based immunotherapy. Mice were inoculated i.p. with 0.5×10^6 AB1 tumour cells at day 0. A *suboptimal* DC-treatment protocol was used. Mice were treated with DC-based immunotherapy at day 10 after tumour injection. Mice received control diet or celecoxib diet from day 1 onwards. All groups consisted of 6 mice. Survival was measured using Kaplan-Meier survival analysis. Combining DC-based immunotherapy with dietary celecoxib improved survival ($p = 0.027$), compared to a single treatment with celecoxib ($p = 0.305$) or DC-based immunotherapy ($p = 0.456$). All compared to no treatment.

The Gr-1^{low} MDSC population can be divided into two subsets. We observed a shift in the Gr-1^{low} MDSC population of celecoxib treated mice leading to less ROS producing cells in this fraction. Greifenberg *et al.* showed that FSC/SSC low subset had suppressive capacity and were able to produce NO after LPS/IFN-γ restimulation. Next to this, we were able to show that this subset is also capable of ROS production whereas the FSC^{high} fraction within the Gr-1^{low} MDSC could not. Furthermore, we showed that celecoxib-treatment not only decreased the amount of immature myeloid cells in tumour-bearing mice, but that treatment also impaired ROS production by the different subpopulations of MDSC. Given the fact that the number of MDSC is

reduced and the production of ROS by these cells is diminished makes them less capable of inducing T cell tolerance. The fact that T cell tolerance was no longer impaired after celecoxib-treatment was confirmed in a cytotoxicity assay showing that AB1 lysis was hampered when cells were co-cultured with splenocytes from mice who had received control diet while tumour specific lysis occurred when co-culture was performed with splenocytes from tumour-bearing mice treated with celecoxib diet.

Histological analyses revealed that COX-2 expression was mainly present in those areas where Gr-1$^+$CD11b$^+$ cells were found. Almost all AB1 tumour cells express the COX-2 enzyme in culture. An explanation for this finding could be that tumour cells secrete high levels of PGE_2 in order to attract MDSC. The other option is that COX-2 expression in culture is necessary for proliferation. However, by adding celecoxib to tumour cells the expression of COX-2 was reduced without affecting the metabolic activity of the cells (data not shown). *In vivo* COX-2 expressing tumour cells were present at the rim of the tumour tissue co-localizing with the Gr-1$^+$CD11b$^+$ areas. COX-2 expression is diminished in tumour when mice were treated with celecoxib. The decrease in COX-2 enzyme expression has been observed in other studies [31-33]. The mechanism by which celecoxib perturbs COX-2 protein expression is not known. It has been suggested that PGE_2 functions as a feedback on COX-2 protein expression and that celecoxib inhibits this loop [34]. It is also possible that the inhibitory effect of celecoxib on the NFκB pathway results in a reduced production of COX-2 proteins [35]. The reduction in COX-2 expression was accompanied by a reduction of PGE_2 levels in peritoneal wash of celecoxib treated mice. Unfortunately no clear division can be made between the MDSC types in tumour tissue. Since Gr-1 (Ly6g) and Lyc6 are both expressed on all subtypes, no histological subdivision can be made using these markers. New histological markers are necessary for better classification of the different MDSC subtypes in tumour tissue.

Currently, we are investigating DC-immunotherapy in mesothelioma patients. Although we were able to induce an immune response in 4 out of 6 patients, the induced anti-tumour responses are opposed by immune suppressive cells like MDSC. Ochoa and colleagues have suggested that prostaglandin E2 produced by tumour cells induced the arginase I expression in MDSC [36]. For this reason, COX-2 inhibition to reduce MDSC in number and function has been proposed as promising strategy to improve immunotherapy. We have previously shown that upon injection of antigen-loaded DCs an effective immune response can be induced in a mouse model for mesothelioma depending on the timing of the immunotherapy [28]. In this study we were able to show that the reduction of MDSC *in vivo* was associated with an improved anti-tumour response and resulted in prolongation of survival in combination with DC-based immunotherapy. Same results were reported by others [37]. We were not able to verify that the improvement of the anti-tumour response in tumour-bearing mice treated with celecoxib was directly caused by the reduction in MDSC. Because MDSC are depending on cytokines, chemokines, prostaglandins and growth factors produced by surrounding cells for their function, we choose to mix total splenocytes instead of sorted Gr-1$^+$CD11b$^+$ cells. For example MDSC are critically depending on IFN-γ production by T cells [2]. To determine that the abolishment of suppression in celecoxib treatment mice was most likely caused by the reduction of MDSC number and function, we screened splenocytes of all mice for other cell-types. No significant differences in the number of B cell, T cell (including Tregs and $\gamma\delta$T cells) or macrophages between untreated and celecoxib treated tumour-bearing mice were observed. The number and function of MDSC did differ between the two groups (as described on in the result section). Therefore we conclude that the improved anti-tumour response is most likely caused be the reduction in MDSC number and function, though no firm conclusions on direct relations can be made.

We determined the effect of celecoxib treatment on MDSC; however, since COX-2 (and PGE_2) is known to contribute to variety of cellular processes it is difficult to determine the solitary effect of a COX-2 inhibitor on MDSC *in vivo*. The role of COX-2 expression and tumour-derived PGE_2 in cancer has been studied intensively. Significant correlations between the levels of COX-2 expression and survival were observed in many human cancers [38-40], including malignant pleural mesothelioma. COX-2 over-expression was found in the majority of mesothelioma (73% epithelial mesothelioma, 50% of mix-variants and 37% sarcomatoid mesothelioma). Survival analysis revealed that over-expression of COX-2 was related to worse prognosis. For this reason COX-2 inhibition has been proposed as potential therapeutic target for mesothelioma [10,11]. Also PGE_2 contributes to proliferation, survival, angiogenesis, migration and invasion of tumour cells [41].

A rapid induction of MDSC was found during tumourigenisis in our mouse mesothelioma model. Although it is generally accepted that MDSC are part of the tumour microenvironment [19], their numbers or function may differ in various tumour cell-lines or other tumour model. This is caused by differences in the cytokine, chemokine and growth factor production of tumour cells that determines the specific microenvironments [42,43]. To determine which patients may benefit

from celecoxib treatment alone or in combination with immunotherapy, more research is needed.

Conclusions

Large numbers of infiltrating MDSC co-localise with COX-2 expression in tumour biopsies. Selective COX-2 inhibition by celecoxib reduced prostaglandin E2 levels *in vitro* and *in vivo*. Treatment of tumour-bearing mice with dietary celecoxib prevented the local and systemic expansion of all MDSC subtypes and also their suppressive function was impaired. Combining celecoxib with DC-based immunotherapy demonstrated highly activated cytotoxic T lymphocytes with superior immunostimulatory potency and anti-tumour activity because of the reduced MDSC expansion. This leads to a significant benefit in overall survival.

We conclude that celecoxib is a powerful tool to reduce the numbers and suppressive function of MDSC, which was associated with a beneficial effect of dendritic cell-based immunotherapy. Future studies will demonstrate the effectiveness of celecoxib treatment combined with dendritic cell-based immunotherapy in a clinical setting for cancer patients.

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Authors' contributions
JV: acquisition and interpretation of data; writing the manuscript. ML: carried out animal studies. MvN: carried out flowcytometric analysis. RH: performed the statistical analysis and data analysis. HH: have given final approval to the manuscript submission, revised the manuscript. JA: have made substantial contributions to conception and design, data interpretation. JH: have made substantial contributions to conception and design, data interpretation, revised the manuscript. All authors read and approved the final manuscript.

Authors' information
Authors have recently published a clinical trial in the American Journal of Respiratory and Critical Care Medicine on the use of dendritic cell-based immunotherapy in mesothelioma patients. We showed for the first time the safety and feasibility of tumor lysate-pulsed dendritic cells as therapeutic adjuvants in mesothelioma patients and found distinct immune responses and antitumor responses in these patients. Now we are focusing on refinement of this approach.

Competing interests
The authors declare that they have no competing interests.

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Chapter 5

Zoledronic acid impairs myeloid differentiation to tumour-associated macrophages in mesothelioma

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Zoledronic acid impairs myeloid differentiation to tumour-associated macrophages in mesothelioma

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BACKGROUND: Suppressive immune cells present in tumour microenvironments are known to augment tumour growth and hamper efficacy of antitumour therapies. The amino-bisphosphonate Zoledronic acid (ZA) is considered as an antitumour agent, as recent studies showed that ZA prolongs disease-free survival in cancer patients. The exact mechanism is a topic of debate; it has been suggested that ZA targets tumour-associated macrophages (TAMs).

METHODS: We investigate the role of ZA on the myeloid differentiation to TAMs in murine mesothelioma *in vivo* and *in vitro*. Mice were intraperitoneally inoculated with a lethal dose of mesothelioma tumour cells and treated with ZA to determine the effects on myeloid differentiation and survival.

RESULTS: We show that ZA impaired myeloid differentiation. Inhibition of myeloid differentiation led to a reduction in TAMs, but the number of immature myeloid cells with myeloid-derived suppressor cell (MDSC) characteristics was increased. In addition, ZA affects the phenotype of macrophages leading to reduced level of TAM-associated cytokines in the tumour microenvironment. No improvement of survival was observed.

CONCLUSION: We conclude that ZA leads to a reduction in macrophages and impairs polarisation towards an M2 phenotype, but this was associated with an increase in the number of immature myeloid cells, which might diminish the effects of ZA on survival.

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Recently, cancer-related inflammation was recognised as the seventh hallmark of cancer (Colotta *et al*, 2009). Infiltration of inflammatory cells into tumour tissues can be detected in many cancers. These infiltrating immune cells can possess immune stimulatory (antitumour activity) or immune suppressive capacity and thus promote tumour progression. In the tumour microenvironment of a progressing tumour, regulatory T cells, myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages (TAMs) have an important role in facilitating tumour growth and immune escape by suppressing antitumour effector cells (Bronte *et al*, 2001; Hegmans *et al*, 2006; Sakaguchi *et al*, 2008; Sica *et al*, 2008; Allavena *et al*, 2008b; Gabrilovich and Nagaraj, 2009).

Regulatory T cells and MDSCs are regarded as immune suppressive, as they are capable of inducing T-cell apoptosis and T-cell tolerance (Nagaraj and Gabrilovich, 2007; Sakaguchi *et al*, 2009), whereas TAMs are involved in a variety of processes, including angiogenesis, tumour invasion and tumour metastasis (Pollard, 2004; Condeelis and Pollard, 2006). Tumour-associated macrophages are derived from circulating monocytic precursors. Recently, it has been shown that this group of immature myeloid cells can be derived in three different populations, carrying MDSC characteristics, and can also function as a pool of precursor cells for macrophages and endothelial cells. Mononuclear-MDSC (MO-MDSC) harbours immune suppressive capacities, but additionally function as TAM precursors, in contrast to polymorph nuclear-MDSC (PMN-MDSC) and $CD11b^{high}Gr\text{-}1^{low}$-MDSC (Bronte and Zanovello, 2005; Nagaraj and Gabrilovich, 2007; Gabrilovich and Nagaraj, 2009; Greifenberg *et al*, 2009).

Tumour-associated macrophages acquire a polarised phenotype of alternatively activated macrophages (M2), whereas classically activated macrophages (M1) are more associated with inflammation. These two types of macrophages differ in receptor expression, effector function, and cytokine and chemokine productions (Mantovani *et al*, 2002). By producing pro-angiogenic enzymes, like matrix metalloproteinase 9 (MMP-9), TAMs are able to breakdown the extracellular matrix, leading to the release of pro-angiogenic proteins and growth factors. In addition, TAMs produce vascular endothelial growth factor (VEGF), promoting angiogenesis and recruitment of cells derived from the myeloid lineage (Kusmartsev and Gabrilovich, 2002; Siveen and Kuttan, 2009).

The recruitment of immature myeloid cells is enhanced by the production of chemokine (C–C motif) ligand-2/monocyte-chemotactic protein-1 (CCL-2/MCP-1), which is produced and expressed by TAMs and tumour cells. Within the heterogeneous group of immature myeloid cells, subpopulations are defined, which can further differentiate into mature macrophages, and hence TAMs provide their own precursor cells, which enhances tumour progression (Rossner *et al*, 2005; Umemura *et al*, 2008; Greifenberg *et al*, 2009; Dolcetti *et al*, 2010).

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Targeting TAMs seems a promising tool to prevent tumour progression, thereby enhancing antitumour therapies. Bisphosphonates have been reported to target macrophages. They were initially prescribed to improve bone density; however, in metastasing cancers, a possible role in prevention of metastasis and prolongation of disease-free survival has been described (Diel *et al*, 2000, 2008; Gnant *et al*, 2009). The exact mechanisms by which bisphosphonates prevent disease progression are still a topic of extensive investigations. It has been proposed that bisphosphonates have a direct apoptotic effect on tumour cells (Yoneda *et al*, 2000; Mundy, 2001, 2002; Fromigue *et al*, 2003). In addition, indirect effects of bisphosphonates have been reported. It has been suggested that bisphosphonates modulate the immune response and may influence macrophage phenotype (Wolf *et al*, 2006; Tsagozis *et al*, 2008; Coscia *et al*, 2009).

Currently, we are investigating novel therapeutic strategies in mesothelioma, like dendritic cell-based immunotherapy. Mesothelioma is a cancer with dismal prognosis deriving from the layers of the pleural cavity or the peritoneal cavity. As high numbers of TAMs are found in pleural effusions and tumour biopsies in human mesothelioma, we determined the efficacy of a bisphosphonate, Zolendric acid (ZA) (Zometa; Novartis Pharma BV, Arnhem, The Netherlands), on TAM formation and tumour progression in a murine mesothelioma model.

In this study, we analysed the effect of ZA on myeloid differentiation both *in vitro* and *in vivo*. In addition, we determined whether ZA changed the phenotype of macrophages. Animal studies were performed to investigate the effect of ZA on survival.

METHODS

Animals and cell lines

CBA-j mice (specific pathogen free, female, 6–8 weeks old) were purchased from Harlan (Huntington, UK) and were housed under pathogen-free conditions at the Erasmus MC animal facility. All experiments were approved by the local ethical committee for animal welfare (Erasmus University Committee of Animal Experts, Rotterdam, the Netherlands) and complied with the guidelines for the welfare of animals in experimental neoplasia by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) and by the Code of Practice of the Dutch Veterinarian Inspection. The AC29 cell line is kindly provided by Professor BWS Robinson (School of Medicine and Pharmacology, Sir Charles Gairdner Hospital Unit, The University of Western Australia, Perth, Australia).

Depletion of macrophages *in vivo*

Macrophages were depleted by intraperitoneal (i.p.) injection of liposome-encapsulated clodronate (Dr Nico Van Rooijen, VUmc, FdG, Amsterdams, The Netherlands) (Claassen, 1992; Van Rooijen and Sanders, 1994).

CBA-j mice were i.p. inoculated with a lethal dose of 20×10^6 AC29 tumour cells on day 0. Mice were i.p. injected with 200 μl liposome-encapsulated clodronate or liposome-encapsulated PBS at days 5 and 10 after tumour injection. At day 12 mice were killed using CO_2. The peritoneal cavity of tumour-bearing mice was washed with 1 ml PBS to obtain peritoneal cells for FACS analysis. All visible tumour material was excised from each mouse and data are expressed as wet weight (accuracy of 0.001 g).

Tumour-associated macrophages were defined by the expression of F4/80, MHCII and CD206 (marker for M2 macrophage phenotype).

Immunohistochemistry on tumour biopsies

Tumour material was obtained 25 days after tumour injection from the peritoneal cavity of tumour-bearing mice. Tumour biopsies were embedded in Tissue-Tek II optimum cutting temperature medium (Miles, Naperville, IL, USA), snap frozen in liquid nitrogen and stored at −80°C. Tissue sections (6 μm) were cut in an HM-560 cryostat (Microm, Heidelberg, Germany). F4/80 (Dr L Boon, Bioceros, Utrecht, The Netherlands) and CD206 (Serotec, Oxford, UK) primary antibodies were incubated for 1 h at room temperature. Binding of antibodies was detected using the immunoalkaline phosphatase antialkaline phosphatase method (DAKO, Glostrup, Denmark). Naphthol-AS-MX-phosphate (0.30 mg ml^{-1}; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and new fuchsine (160 mg ml^{-1} in 2 M HCl; Chroma-Gesellschaft, Köngen, Germany) were used as substrate. The specificity of the antibodies was checked using a protein concentration-matched non-relevant monoclonal antibody and PBS.

Flow cytometry

Spleens were aseptically removed and mechanically dispersed in cold PBS. Cell suspensions were filtered through a 100-μm nylon cell strainer (BD Biosciences, Bedford, MA, USA), depleted of erythrocytes by osmotic shock, washed twice in RPMI and adjusted to a concentration of 1×10^6 cells ml^{-1} in FACS buffer.

Splenocytes were stained with the following monoclonal antibodies: Ly6C (FITC), F4/80 (FITC), MHCII (PE), CD11c (PE-Texas red), CD11b (PerCP-Cy5.5), CD31 (PE-Cy7), CD206 (Alexa 647), Ly6G (Alexa Fluor 700), Gr-1 (APC-Cy7) and a fixable live/dead marker in DAPI (Invitrogen, Breda, The Netherlands). The final analysis and graphical output were performed using FlowJo software (Tree Star Inc., Costa Mesa, CA, USA).

Macrophage culture

Single-cell suspensions of bone marrow, isolated from femurs and tibias of naïve mice, were cultured in RPMI supplemented with 10% foetal calf serum, 2.5 ml gentamicin (10 mg ml^{-1}) (Gibco, Breda, the Netherlands) and β-mercaptoethanol (Sigma-Alderich) (further referred to as culture medium).

Bone marrow cells were isolated from the femurs and tibias of naïve mice under sterile conditions (Inaba *et al*, 1992). In short, all muscle tissues are removed with gauze from the bones and placed in a 60-mm dish with 70% alcohol for 1 min, washed twice with PBS and transferred into a fresh dish with RPMI 1640. Both ends of the bones were cut with scissors in the dish, and then the marrow was flushed out using 2 ml of RPMI 1640 with a syringe and 25-gauge needle. The tissue was suspended, passed through nylon mesh to remove small pieces of bone and debris, and red cells were lysed with ammonium chloride. A concentration of 10 ng ml^{-1} M-CSF (R&D systems, Oxon, UK) was used in our bone marrow cultures according to the protocol of Wan *et al* (2007, 2009)) or 30% tumour supernatant from AC29 cell culture (at 80% confluency) was added on day 0 to a culture of 2×10^6 bone marrow-derived cells. AC29 was cultured in RPMI-1640 (Gibco, Paisley, UK) supplemented with GlutaMax, 10 mM HEPES and 5% heat-inactivated foetal bovine serum. When confluency reached 80% (typically 3–4 days), supernatant was collected, centrifuged for 10 min at 1000 **g** to remove cells/cell debris. Supernatant is added to the bone marrow culture at a concentration of 30%. This concentration was selected based on findings by Tsagozis *et al* (2008), who described that 30% tumour supernatant generated macrophages with a characteristic TAM phenotype. Zoledronic acid (ZA) (Zometa, Novartis Pharma BV) was added on day 0 to the culture conditions in different concentrations: 0.03, 0.15 or 0.3 μM. All culture experiments have been repeated five times under comparable conditions.

Mononuclear-MDSCs were isolated from the spleen of tumour-bearing mice. Four to six colour samples were sorted using a FACSAria equipped with FACSDIVA software (BD Biosciences).

After sorting, cells were cultured with M-CSF for 6 days. This experiment has been repeated twice under comparable conditions.

Zoledronic acid treatment protocol

CBA-j mice were divided into two groups: each group consisting of 10 mice. On day 0, all mice were i.p. injected with a lethal dose of 20×10^6 AC29 tumour cells. One group was treated daily with subcutaneous (s.c.) ZA injections ($100\,\mu g\,kg^{-1}$, $\sim 2.5\,\mu g$ per mice) in $100\,\mu l$ PBS and the other group was treated with $100\,\mu l$ PBS as a control starting at day 5. This dosage was proven effective and non-toxic in mice (Stathopoulos *et al*, 2008).

Of both groups, five mice were killed at day 25. The others were killed when found profoundly ill. The occurrence of tumour growth, body weight, physical well-being and survival were measured for 2 months as described previously (Hegmans *et al*, 2005). Survival experiments were repeated three times under comparable conditions.

Enzyme-linked immunosorbent assay

To measure cytokine levels, effusion fluid from the peritoneal cavity of tumour-bearing mice was obtained at day 25 after tumour injection. IL-10, IL-12, TNFα, IL-6, IL-1β, CCL-2 (MCP-1) and

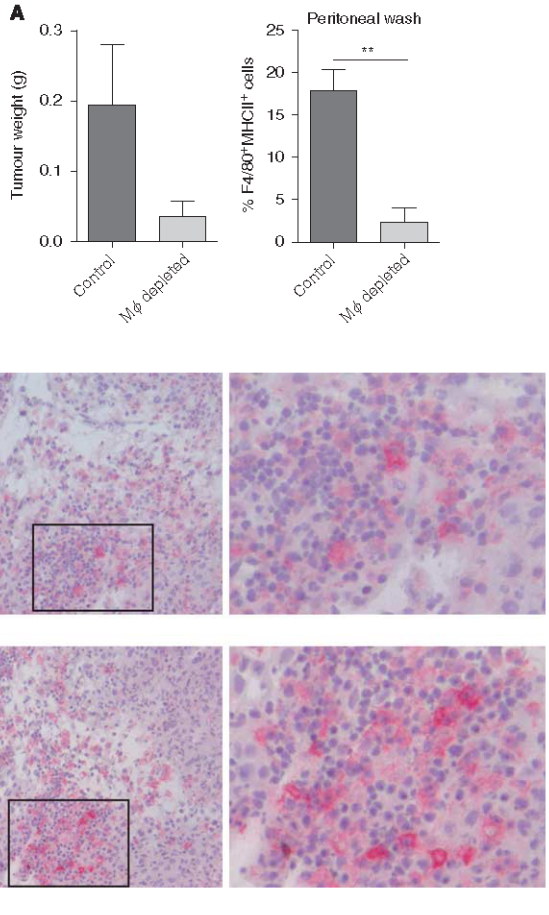


Figure 1 Depletion of macrophages inhibits tumour development. On day 1, mice were i.p. injected with a lethal dose of AC29 mesothelioma tumour cells and were treated twice with liposome encapsulated clodronate (macrophage depletion) or liposome encapsulated PBS (control) on days 5 and 10 after tumour injection ($n = 10$). Twelve days after tumour injection, mice were killed and tumour weight was measured. All visible tumour material was excised from each mouse and data are expressed as wet weight (accuracy of 0.001 g). FACS analysis was performed to verify the effectiveness of macrophage depletion using liposome encapsulated clodronate. Tumour biopsies were embedded in Tissue Tek II and snap frozen in liquid nitrogen. Tissue sections (6 μm) were analysed for the presence of macrophages. (**A**) Effect of macrophage depletion on tumour growth. Tumour growth was observed in all five mice treated with control liposomes; in contrast, only two out of five mice treated with macrophage depleting liposomes developed visible tumour growth on day 12. Significant reduction in the percentage of $F4/80^+MHCII^+$ cells was found in the peritoneal cavity of macrophage depleted mice ($P = 0.0015$). Tumour weight was found to be lower in macrophage depleted animals ($P = 0.077$). (**B**) TAMs in murine mesothelioma. Tumour biopsies of control treated mice showed infiltration of $F4/80^+$ and $CD206^+$ cells (magnification: upper, $\times 200$; lower, $\times 400$). $^{**}P < 0.001$.

VEGF levels in the effusion fluid were determined using a specific ELISA assay (IL-10, IL-12 and TNFα: R&D systems, Abingdon, UK; IL-6, IL-1β and CCL-2 (MCP-1): BD Biosciences; VEGF: Calbiochem, Darmstadt, Germany). Protocols were followed as per the manufacturer's instructions. Samples were diluted appropriately to ensure that readings were within the limits of accurate detection. Results are expressed as pg ml^{-1} of effusion fluid.

Statistical analysis

Data are expressed as mean ± s.d. Comparisons between groups were made using *t*-tests. A two-tailed *P*-value <0.05 was considered significant. Data presented as a percentage of tumour-free animals were analysed with Kaplan–Meier survival curves, using the log-rank test to determine significance.

RESULTS

Macrophages are essential in the onset and tumour development

We investigated the effect of the depletion of macrophages on tumour progression in a murine model for mesothelioma by treating mice with liposome-encapsulated clodronate. These liposomes are readily taken up by phagocytic cells, including macrophages, and induce cell-specific apoptosis after clodronate is set free into the cytoplasm of cells (Claassen, 1992; Van Rooijen and Sanders, 1994).

Mice were i.p. inoculated with a lethal dose of AC29 tumour cells and mice were treated with liposome-encapsulated clodronate or liposome-encapsulated PBS on days 5 and 10 after tumour injection. Mice were killed 12 days after tumour injection. All visible tumour material was excised from each mouse and wet weight was measured (accuracy of 0.001 g. FACS analysis was performed to verify the effectiveness of macrophage depletion (Mantovani *et al*, 2002; Sica *et al*, 2006).

Treatment with liposome-encapsulated clodronate significantly reduced the number of macrophages in the peritoneal cavity of tumour inoculated mice ($P=0.0015$). All mice ($n=5$) treated with liposome-encapsulated PBS showed profound tumour growth at day 12. Three of the five mice treated with liposome-encapsulated clodronate had no visible tumour. In the case of mice that did develop tumour, tumour growth was less profound (Figure 1A). Macrophages (M1/M2) were found scattered throughout the tumour of a control mice (Figure 1B).

These data show that macrophages have a significant role in the onset and progression of tumour in our murine mesothelioma model.

Zoledonic acid inhibits myeloid differentiation *in vitro*

To investigate the effect of ZA on myeloid differentiation *in vitro*, total bone marrow cells were cultured with M-CSF or 30% tumour supernatant in the presence or absence of ZA for 6 days using similar culture conditions as described by others (Wan *et al*, 2007, 2009; Tsagozis *et al*, 2008). FACS analysis was performed daily. ZA was added in different concentrations: 0.03, 0.15 or 0.30 μM.

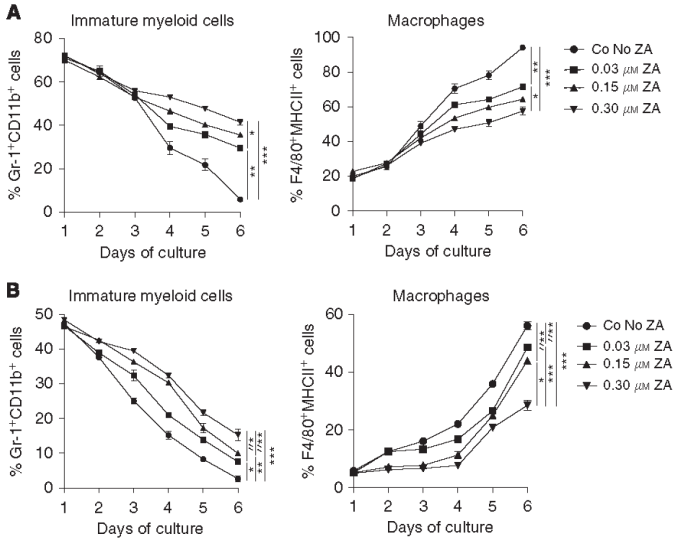


Figure 2 ZA inhibits differentiation of myeloid cells *in vitro*. The effect of ZA on myeloid differentiation was determined by cultured bone marrow-derived cells with 0.5 μg ml^{-1} M-CSF or RPMI containing 30% tumour supernatant. Cells were cultured for 6 days. ZA was added to the cultures on day 0 in different concentrations (0.03, 0.15 or 0.30 μM). FACS analysis was performed daily. ZA inhibits the downregulation of Gr-1$^+$ cells and the upregulation of F4/80$^+$ and MHCII$^+$ cells in a dose-dependent manner. Experiments were repeated and data of five individual experiments were then combined. No significant differences were observed in the number of cells between the different culture conditions. A significant difference was found in the percentage of immature myeloid cells and the percentage of macrophages after 6 days of culture in both, resulting in a higher number of immature cells and a lower number of macrophages in ZA culture conditions. (**A**) Bone marrow culture with M-CSF: immature myeloid cells, $^*P=0.004$; $^{**}P=0.0001$; $^{***}P<0.0001$; macrophages, $^*P=0.0003$; $^{**}P<0.0001$; $^{***}P=0.005$. (**B**) Bone marrow culture with 30% tumour supernatant: immature myeloid cells, $^*P=0.016$; $^{**}P=0.0014$; $^{***}P=0.0025$; $^{*\prime\prime}P=0.015$; $^{**\prime\prime}P=0.036$; macrophages, $^*P=0.002$; $^{**}P=0.0011$; $^{***}P=0.006$; $^{*\prime\prime}P=0.0004$, $^{**\prime\prime}P=0.001$.

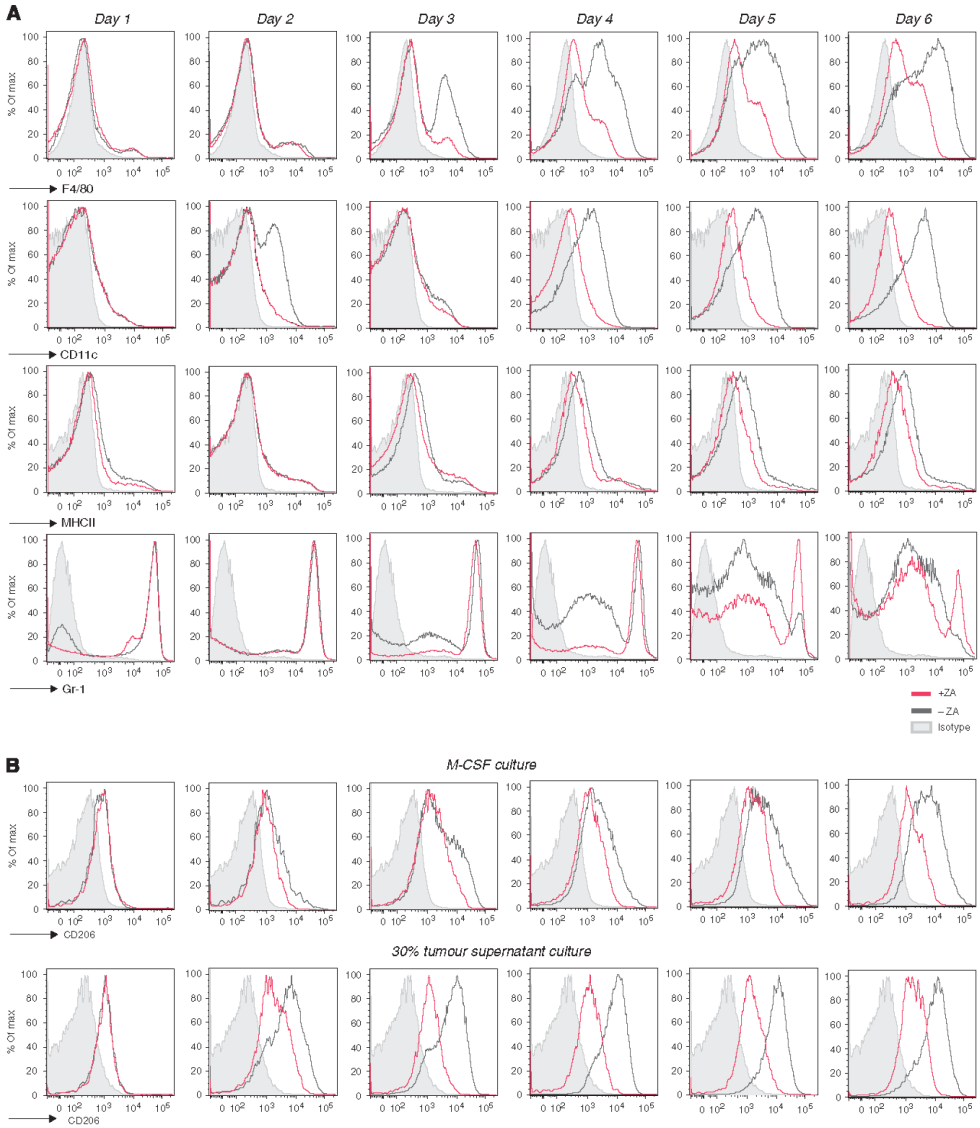


Figure 3 ZA inhibits the upregulation of extracellular markers *in vitro*. Expression profiles of M-CSF and RPMI containing 30% tumour supernatant cultured cells were measured by FACS to determine the effect of ZA addition to the culture (0.5 μg ml^{-1} M-CSF; 0.30 μM ZA was added on day 0). CD206 on macrophages was analysed to determine changes in macrophage phenotype. (**A**) M-CSF culture. F4/80, CD11c and MHCII were upregulated within 6 days. The immature myeloid marker Gr-1 was rapidly downregulated. The addition of ZA to the culture supernatant reduced the upregulation F4/80 and MHCII and CD11c, leading to a significant difference in MFI of these markers on day 6 of culture ($P = 0.003$, 0.0023, 0.0003, respectively). As a consequence, the expression of Gr-1 was still high in a majority of the cells after 6 days of culture. (**B**) CD206 expression on macrophages (M-CSF culture and 30% tumour supernatant culture). After day 5, almost all F4/80$^+$MHCII$^+$ cells in the M-CSF culture expressed CD206. The upregulation of CD206 on cells cultured in the presence of tumour supernatant was more explicit. The addition of ZA to the cultures reduced the expression of CD206 on macrophages in both conditions and a significant reduction in the MFI of CD206 on macrophages after 6 days of culture ($P < 0.0001$). Experiments were repeated several times under comparable conditions ($n = 5$). Determination of the significance of peak shifts was based on calculation of the MFI.

In both culture conditions there was a rapid decline in the number of cells expressing the immature marker Gr-1; however, the downregulation of Gr-1 was delayed when ZA was added to the culture. Simultaneously, a delay in the upregulation of macrophage markers was observed when ZA was added. The delay in the downregulation of Gr-1 and the upregulation of F4/80 and MHCII was dose dependent, leading to a reduced number of mature macrophages after 6 days of culture when ZA was added to the culture (Figure 2). The total number of cells within the different culture conditions did not significantly change over time.

To summarise, we observed an inhibition of myeloid differentiation to macrophages when ZA was added to the culture conditioned for macrophages. This inhibitory effect on differentiation was dose dependent and led to significant differences in the number of macrophages and immature cells between the different culture conditions on day 6. Furthermore, we showed that tumour-derived factors present in tumour supernatant induced the development of macrophages from bone marrow-derived cells.

Zoledronic acid shifts the balance from M2 macrophages to M1 macrophages *in vitro*

To investigate the influence of ZA on the differentiation of myeloid progenitor cells to macrophage phenotypes, bone marrow cells were cultured in the presence or absence of 0.30 μM ZA. Cells were cultured with M-CSF or 30% tumour supernatant as described previously (Wan *et al*, 2007, 2009; Tsagozis *et al*, 2008). The mannose receptor CD206 is a specific marker for M2 macrophages; therefore, the expression of this marker was analysed during culture to observe changes in macrophage phenotype (Mantovani *et al*, 2002).

Both M-CSF and 30% tumour supernatant culture conditions revealed that macrophage markers, F4/80, CD11c and MHCII, were rapidly upregulated within 6 days of culture. Simultaneously, the marker for immature myeloid cells Gr-1 was downregulated. However, the addition of ZA to the culture significantly inhibits the upregulation of F4/80, MHCII and especially CD11c, leading to a significant difference in mean fluorescent intensity (MFI) of these markers on day 6 of culture ($P = 0.003$, 0.0023 and 0.0003, respectively). The expression of Gr-1 remained high in both these culture conditions (Figure 3A).

On day 1, cells expressed CD206 at low levels; however, at day 2 of culture, a slight increase was found in both conditions. This upregulation persisted in the following days in the M-CSF culture conditions. In those conditions where ZA was added to the M-CSF culture, the upregulation of CD206 was abolished. The upregulation of CD206 took place earlier in the tumour supernatant conditions compared with the M-CSF culture. As a consequence, the blocking effect of ZA on the generation of M2 macrophages was more profound, leading to a complete inhibition in the upregulation of CD206 by ZA and a significant reduction in the MFI of CD206 on macrophages after 6 days of culture ($P < 0.0001$) (Figure 3B).

In conclusion, these 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 addition of tumour supernatant leads to polarisation of the macrophage phenotype towards M2, and that ZA can prevent this polarisation *in vitro*, leading to a significant reduction in the MFI of CD206 on macrophages cultured in the presence of ZA ($P < 0.0001$).

Zoledronic acid does not improve survival in a murine tumour model

Next, we investigated whether treatment with ZA had an effect on survival. Mice were i.p. inoculated with AC29 tumour cells and

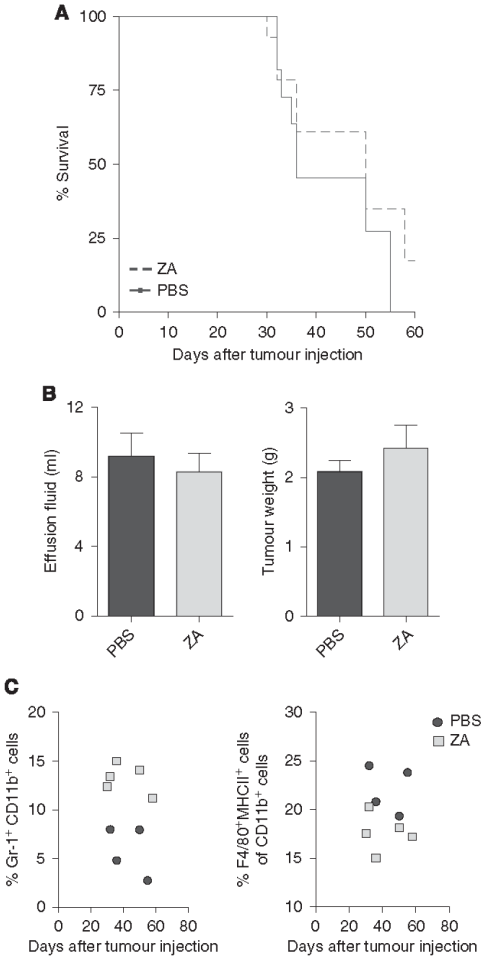


Figure 4 Treatment with ZA does not improve survival. Mice were divided into two groups ($n = 10$ mice per group). Mice were treated daily with s.c. ZA (100 μg kg^{-1}, ~2.5 μg per mice) or PBS injections starting on day 5 after tumour injection. This dosing schedule was proven effective and non-toxic (Stathopoulos *et al*, 2008). Mice were killed when found profoundly ill. No significant improvement of survival was measured. (**A**) Kaplan Meier survival curve. No significant differences in survival were observed between mice treated daily with s.c. injection of ZA compared with untreated mice ($P = 0.3675$). (**B**) Malignant effusions and tumour weights. Tumour weight and the amount of malignant effusion were measured when mice were killed. The effusion fluid was removed from the peritoneal cavity by fine-needle aspiration and all visible tumour material was collected. No significant differences in tumour weight or the amount of malignant effusion were observed ($P = 0.42$ and 0.61). (**C**) Myeloid cell types in the spleen of tumour-bearing mice. Long-term treatment effects were observed in the number of myeloid cells within the spleen of tumour-bearing mice, implicating that higher numbers of myeloid precursors and lower numbers of TAMs were detected in mice treated with ZA compared with untreated mice.

treated daily with s.c. ZA injections or PBS as a control. The dosing schedule was chosen according to the literature, in which it was proven effective and non-toxic (Stathopoulos *et al*, 2008). Mice were weighted on a daily basis and killed when found profoundly ill. The effusion fluid was collected when mice were killed.

No significant prolongation of survival was observed upon treatment with ZA ($P=0.2183$) (Figure 4A). No significant differences were found in the total body weight or in the amount of effusion fluid volume during the experiment ($P=0.4178$ and 0.6103, respectively) (Figure 4B). Long-term treatment effects showed that higher numbers of myeloid precursors and lower numbers of TAMs were detected in mice treated with ZA compared with untreated mice; however, no effects were found in tumour burden and survival (Figure 4C).

Taken together, although no significant differences on tumour progression and survival could be observed between untreated mice and mice treated with ZA, a reduction in the number of macrophages and an increase in the number of immature myeloid cells was detected.

Identification of myeloid cells *in vivo*

To establish whether ZA inhibits myeloid differentiation in tumour-bearing mice, myeloid cell types within the spleen, tumour and peritoneal cavity of tumour-bearing mice were identified. Recently, it has been shown that the heterogeneous group of MDSC consists of three major groups: polymorph nuclei $CD11b^{high}Gr\text{-}1^{high}$ MDSC (PMN-MDSC), mononuclear $CD11b^{high}Gr\text{-}1^{int}$ MDSC (MO-MDSC) and the $CD11b^{high}Gr\text{-}1^{low}$-MDSC (Greifenberg *et al*, 2009). Identification of MDSC subtypes was carried out on cells of untreated tumour-bearing mice 25 days after tumour inoculation. The population of PMN-MDSC produces high levels of reactive oxygen species leading to the downregulation of the ζ-chain of T cells, resulting in T-cell tolerance, whereas MO-MDSC can directly inhibit T-cell expansion by the production of nitric oxide-inducing T-cell apoptosis (Bronte and Zanovello, 2005; Nagaraj and Gabrilovich, 2007; Gabrilovich and Nagaraj, 2009).

A massive increase in MDSC was found in the spleens and effusion fluids from the peritoneal cavity of tumour-bearing mice on day 25 after tumour injection. MDSCs were further subdivided into three groups: PMN-MDSC, MO-MDSC and $Gr\text{-}1^{low}$-MDSC. All subgroups of MDSC stained intermediate positive for the marker CD206 (Figure 5A). Expression of the markers F4/80 and MHCII was found on MO-MDSC and $Gr\text{-}1^{low}$-MDSC, but not on PMN-MDSC. A small subpopulation of the MO-MDSC was strongly positive for F4/80, MHCII and CD206 (Figure 5A).

In the peritoneal cavity and in the spleen, M1 and M2 macrophages were identified based on the expression of F4/80, MHCII and CD206, and forward–sideward scatter patron. M2 macrophages express higher levels of CD206 and F4/80 and lower levels of MHCII compared with the M1 macrophage population (Figure 5B).

New insights have revealed that MO-MDSC are pluripotent and under certain conditions are able to differentiate into TAMs (Rossner *et al*, 2005; Umemura *et al*, 2008; Greifenberg *et al*, 2009; Dolcetti *et al*, 2010). This is also reflected *in vivo*, as the population of MO-MDSC already showed partial expression of F4/80 and MHCII in an early stage compared with PMN-MDSC. To confirm that MO-MDSC could further differentiate into macrophages, we sorted this population from the spleen of tumour-bearing mice and cultured the cells for 5 days in the presence of M-CSF. Mononuclear-MDSC upregulated the expression of F4/80 and MHCII rapidly, in contrast to the PMN-MDSC that were not able to upregulate F4/80 under the influence of M-CSF (Figure 5C).

To summarise, we were able to identify three populations of MDSC as described in the literature in our tumour model. The MO-MDSCs were found to be able to differentiate into macrophages. Two populations of macrophages based on CD206 expression were found both in the spleen and in the peritoneal cavity of tumour-bearing mice.

Zoledronic acid reduces macrophages but increases the number of immature myeloid cells during tumour development

As we observed a difference in the number of macrophages and immature myeloid cells during ZA treatment, we next wanted to know whether ZA treatment also affects macrophage phenotype *in vivo*. To investigate whether ZA influences the development of macrophages during tumour progression *in vivo*, mice were i.p. inoculated with AC29 tumour cells and treated daily with s.c. ZA injections ($100\,\mu g\,kg^{-1}$, $\sim 2.5\,\mu g$ per mice) or PBS as a control. Mice were killed 25 days after tumour injection.

An increase in $CD11b^{+}Gr\text{-}1^{+}$ cells was found in the spleen and effusion fluid of tumour-bearing mice of both groups. A significant increase was found in MO-MDSC both in the spleen and in the effusion fluid from the peritoneal cavity (spleen: $P=0.0312$; effusion fluid: $P=0.034$), whereas no significant differences were found in the proportion of PMN-MDSC and $Gr\text{-}1^{low}$-MDSC (spleen: $P=0.77$ and 0.75; effusion fluid: $P=0.74$ and 0.72). The proportion of macrophages in the spleen of ZA-treated mice was significantly lower compared with PBS-treated mice ($P=0.0091$). There was a trend towards a shift in macrophage phenotype from M2 to M1; however, the reduction in M2 macrophages was not significant ($P=0.18$). However, we found that the expression levels of CD206, expressed as the MFI, were significantly lower on M2 macrophages of ZA-treated mice compared with M2 macrophages in the spleen of PBS-treated mice ($P=0.0095$) (Figure 6A). No significant decrease was found in the percentage of macrophages in the effusion fluid of ZA-treated mice ($P=0.75$). However, a significant increase in M1 macrophages was found, while M2 macrophages were reduced ($P=0.035$ and 0.33). A shift from M2 macrophage to M1 macrophage phenotype was found based on the MFI expression of CD206, confirming the findings in the spleen ($P=0.14$). As a result, a difference in ratio of M1:M2 was found ($P=0.011$) (Figure 6B).

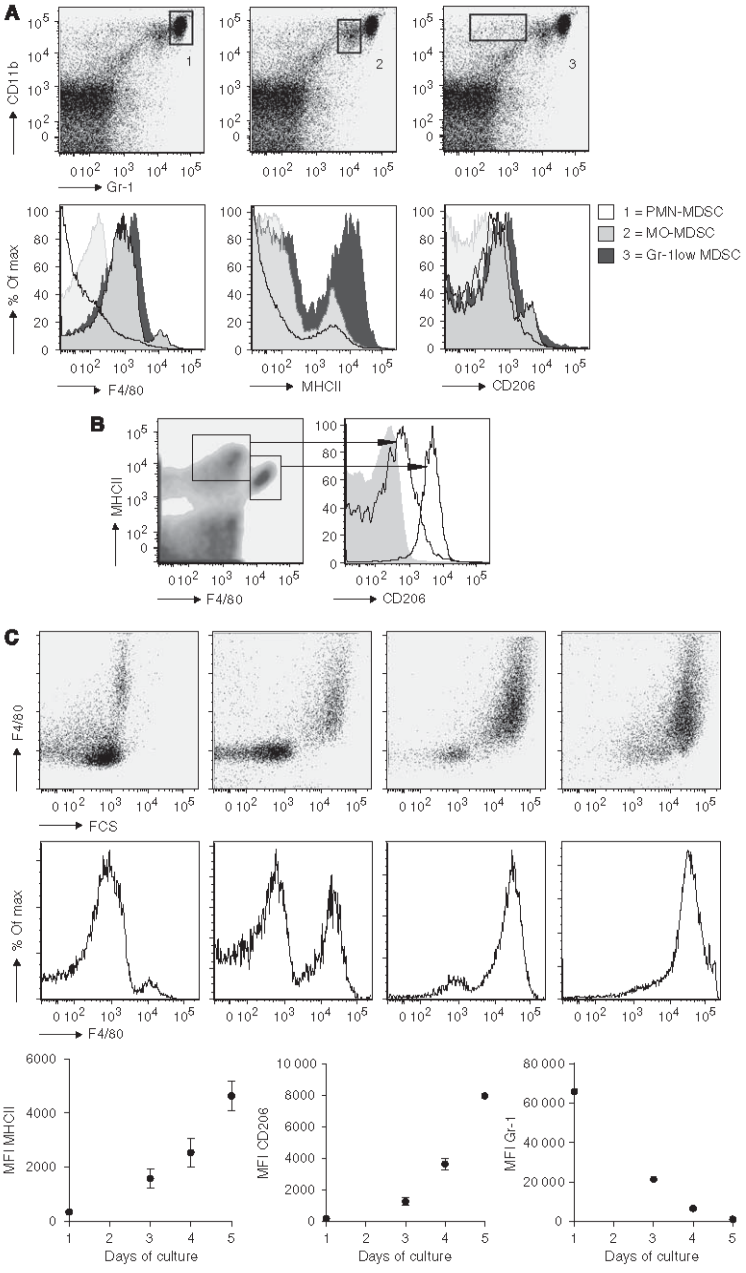
Enzyme-linked immunosorbent assay on the effusion fluid was performed to determine whether the reduction on CD206 expression on macrophages in ZA-treated mice was accompanied by changes in cytokine, chemokine and growth factor production. A significant increase was found in the levels of IL-6, IL-12 and IL-1β in the effusion fluid of ZA-treated mice ($P=0.049$, 0.042 and 0.005, respectively). In addition, we observed a significant reduction in VEGF and CCL-2 (MCP-1) in the effusion fluid of ZA-treated animals ($P=0.05$ and 0.039). Levels of IL-10 and TNFα were not detectable in the effusion fluid.

In conclusion, we have shown that treatment with ZA reduces the number of macrophages, but at the same time, we observed higher levels of immature myeloid cell types. When we further defined the population of immature myeloid cells, significantly more MO-MDSC were found. In addition, we found that the expression of CD206 on macrophages was lower in ZA-treated animals. This reduced expression of the M2 macrophages marker was accompanied with a significant reduction in VEGF and CCL-2 (MCP-1) levels and a significant increase in the levels of IL-6, IL-12 and IL-1β (Figure 6C).

DISCUSSION

Many effects of bisphosphonates have been reported in literature. Treatment with ZA prolongs the 1-year survival rate in breast cancer, prostate cancer and bladder cancer (Gnant, 2009; Rajpar *et al*, 2010; Zaghloul *et al*, 2010). However, exact mechanisms by which ZA prevents disease progression is still a topic of investigation. The role of bone formation and mineralisation in

relation to adhesion and tumour outgrowth in these structures has gained interest after treatment with bisphosphonates appear to have protective effects on the prevention of bone metastasis (Boissier *et al*, 1997, 2000; Magnetto *et al*, 1999; Montague *et al*, 2004). Besides the reduction in bone metastasis, studies have shown that ZA also prevents metastasis to secondary organs



(Hiraga *et al*, 2004). Therefore, other mechanisms, during tumour progression, may be influenced by ZA treatment. Recently, it has been suggested that ZA may work as immune modulator and may therefore be applicable as and antitumour agent (Clezardin, 2005; Tsagozis *et al*, 2008; Santini *et al*, 2010).

ZA is an aminobisphosphonate that targets the mevalonate pathway in myeloid cells (Wolf *et al*, 2006). Recently, several clinical trails have been performed to investigate the role of ZA in tumour progression (Saad *et al*, 2002, 2004; Gainford *et al*, 2005). It has been suggested that ZA has direct antineoplastic activity on tumour cells (Santini *et al*, 2003; Clezardin, 2005; Ohtsuka *et al*, 2005; Caraglia *et al*, 2007). However, other studies have shown that clinically observed effects of ZA treatment may also be explained by indirect mechanisms involving immune modulation.

Studies have shown that ZA eventually leads to stimulation and proliferation of gamma/delta T cells (Kunzmann *et al*, 2000; Miyagawa *et al*, 2001; Sato *et al*, 2005). Besides, direct effects of ZA on tumour cell have been reported in breast cancer, prostate and bladder cancer (Gnant *et al*, 2009; Rajpar *et al*, 2010; Zaghloul *et al*, 2010). However, more recently the effect of ZA on macrophages has gained interest. Macrophages are known to have a dominant role within the tumour microenvironment. Several studies have reported the importance of TAMs on tumour progression (Pollard, 2004; Sica *et al*, 2006, 2008; Sangaletti *et al*, 2008; Allavena *et al*, 2008b; Coffelt *et al*, 2009). We and others have shown that depleting TAMs with liposome-encapsulated clodronate inhibits tumour growth and prolongs survival (Zeisberger *et al*, 2006; Miselis *et al*, 2008).

As our *in vitro* data revealed that ZA inhibits the differentiation towards macrophages, our aim was to determine whether this inhibition in differentiation also led to a reduction in TAMs *in vivo*.

The percentage of macrophages in the spleen was significantly reduced in ZA-treated animals compared with the control group. In the effusion fluid, no significant difference was found in the total number of macrophages; however, a shift towards a more M1 macrophage phenotype was observed. Recent studies have shown that TAMs can be derived from certain subpopulations within the heterogeneous MDSC population (Rossner *et al*, 2005; Umemura *et al*, 2008; Greifenberg *et al*, 2009; Dolcetti *et al*, 2010). From these studies, it has been established that TAMs were mainly derived from the MO-MDSC population. A significant increase in MO-MDSC was found both in the spleen and in the effusion fluid of mice treated with ZA. As we found that ZA inhibits myeloid differentiation *in vitro*, it can be assumed that the significant increase in the MO-MDSC population found in ZA-treated mice compared with untreated mice are caused by similar inhibitory effects on myeloid differentiation. Inhibition of myeloid differentiation by ZA has also been reported by others (Wolf *et al*, 2006; Melani *et al*, 2007; Chen *et al*, 2009).

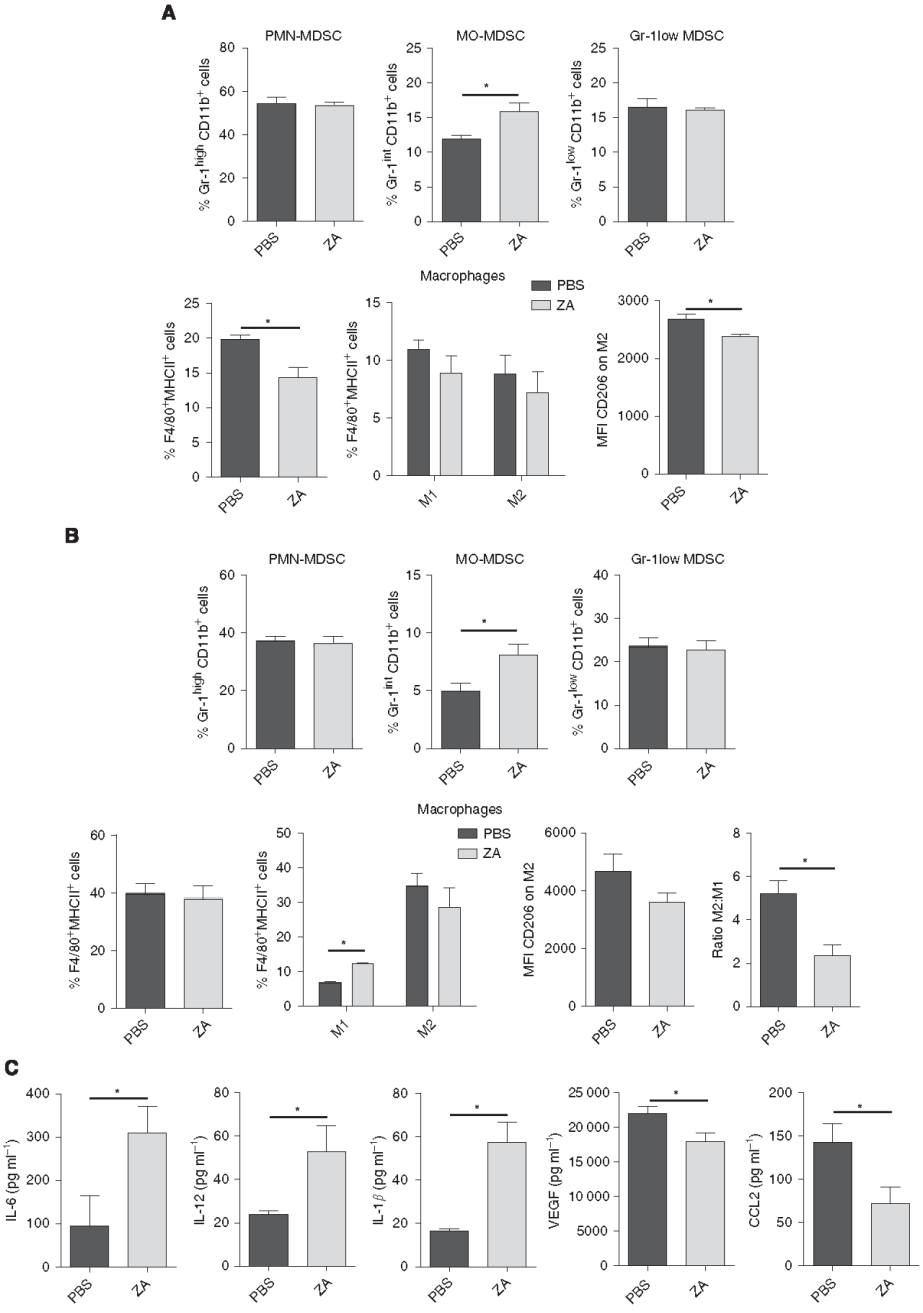
Tsagozis *et al* (2008) reported that ZA shifts TAMs from an M2-like phenotype to an M1-like phenotype, resulting in a reduction in TAM-associated cytokine production *in vitro*. We were able to confirm these data *in vitro*, but were also able to show that these changes also occur *in vivo*. A reduction in the M2-associated receptor (CD206) was found on macrophages in tumour-bearing mice treated with ZA. Changes in M2-associated cytokines, chemokines and growth factors were observed in the effusion fluid after ZA treatment. We showed that ZA lowered the levels of VEGF in the effusion fluid of tumour-bearing mice. The reduction in VEGF and MMP-9 expressions by macrophages under the influence of ZA has been described to prevent tumour neovascularisation (Santini *et al*, 2002; Giraudo *et al*, 2004; Vincenzi *et al*, 2005; Melani *et al*, 2007; Tsagozis *et al*, 2008). The production of MMPs and VEGF is known to be essential during tumour progression and especially facilitating metastases (Allavena *et al*, 2008b; Coffelt *et al*, 2009).

Even though it was found that ZA inhibits myeloid differentiation and shifts the balance from M2 macrophage phenotype towards an M1 macrophage phenotype, this did not improve survival in our model. There are several explanations for this finding. One of the most striking findings was the increased levels of immature myeloid cells (MDSC) due to treatment with ZA. Recently, studies have reported the role of MDSC in tumour progression. MDSC are most known for their immune suppressive function and induction of tumour-specific T-cell tolerance facilitating tumour immune escape (Bronte and Zanovello, 2005; Nagaraj and Gabrilovich, 2007; Gabrilovich and Nagaraj, 2009). Therefore, the inhibition of myeloid development may lead to reduction in the number of TAMs, resulting in a higher number of MDSC, which also promote tumour progression.

A second reason as to why ZA has no effect on survival may be assigned to the shift in macrophage phenotype. As most studies showed that TAMs are polarised to an M2 phenotype that contribute to carcinogenesis, shifting the phenotype of macrophages to M1 seems promising. M1 macrophages produce less tumour growth-promoting soluble factors and can potentially kill tumour cells. However, the question rises whether shifting the phenotype in full-blown tumours is possible and whether repolarisation is as promising as believed previously (Mills *et al*, 1992; Mantovani *et al*, 2002; Weigert and Brune, 2008). Negative aspects of iNOS+ M1 macrophages were also observed. Actually, these cells can kill tumour cells, but as a consequence pro-carcinogenic substances are released from dying cells, leading to enhanced angiogenesis and enhanced polarisation of M1 macrophages to an M2 phenotype (Weigert and Brune, 2008). Coscia *et al.* reported similar observation. In their study, repolarisation of the macrophage phenotype was most promising during early tumour development, resulting in prolonged tumour-free survival and overall survival. In addition, they notify that shifting the phenotype in a host with a large tumour burden can be hazardous and no effects of ZA administration were observed when *in situ* carcinomas had progressed. From their data, it appears that tumour development is inhibited when the induction of M2 macrophages can be post-poned. We observed similar findings: shifting the phenotype from M2 to M1 at later stages of tumourigenesis seems less effective. Therefore, it can be hypothesised that preventing the formation of M2 macrophages is a critical key in preventing tumour outgrowth during early stages of disease development.

A third explanation for the limited effect on survival could be that ZA has effects on processes during tumour development that

Figure 5 Identification of myeloid cells in tumour-bearing mice. To identify the effect of tumour growth on the recruitment of myeloid cells during tumour progression, mice were inoculated with tumour cells and killed on day 25 ($n = 12$). (**A**) Identification of myeloid cell types in splenocytes of tumour-bearing mice. Immature myeloid cells could be divided into three groups, as described in the literature (Greifenberg *et al*, 2009). The Gr-1^{low}-MDSC showed intermediate expression of F4/80, MHCII and CD206. F4/80 expression was found in MO-MDSC, but not in PMN-MDSC. A small number of MO-MDSC expressed high levels of F4/80 MHCII and CD206. MHCII and CD206 expressions were low in PMN-MDSC. (**B**) Identification of type I and type II macrophages in splenocytes of tumour-bearing mice. Two populations of macrophages could be identified; a high expression of CD206 was found in the membrane of the population with a high expression of F4/80 but lower expression of MHCII. (**C**) M-CSF culture of MO-MDSC-sorted cell fraction. MO-MDSC were sorted from splenocytes of tumour-bearing mice and cultured with 0.5 μg ml^{-1} M-CSF. The expression of F4/80, MHCII, CD206 and Gr-1 were measured on consecutive days.


A
PMN-MDSC
MO-MDSC
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PBS
ZA
Macrophages
% F4/80+MHCII+ cells
MFI CD206 on M2
M1
M2
B
Ratio M2:M1
C
IL-6 (pg ml−1)
IL-12 (pg ml−1)
IL-1β (pg ml−1)
VEGF (pg ml−1)
CCL2 (pg ml−1)

Figure 6 ZA changes M1:M2 ratio and increases MO-MDSC in tumour-bearing mice. Mice were i.p. inoculated with AC29 tumour cells and treated daily with s.c. injection of ZA. (100 $\mu g\,kg^{-1}$) or PBS as a control ($n = 6$ each group). Mice were killed 25 days after tumour injection. The number of MDSC was analysed according to the subdivision as described in Figure 5. Macrophages were subdivided into M1 and M2 macrophages based on the co-expression of CD206, F4/80 and MHCII on the membrane. (**A**) Myeloid cell types in spleen of tumour-bearing mice. MO-MDSC were significantly increased in the spleen of ZA-treated animals (*$P = 0.0312$). No difference was found in the percentage of PMN-MDSC and Gr-1^{low}-MDSC (*$P = 0.77$ and 0.75). The percentage of total macrophages in the spleen of ZA-treated mice was significantly lower compared with untreated mice (*$P = 0.0091$). In the spleen of tumour-bearing mice, although not significant there was a trend towards a reduction in both M1 and M2 macrophages in ZA-treated mice. In addition, ZA treatment significantly lowers the MFI of CD206 on M2 macrophages (*$P = 0.0095$). (**B**) Myeloid cell types in effusion fluid of tumour-bearing mice. MO-MDSCs were significantly increased in the effusion fluid of ZA-treated animals (*$P = 0.034$). No difference was found in the percentage of PMN-MDSC and Gr-1^{low}-MDSC or macrophages (*$P = 0.72$ and 0.74). A significant increase in M1 macrophages was found (*$P = 0.035$), and also an increase was found in the number of M2 macrophages (*$P = 0.33$). ZA shifts the balance, leading to a significant difference in the ratio of M1:M2 macrophages (*$P = 0.011$); a trend towards a lower MFI of CD206 was observed (*$P = 0.114$). (**C**) Cytokines in effusion fluid of tumour-bearing mice. ELISA was performed on effusion fluid of tumour-bearing mice treated with ZA or PBS as a control. A significant increase in IL-6, IL-12 and IL-1β was found in ZA-treated mice (*$P = 0.049$, 0.042 and 0.005, respectively). A significant reduction in VEGF and CCL-2 (MCP-1) expressions was found in ZA-treated mice (*$P = 0.05$ and 0.039).

do not have a dominant role in our tumour models. Most investigations are carried out in tumours with high metastasis rates in combination with other therapeutic approaches (like adjuvant endocrine therapy) (Saad *et al*, 2002; Hiraga *et al*, 2004; Gnant, 2009). In these tumours, metastasis may be the cause of death, in contrast to our model. As TAMs do have an essential role in metastasis, ZA may be beneficial in these types of tumours (Allavena *et al*, 2008a). However, in both human studies and animal models, survival is only marginally influenced by ZA, and also in tumours with high metastases rates (Hiraga *et al*, 2004; Stathopoulos *et al*, 2008; Pandya *et al*, 2010; Ottewell *et al*, 2010).

To study the effects of ZA on macrophages in the primary tumour, dose levels are higher than clinically advised. Doses selected for clinical trials were based on changes in bone resorption markers (including serum COOH-terminal telopeptide and urinary *N*-telopeptide/creatinine ratio) and preliminary evidence of efficacy on skeletal-related events; however, no studies were performed comparing dose–effect relations on the tumour microenvironment. Although there are some concerns on side effects caused by ZA at higher levels (Ibrahim *et al*, 2003), additional studies are required to determine the safety and efficacy of increased dosages for this new application.

In conclusion, our data show that daily administration of ZA in tumour-bearing mice inhibits myeloid differentiation and shifts the balance from M2 macrophage phenotype to M1 macrophage phenotype. However, as a consequence the number of immature myeloid cells remains high in the spleen and in the effusion fluid, especially in the MO-MDSC population. This MO-MDSC population was found to have an immunosuppressive effect to which we ascribe the fact that survival was not improved with ZA in our study.

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Chapter 6

Low-Dose Cyclophosphamide Synergizes with Dendritic Cell-Based Immunotherapy in Antitumor Activity

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Clinical immunotherapy trials like dendritic cell-based vaccinations are hampered by the tumor's offensive repertoire that suppresses the incoming effector cells. Regulatory T cells are instrumental in suppressing the function of cytotoxic T cells. We studied the effect of low-dose cyclophosphamide on the suppressive function of regulatory T cells and investigated if the success rate of dendritic cell immunotherapy could be improved. For this, mesothelioma tumor-bearing mice were treated with dendritic cell-based immunotherapy alone or in combination with low-dose of cyclophosphamide. Proportions of regulatory T cells and the cytotoxic T cell functions at different stages of disease were analyzed. We found that low-dose cyclophosphamide induced beneficial immunomodulatory effects by preventing the induction of Tregs, and as a consequence, cytotoxic T cell function was no longer affected. Addition of cyclophosphamide improved immunotherapy leading to an increased median and overall survival. Future studies are needed to address the usefulness of this combination treatment for mesothelioma patients.

1. Introduction

Malignant mesothelioma (MM) is a cancer arising from mesothelial cells that lines the body's serous cavities (pleural, pericardial, and peritoneal) and the internal organs and is characterized by poor prognosis [1]. Chemotherapy or surgical treatments result in only limited improvements in response and survival. Novel therapeutic strategies are therefore needed. Immunotherapy is a promising but also challenging approach in the treatment of cancer.

Dendritic cells (DCs) are highly mobile antigen-presenting cells, capable of instructing and controlling the activation of NK cells, NKT cells, and B and T lymphocytes [2–4]. Previously we showed that DC-based immunotherapy in a murine MM model leads to protective immunity as well as regression of established tumors [5]. Currently we are investigating DC-based immunotherapy in MM patients. Although DC-vaccines are well tolerated by patients, further optimization is necessary to exploit the full potential of this therapeutic strategy [6].

It is becoming evident that immune suppression plays a crucial role in tumor progressing. Tumors secrete several mediators to recruit and/or activate suppressive cells. Regulatory T cells (Tregs) are prominent cells in this suppressive environment. These cells are instrumental in allowing a growing cancer to evade immunological attack by impairing T cell function [7]. Elevated levels of Tregs have been reported in many tumors and their presence predicts for poor survival. We have demonstrated previously the presence of Tregs within the tumors of MM patients [8]. It has also been described that Tregs are increased in the peripheral blood [9] and pleural effusions [10] of these patients.

Recent clinical studies have shown that low-dose cyclophosphamide (CTX) induces beneficial immunomodulatory effects in the context of active or adoptive immunotherapy [11–21]. CTX is widely used to treat various types of malignancies and some autoimmune disorders. It displays either immunosuppressive or immunopotentiating effects, depending on the dosage and the timing of drug administration [22]. Although the mechanisms underlying

these modulations are not fully understood, low-dose CTX might prevent the development and functionality of the Tregs [23–27].

Van der Most et al. reported that CTX enhances the effectiveness of gemcitabine treatment in murine mesothelioma by reducing the amount of Tregs [28, 29]. Thereby they underline the immunogenic role of Tregs in the suppression of activated target cells. On the other hand, Jackaman et al. recently stated that Tregs are no potent regulators of antimesothelioma immunity in their murine model and that targeting of these cells will not improve results [30]. Taken together, the role of Tregs in mesothelioma is controversial and whether these cells will influence the outcome of immunotherapy is still unclear.

Here we investigated the effect of CTX on immunosuppression and the combination of CTX and DC-based immunotherapy was studied in a murine MM model. We found that CTX reduced the levels of Tregs and this induced beneficial immunomodulatory effects in the context of DC-based immunotherapy. These results anticipate that antitumor immune responses elicited by DC-based immunotherapy in humans might be improved by simultaneously depleting Tregs using low-dose CTX.

2. Methods

2.1. Animals and Cell Lines. Female 6–10 week old BALB/c (H-2d) mice (Harlan, Zeist, The Netherlands) were housed under pathogen-free conditions at the animal care facility of the Erasmus MC, Rotterdam. Experiments were approved by the local Ethical Committee for Animal Welfare and complied to the Guidelines for the Welfare of Animals in Experimental Neoplasia by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) and by the Code of Practice of the Dutch Veterinarian Inspection. The AB1 cell line, a mouse mesothelioma cell line, was kindly provided by Professor Bruce W.S. Robinson of the Queen Elizabeth II Medical Centre, Nedlands, Australia. The cell line was derived from tumors induced by Wittenoom Gorge crocidolite asbestos injected intraperitoneally into a BALB/c mouse [31]. Cells were cultured in RPMI 1640 medium containing 25 mM HEPES, Glutamax, 50 μg/ml gentamicin, and 5% (v/v) fetal bovine serum (FBS) (all obtained from GIBCO/Invitrogen, Breda, The Netherlands) in a humidified atmosphere at 37°C and 5% CO_2, in air. AB1 cells were passaged once or twice a week to a new flask by treatment with 0.05% trypsin, 0.53 mM EDTA in phosphate buffered saline (PBS, all GIBCO/Invitrogen). The cell line was regularly tested and remained negative for mycoplasma contamination.

2.2. Reagents. Cyclophosphamide ([CTX], the generic name for Endoxan) was purchased from Baxter B.V., Utrecht, The Netherlands. A stock solution was prepared by dissolving 1 gram into 50 ml of PBS. It was further diluted in drinking water at a concentration of 0.13 mg/ml.

Every 4 days bottles were carefully examined (volume was determined) and replaced with fresh bottles containing 100 ml of drinking water or CTX-containing water. No changes in the drinking pattern of mice were observed between tumor-bearing mice with normal water or with CTX-containing drinking water (+/−3 ml per day per mouse). This equals approximately 20 mg CTX/kg body weight/day and is considered as a low-dose. Dehydration, being one of the points for signs of illness, was routinely checked. No signs of dehydration were observed during experiments except for a few cases with ill health or overt tumor growth.

2.3. Source of Tumor Antigen Derived from AB1 Tumor. AB1 cell line-derived tumor lysate was prepared from 50×10^6 cells suspended per ml PBS. The cell suspension was frozen in liquid nitrogen and disrupted by four cycles of freeze-thawing followed by sonication for 4×10 seconds with an amplitude of 10 microns, using a Soniprep 150 ultrasonic disintegrator equipped with a microtip (Sanyo Gallenkamp BV, Breda, The Netherlands) on ice. Cell lysate was aliquoted and stored at −80°C.

2.4. Culture Conditions of Bone Marrow-Derived DC Used for Vaccination. DCs were generated with only minor adaptations from a previously described protocol by Lutz [32]. After flushing femurs and tibias and red blood cell lysis, resulting bone marrow cells (2×10^6) were seeded in 100-mm Petri dishes (day 0) and cultured in 10 ml DC Culture Medium [DC-CM]: RPMI 1640 containing glutamax-I (GIBCO/Invitrogen) supplemented with 5% (v/v) FBS, 50 μM β-mercaptoethanol (Sigma-Aldrich), 50 μg/ml gentamicin (Invitrogen), and 20 ng/ml recombinant murine granulocyte macrophage-colony-stimulating factor [GM-CSF, kindly provided by Professor K. Thielemans, Free University Brussels, Belgium]. Cells were cultured at 37°C in a humidified atmosphere at 5% CO_2, in air. At day 3, 10 ml of fresh DC-CM was added. On day 6, 10 ml of each plate was replaced with 10 ml of fresh DC-CM. After 8 days of culture, AB1 cell lysate was added to the DC cultures, to the equivalent of three AB1 cell-equivalents per DC. After 8 hours, 100 μg/ml LPS (E. coli 026:B6, Sigma-Aldrich) was added to the culture to allow complete maturation while incubated overnight. The next day, DCs were harvested by gentle pipetting and purified by Lympholyte-Mammal (Cedarlane, Hornby, ON, Canada) density gradient centrifugation, the interphase washed three times in PBS and resuspended at a concentration of 1×10^6 viable cells in 500 μl PBS. The quality of the DC preparation was determined by cell-counting, morphologic analysis and cell surface marker expression by flow cytometry, as previously described [5]. DCs (1×10^6) were delivered into the peritoneal cavity of BALB/c mice; control mice received 500 μl PBS.

2.5. Treatment with Tumor Lysate-Pulsed DCs and Cyclophosphamide on Outcome. Initial experiments were performed to determine the optimal concentration of CTX necessary to reduce Tregs in vivo. Continuous administration via the drinking water of low concentrations ("metronomic") CTX had our preference because a single administration

of a higher dose or multiple administrations i.p. engenders worse life expectancy as a consequence of peritoneal damage caused by the needle. Approximately 20 mg CTX/kg body weight/day in the drinking water of mice was the optimal concentration for further studies in mice, concentrations below 100 mg/kg are considered as a low dose. No effect on feeding, body weight, and physical well-being or other side effects were observed at this concentration.

On day 0, BALB/c mice (4 groups each consisting of 6 mice) were inoculated intraperitoneally with 0.5×10^6 AB1 tumor cells in 500 μl PBS. On day 12, 12 mice (2 groups) were vaccinated with 1×10^6 LPS-matured DCs pulsed with AB1 tumor cell line lysate in 500 μl PBS. From day 3 till day 10 and/or day 14 till day 21 mice were given drinking water supplemented with 0.13 mg/ml CTX. Mice were examined daily for evidence of ill health or overt tumor growth. Mice were killed if profoundly ill, according to UKCCCR regulations, and were scored as a death in survival analysis. All mice underwent extensive autopsy.

2.6. Immunohistology on Tumor Biopsies. Tumor biopsies were embedded in Tissue-Tek II optimum cutting temperature medium (Miles, Naperville, IL, USA), snap-frozen, and stored at −80°C. Tissue sections (6 μm) were cut on an HM-560 cryostat (Microm, Heidelberg, Germany) and immunostaining was carried out using a rat antimouse Foxp3 antibody (eBioscience [#14-5773], San Diego, CA, USA). Binding of antibody was detected using alkaline phosphatase- (AP-) conjugated goat antirat (Sigma-Aldrich Chemie B.V.). Naphtol-AS-MX-phosphate (0.30 mg·mL^{-1}; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) and new fuchsine (160 mg·mL^{-1} in 2 M HCl; Chroma-Gesellschaft, Köngen, Germany) were used as substrate. The specificity was checked using a protein concentration-matched nonrelevant rat antibody and PBS. Double staining of Foxp3 and CD8 was performed using the AP-conjugated goat antirat followed by rabbit antiphycoerythrin (AbD Serotec, Düsseldorf, Germany) followed by incubation with horseradish peroxidase- (HRP-) conjugated swine antirabbit. Naphtol-AS-MX-phosphate and 1 mM Fast Blue substrate were used as substrate for AP, and NovaRed was used as substrate for HRP, according to the manufacturer's instructions (Vector, Burlingame, CA, USA). Foxp3 and phycoerythrin-labeled antimouse CD8 (BD Biosciences, San Jose, CA, USA) were both used at a dilution of 1 : 10. Signals were captured on a Leica microscope (Leica Microsystems B.V., Rijswijk, The Netherlands).

2.7. Preparation of Lymphocytes from Lymphoid Organs or Blood. Lymphocytes were collected from the spleens, lymph nodes, and blood of mice from each group. Briefly, spleens and lymph nodes were aseptically removed and mechanically dispersed in cold PBS. Cells suspensions were filtered through a 100 μm nylon mesh cell strainer (BD Biosciences, Bedford, MA, USA). Resulting suspensions and blood were depleted of erythrocytes by density gradient centrifugation, washed twice with PBS +2% BSA, and resuspended at a concentration of 1×10^6 cells/ml for flow cytometric analysis.

2.8. Flow Cytometry. The following antimouse antibodies were used: fluorescein isothiocyanate- (FITC-) conjugated anti-CD4 (1 : 800), anti-CD19 (1 : 200), phycoerythrin- (PE-)-conjugated anti-CD3 (1 : 25), anti-CD25 (1 : 200) (all from BD Biosciences), allophycocyanin- (APC)conjugated anti-Foxp3 (1 : 25) (eBioscience), and appropriate isotype-matched controls.

For cell surface marker staining, cells were washed with FACS-wash (0.05% NaN_3, 2% BSA in PBS) and Fcγ II/III receptor blocking was performed using antimouse 2.4G2 antibody (1 : 100; kindly provided by L. Boon, Bioceros, Utrecht, The Netherlands) for 15 minutes on ice. After the blocking procedure, properly diluted antibodies for cell surface staining were added into each sample and placed on ice for 30 minutes protected from light. After two additional washes with FACS-wash, Fix/Perm buffer (eBiosience) was added and cells were incubated for 1 hour at 4°C in the dark. Cells were washed twice with Perm buffer. Intracellular staining was performed for Foxp3 according to the manufacturer's instructions (eBioscience). After blocking for 15 minutes with blocking antibody 2.4G2, properly diluted APC-conjugated Foxp3 antibody in Perm buffer was added and incubated for 30 minutes on ice protected from light. After washing the cells twice with Perm buffer, cells were washed with FACS-wash and measured. Data acquisition was performed by flow cytometry (LSR II; BD Biosciences) and data analysis was performed with FlowJo software (Tree Star, Inc.).

2.9. Coculture of Tregs and Activated Splenocytes. Splenocytes obtained from tumor-bearing mice were stained for CD3, CD4, CD8, and CD25. Four colour sample sorting was done on a FACS ARIA (BD Biosciences). Tregs were defined as $CD3^+CD4^+CD25^{+bright}$ cells and sorted with a purity >90% as determined by intracellular staining on Foxp3 expression. Splenocytes of DC-treated mice were cocultured with sorted Treg cells at different ratios. Splenocytes were restimulated for 4 hours using anti-CD3 and stained with mAbs directed against CD3, CD8, intracellular Granzyme B, and IFN-γ.

2.10. Statistical Analysis. Data are expressed as mean ± SD. Comparisons between groups were made using the Mann-Whitney U-test for independent samples. A two-tailed P-value < .05 was considered significant. Data presented as a percentage of tumor-free animals were analyzed with Kaplan-Meier survival curves, using the log-rank test to determine statistical significance.

3. Results

3.1. Regulatory T Cells in the Mouse Mesothelioma Model. The mesothelioma mouse model described in our previous study was used to examine the presence of $CD4^+CD25^+Foxp3^+$ T cells in tumor progression [8]. In this protocol, BALB/c mice were intraperitoneally (i.p.) injected with a lethal dose of 0.5×10^6 AB1 tumor cells or 500 μl PBS as control. First signs of terminal illness (typically formation of ascites, ruffled hair, or marked loss of

condition) appeared between 20 days and 30 days in tumor cell inoculated mice. Mice were sacrificed and subjected to extensive autopsy. All tumor inoculated mice showed solid tumor formation within the peritoneal cavity. The nature of these solid tumors varied from small nodules spreading throughout the mesentery and peritoneal lining to a single large mass. Tumor tissue, blood, spleen, and lymph nodes were collected from tumor-bearing mice for further analyses at day 10 or at first signs of terminal illness. No tissue abnormalities or formation of tumors could be detected in naive (PBS treated) mice.

The presence of Tregs in mesothelioma tissue sections at day 10 was analyzed by immunohistochemistry for the phenotypic evidence of the transcription factor Foxp3, a hallmark of naturally arising $CD4^+CD25^+$ Treg cells [33–37]. Stainings confirmed that mouse tumor tissue obtained from AB1 inoculated mice contained significant amounts of $Foxp3^+$ regulatory T cells, consisting with previous findings in human mesothelioma tissue [8]. Foxp3-expressing cells were located in the proximity of the tumor areas (Figure 1(a)). Double staining showed that the presence of these cells was occasionally in the vicinity of $CD8^+$T cells, suggesting their possible direct suppressive role in antitumoral responses (Figure 1(b)).

We then examined the blood and peripheral lymphoid organs for the presence of Tregs, determined by CD4, CD25, and Foxp3 positive expression using flow cytometry. In peripheral blood of control mice, the mean proportion of Treg cells was 4.9% of all $CD4^+$ T cells. The percentage increased in the blood to 6.1% ten days after the inoculation of AB1 tumor cells (Figure 1(c)). Also the $CD4^+CD25^+Foxp3^+$ Tregs were increased in the lymph nodes from 8.9% to 11.5% of the total $CD4^+$ T cell population in tumor-bearing mice (Figure 1(d)). In the splenocytes from tumor-inoculated mice, the presence of $Foxp3^+$ Tregs increased to 11% compared to 9% in the control group treated with PBS alone (data not shown).

To determine the impact of a growing tumor on Tregs, the percentage of $Foxp3^+$ T cells in the draining lymph nodes was measured. Total weight of excised tumor tissue and the percentage of Tregs in the draining lymph nodes of corresponding mice are depicted in Figure 1(e). At ten days after AB1 inoculation ($n = 5$), the weight of the total tumor mass collected varied from 0.1 gram to 1 gram, or larger (1.5 to 2.1 gram) when mice showed evidence of ill health or overt tumor growth ($n = 3$).

In conclusion, Tregs were found within the tumor area and increased proportions of Tregs were found in the tumor-bearing mice. Moreover we show a significant increase in the proportion of Tregs that correlates with tumor burden ($P = .0039$).

3.2. Reduction of Tregs Improves Cytotoxic T Cell Function In Vivo. To demonstrate that cytotoxic T cell function is inhibited by Tregs, the intracellular expression of granzyme B and IFN-γ in activated $CD8^+$ T cells was measured. Tregs from tumor-bearing mice were isolated from spleen. These $CD4^+CD25^+$ T cells were then added in different ratios to the activated splenocytes isolated from DC-treated mice. Intracellular granzyme B expression by $CD8^+$ T cells decreased in the fractions containing high amounts of $CD4^+CD25^+$ T cells. The same was observed for the IFN-γ expression (Figure 2). When $CD4^+CD25^+$ T cells are present in a ratio higher than 1 : 100, the function of $CD8^+$ T cell is impaired.

Taken together, these data provide evidence that the addition of Tregs leads to impaired CD8 T cell activation.

3.3. Effects of Low-Dose Cyclophosphamide on the T and B Cell Populations. To examine the immunomodulating effects of CTX on Tregs, BALB/c mice were inoculated i.p. with 0.5×10^6 AB1 mesothelioma tumor cells and given drinking water with or without CTX. After 10 days peripheral blood and spleen were analyzed. Metronomic CTX treatment resulted in a significant decrease in the proportions of $CD19^+$ B cells and an increase of $CD3^+$ T cells (Figures 3(a) and 3(b)), in agreement with reports by others [13]. In contrast to the significant increase in the proportions of total $CD3^+$ T cells, we observed a significant reduction in the fractions of $Foxp3^+$ T cells (Figures 3(a) and 3(b)).

In summary, these data show that metronomic administration of low-dose CTX has a strong immune-modulating effect in vivo, causing a shift in ratio between $CD19^+/CD3^+$ cells. Addition of CTX to drinking water of mice leads to a significant increase in the proportion of $CD3^+$ T cells in the peripheral blood and the spleen, whereas the proportion of Tregs is reduced.

3.4. CTX Improves Suboptimal Dc-Based Immunotherapy. Next, we then investigated if combining CTX and DC vaccination enhanced the efficacy of the DC treatment as an antitumor treatment. In contrast to *optimal* DC-treatment protocols (as described by Hegmans et al. [5]), DCs are given at a relative late time point (day 12) in order to achieve suboptimal survival of DC-treated mice. Twelve days after i.p. tumor cell inoculation, mice were vaccinated with 1×10^6 LPS-matured DCs pulsed with AB1 tumor lysate. Mice that received CTX had an increased survival, as seen in our earlier experiments, compared to untreated mice.

When mice were given drinking water supplemented with 0.13 mg/ml CTX from day 3 till day 10 and day 14 till day 21, an increased survival was measured. However, the combination of DC-based immunotherapy and CTX administration significantly improved survival compared to DC-based immunotherapy ($P < .0035$) or CTX administration alone ($P < .0056$) (Figure 4).

Therefore, we conclude that CTX is a powerful tool to optimize suboptimal DC-based immunotherapy. Although CTX alone also improves survival, the combination of both was significantly better.

3.5. Long-Term Administration of CTX Improves Survival Compared to Pre- or Postimmunotherapy Treatment. It has been reported that the timing of CTX administration is critical [38, 39], therefore we investigated the consequences of administration at different time points. To evaluate the effect of timing of CTX administration, mice were divided

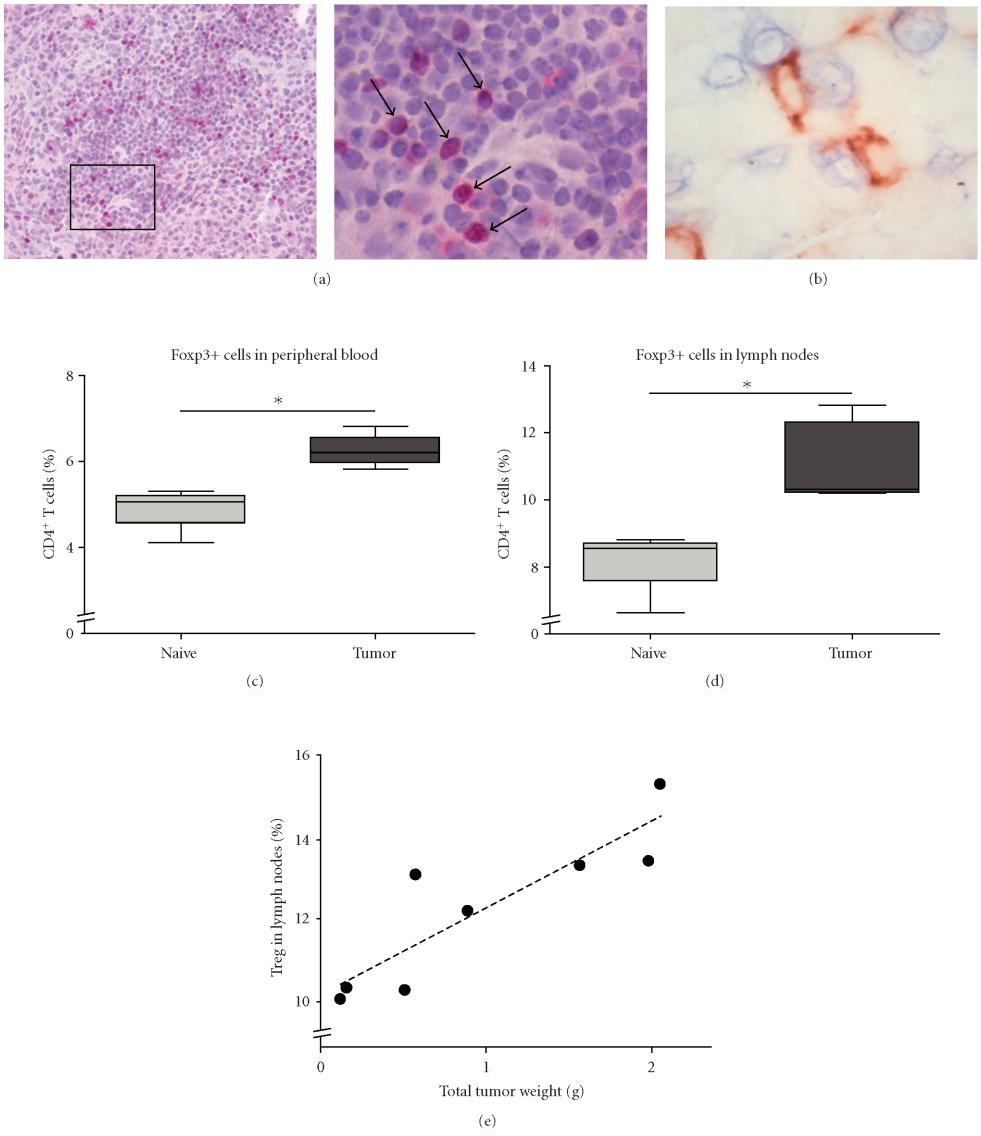

Figure 1: Increase in the proportion of regulatory T cells in a murine model for mesothelioma. (a) Left: The transcription factor Foxp3, a hallmark of naturally arising $CD4^+CD25^+$ Tregs, was expressed inside the tumor (red staining). Cells were counterstained with haematoxylin. [Magnification 200×] Right: A higher magnification (400×) shows the appearance of $Foxp3^+$ cells as indicated by arrows. (b) Tregs (blue staining) were occasionally present in the near vicinity of $CD8^+$ cells (red staining) [Magnification 1000×]. (c) and (d) Percentage of Tregs (defined as $CD4^+CD25^+Foxp3^+$ cells) from total $CD4^+$ T cells in the peripheral blood and draining lymph nodes is significantly higher at day 10 in tumor-bearing mice compared to healthy mice as observed by flow cytometry. (e) A positive correlation was found between the total tumor weight and the percentage of Tregs, measured in the lymph nodes of tumor-bearing mice. Five mice were euthanized at day 10 (tumor weight varied from 0.1 gram to 1 gram) and three mice at stage with ill health (tumor weight 1.5 to 2.1 gram). Correlation coefficient of the trend line (R-squared) is 0.780 (P value is .0039).

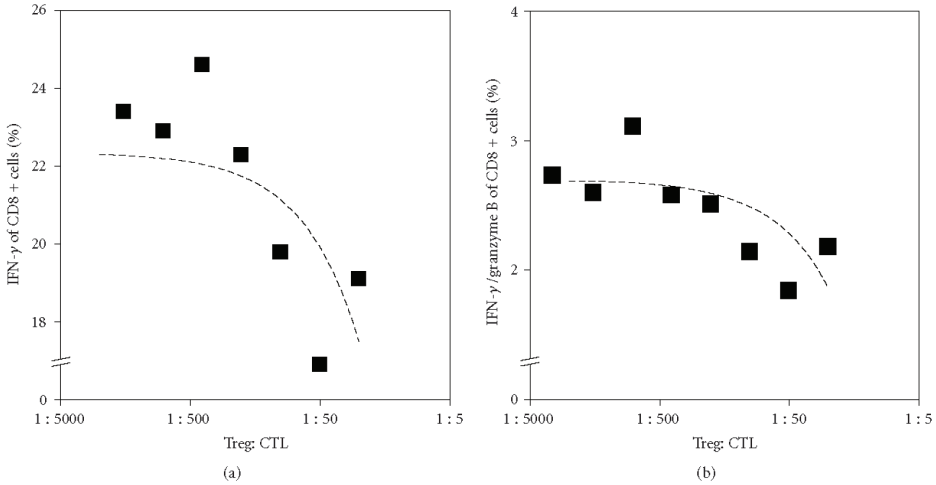

FIGURE 2: Tregs inhibit CTL function in vitro. (a) Activated splenocytes from DC-treated mice were cocultured with $CD4^+CD25^+Foxp3^+$ cells (purified from tumor-bearing mice) in different ratios. The percentage of intracellular IFN-γ of CD8+ cells was measured using flow cytometry. And (b) the percentage of intracellular IFN-γ and granzyme B expression was measured using FACS. A reduction in the IFN-γ and granzyme B expression was found especially in those fractions where increasing doses of Treg were added to the $CD8^+$ T cells.

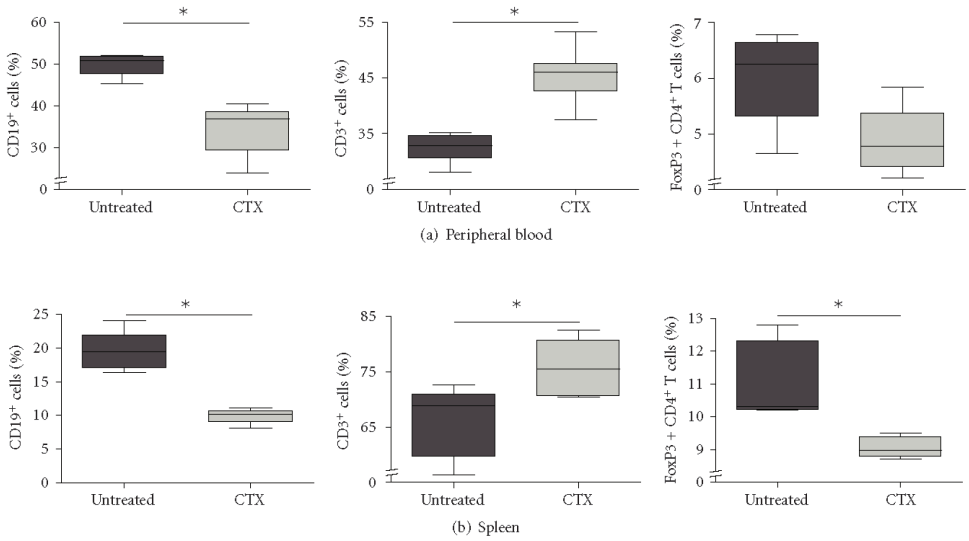

FIGURE 3: CTX influences lymphocyte subsets in peripheral blood and draining lymph nodes of tumor-bearing mice. (a) The percentage of $CD19^+$ cells was significantly decreased ($P < .05$) in CTX-treated tumor-bearing mice. $CD3^+$ cells were significantly increased ($P = .0325$) in the peripheral blood of CTX-treated tumor-bearing mice compared to untreated tumor-bearing mice. (b) $CD19^+$ cells were significantly decreased ($P < .05$) while $CD3^+$ cells were significantly increased ($P < .05$), while the percentage of $CD4^+CD25^+Foxp3^+$ cells was significantly decreased ($P < .05$) in the splenocytes of CTX-treated mice compared to untreated tumor-bearing mice.

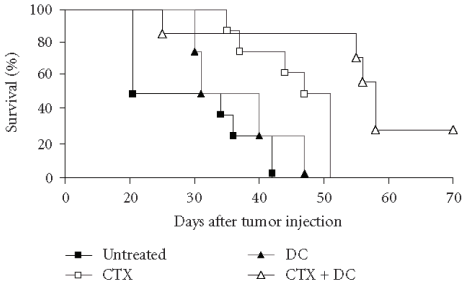

FIGURE 4: CTX combined with DC-based immunotherapy prolongs survival. Kaplan-Meier survival plot showing the effect of the combination of CTX and DC-based immunotherapy. Mice were given drinking water supplemented with 0.13 mg/ml CTX from day 3 till day 10 and day 14 till day 21. Tumor-lysate-pulsed DCs were given at day 12. Survival significantly improved when CTX and DC-based immunotherapy were combined compared to DC-based immunotherapy ($P = .0035$) or CTX administration alone ($P = .0056$). Each group contained 8 mice.

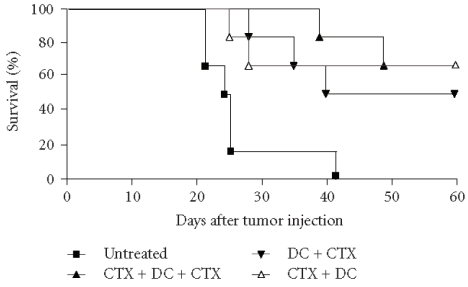

FIGURE 5: Long-period administration of CTX improves survival compared to pre- or post-immunotherapy treatment. Mice were divided into four groups ($n = 6$ per group) and inoculated with a lethal dose of AB1 tumor cells on day 0. Mice received low-dose CTX before (day 3 till 10) or after (day 14 till 21) immunotherapy or metronomic dosed CTX (day 3 till 10 and day 14 till 21). Groups 2, 3, and 4 were treated with DC-immunotherapy on day 12. Group 1 functioned as a tumor control group and did not receive any treatment. Administration of metronomic dosed CTX was not significantly better than CTX treatment before ($P = .840$) or after ($P = .454$) immunotherapy. However, the combination of CTX and immunotherapy was significantly better than no treatment (CTX before immunotherapy compared to untreated $P = .0081$, CTX after immunotherapy compared to untreated $P = .0147$, metronomic dosed CTX and immunotherapy compared to untreated $P = .0018$).

into three groups, receiving low-dose CTX before (day 3 till 10) or after (day 14 till 21) immunotherapy or long-term CTX (day 3 till 10 and day 14 till 21). Mice were treated with DC-immunotherapy on day 12. No significant differences were found between the groups (before [$P = .37$] , after treatment [$P = .84$]). However long-term CTX administration may prolong the survival beneficial since no side effects of this low dose were observed (Figure 5).

4. Discussion

Cancer immunotherapy attempts to harness the exquisite power and specificity of the immune system to recognize and destroy tumor cells or to prevent tumor recurrence. The finding that some patients with malignant pleural mesothelioma (MM) have tumors that regress spontaneously [40–43] or respond to immunotherapy [44–48] suggests that the immune system can generate antitumor reactivity under specific circumstances [41, 49].

DCs are extremely potent antigen-presenting cells specialized in inducing activation and proliferation of lymphocytes, which are essential for tumor killing [50]. Patient's own DCs can be used to present tumor-associated antigens and thereby generate tumor-specific immunity [3, 4]. Previously we used the mesothelioma mouse model to examine the impact of dendritic cell immunotherapy [5]. For malignant mesothelioma, as for most other cancer types, only a few tumor-associated antigens (TAAs) are known. These antigens are not expressed on the membranes of all tumors and therefore less suitable for antigen source for DC pulsing. Furthermore, none of these TAAs have been evaluated as a source of peptides to pulse DC or in a cancer vaccine trial. Tumor lysate priming strategies are advantageous in providing the full antigenic repertoire of the tumor and, particularly, unique individual tumor antigens, which will theoretically decrease the ability of tumors to evade the immune response by downregulation of a single antigen. Therefore, DCs were pulsed with autologous total tumor lysate fractions prepared by freeze thawing and sonication of AB1 tumor cells. Mice receiving tumor lysate-loaded DCs developed a protective antitumor immunity when animals were vaccinated before tumor inoculation. They showed no signs of tumor growth even after 3 months and after repeated injection of tumor cells (2nd tumor challenge). MM had a better outcome when DCs were injected early in tumor development indicating that tumor load played an important role in survival.

There are multiple levels to explain the limited benefit from DC vaccinations in mice with high tumor load. It has been suggested that immune suppression by tumor-derived factors is one of the main reasons for immunotherapy failure in general [7, 51]. Tumors escape immune recognition by attracting immune suppressive cells like Treg cells and myeloid-derived suppressor cells. Others and we have shown that mesothelioma cells are potent sources of a number of cytokines (e.g., IL-6, IL-8, VEGF, GRO, and RANTES) that might directly suppress immune activating cells, like DCs and/or recruit suppressive cells and thereby abolish an efficient immune response [8]. It has become evident that while protecting the host against cancer development, the immune cells also promote the emergence of tumors with reduced immunogenicity leading to a complex interplay of tumor growth and tumor regression mechanisms.

Like in human mesothelioma biopsies, a growing AB1 tumor in vivo contains significant amounts of $CD4^+CD25^+Foxp3^+$ regulatory Tcells, which were previously shown to promote tumor progression in other cancer models. Exact mechanisms are not yet fully understood; however one of the mechanisms by which Tregs can abolish the effectiveness of immunotherapy may be by their capacity to produce granzyme B. This can lead to killing of cytotoxic T cells [52–54]. Depletion of Tregs by using a blocking anti-CD25 antibody (PC61), capable of specifically binding to the IL-2 receptor α-chain, led to an increased survival in mice; however, this depleting antibody is not suitable for human clinical use. Cyclophosphamide (CTX) is an alkylating cytotoxic cancer drug that, depending on its dose and timing of administration, has been used as a chemotherapeutic and disease-modifying agent or to enhance immune responses [14, 26, 27, 55–57]. Reports dating from the 1980s have shown that under some conditions low-dose CTX can potentiate antitumor immunity in mouse models [55]. Recently it has been suggested that this effect is caused by the depletion of Tregs [39]. Reductions in the amount of Tregs by using CTX were also found in mesothelioma [28]. In addition, Van der Most et al. showed that the combination of CTX and gemcitabine eradicates established murine mesothelioma whereas single treatment with gemcitabine did not lead to total tumor regression [29]. However the benefit of combining Treg depletion with DC-based immunotherapy for mesothelioma was not established till now.

$CD4^+CD25^+Foxp3^+$ regulatory T cells comprise between 5 and 10% of the total $CD4^+$ population in the blood of mice, and are important in controlling tolerance to self-antigens and thereby maintain immune system homeostasis [54, 58]. We found that the prevalence of Tregs increases in tumor-bearing mice in the tumor, peripheral blood, and lymph nodes (Figures 1 and 3) as compared with normal controls. Prolonged, lower-dose ("metronomic") CTX inhibits the rise of Tregs (Figure 3). This effect augments the efficacy of dendritic cell-based immunotherapy (Figures 4 and 5). DC-based immunotherapy leads to an increase in survival that is further improved by depleting Treg cells.

5. Conclusion

We found that CTX is an applicable agent to reduce the proportion of Tregs in tumor-bearing mice. In addition, we showed that combining CTX and DC-based immunotherapy significantly prolongs the mean and overall survival in murine model for mesothelioma. We anticipate that CTX allows the host immune system to overcome the immunosuppressive mechanisms of Tregs, thereby allowing for a more complete and robust antitumor immune response initiated by DC-vaccination that is paramount to eliminate the tumor in vivo. Our findings suggest that CTX may also potentiate the immunogenicity of DC-immunotherapy in mesothelioma patients. In fact, these findings now form the basis of a new trial aiming the depletion of Tregs by CTX in combination with DC-based immunotherapy as a multimodality treatment in mesothelioma patients after chemotherapy.

Non-Standard Abbreviations

CTL(s): Cytotoxic T lymphocyte(s)
CTX: Cyclophosphamide
MM: Malignant mesothelioma
DC(s): Dendritic cell(s)
Treg(s): Regulatory T cell(s).

Conflict of Interest

None of the listed authors on this paper had any contact with people or organizations that could inappropriately influence (bias) their work.

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Chapter 7

Dendritic cell-based immunotherapy combined with regulatory T cell depletion for the adjuvant treatment of malignant mesothelioma

Joris D. Veltman[1], Joachim G.J.V. Aerts[1,2], Margaretha E.H. Lambers[1], Robin Cornelissen[1], Femke Muskens, Rudi W. Hendriks[1], Henk C. Hoogsteden[1], and Joost P.J.J. Hegmans[1]

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Study design of the newly initiated trial

Dendritic cell-based immunotherapy combined with regulatory T cell depletion for the adjuvant treatment of malignant mesothelioma.

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SPECIFIC AIMS: **In 2010, we published the first clinical study demonstrating that injection of tumor lysate-pulsed autologous DCs injected in patients with MM after chemotherapy was safe and well-tolerated. There were no grade 3 or 4 toxicities associated with the vaccines or any evidence of autoimmunity. Local accumulations of infiltrating T cells were found at the site of vaccination. The vaccinations induced distinct immunological responses to KLH, both in vitro and in vivo. Importantly, after three vaccinations, cytotoxic activity against autologous tumor cells was detected in a subgroup of patients (CHAPTER 3).**
It is known that the induced anti-tumor immunity in cancer patients is created in an unfavorable highly suppressive tumor environment, influencing the therapeutic efficacy of DC-based immunotherapy negatively. We believe that the full therapeutic potential of DC-based immunotherapy for mesothelioma patients can therefore be further exploited. Regulatory T cells (Tregs) play a pivotal role in the suppressive environment created by the tumor. We have shown in a mouse mesothelioma model that modulation of Tregs using metronomic cyclophosphamide (CTX) could enhance the anti-tumoral immune responses elicited by DC vaccines (CHAPTER 6). In our current study, ten patients with stable disease or partial response after chemotherapy are subjected to a combined treatment with DC-based immunotherapy and simultaneously oral CTX administration.
STUDY ENDPOINTS: **the primary endpoint of the study is: to determine the effect of metronomic cyclophosphamide during DC-based immunotherapy on the reduction of regulatory T cells in peripheral blood of MM patients (by laboratory measurements). Secondary endpoints: A) Determination of the effect of regulatory T cell depletion on specific anti-tumor activity (laboratory measurements), B) Determination of clinical responses (CT scans, time-to-progression, and survival) and to define the safety and toxicity of this combined treatment in MM patients.**
CLINICAL RELEVANCE: **The findings of this study are important to demonstrate if the immune system can be subverted from a suppressive environment to a stimulating situation in cancer patients.**

The study is approved by the Central Committee on Research involving Human Subjects (CCMO) as defined by the WMO (Medical Research Involving Human Subjects Act 1999) [MEC-2008-109/CCMO NL24050.000.08, EudraCT number 2008-000957-36

Keywords: DC-based immunotherapy, mesothelioma, cyclophosphamide, regulatory T cells

PATIENT ELIGIBILITY

PATIENT INCLUSION
Patients with suspicion of pleural MM and who are considered to be fit enough to be treated with chemotherapy will be asked to participate in this study. Also treatment naive patients who have already been diagnosed with MM with a medical need for a pleural puncture are asked to participate. When patients are diagnosed with MM of the epithelial subtype, they will be informed about standard treatment and this experimental study protocol. Ages eligible for study: 18 years - 85 years (both genders).
Standard treatment at this moment includes 4 courses of chemotherapy with Alimta ([pemetrexed disodium, FDA approval February 5, 2004], Eli Lilly and company, Indianapolis, U.S.A.). Then, ten patients will be asked to participate in adjuvant treatment with low dose of CTX in combination with DC-based immunotherapy. Only patients will be included in this trial when a stable disease or partial response occurs after chemotherapy. Response assessment will be done according to modified RECIST criteria.
In case of progressive disease after chemotherapy, both for ethical and study related reasons, patients will not be treated with DC-immunotherapy and will be treated according to patient and clinicians decision. Inclusion and exclusion criteria are summarized in table 1. In addition, table 2 overviews pre-study screening that needs to be performed before inclusion.

STUDY DESIGN

The working plan is to inform patients with suspicion for MM to participate in our study protocol, only when they are fit enough to be treated with chemotherapy. Patients will be informed about the suspicion of MM and the need to perform further diagnostic analysis. The diagnostic process of MM includes the removal of pleural fluid by pleural puncture. Patients will be asked to agree that the excess of the pleural fluid, which is normally discarded, is sent to the laboratory for cell culture and preparing tumor lysate. When a patient is not diagnosed for MM, all patients' corresponding material will be destroyed. In case the diagnosis is not obtained from the cytology of the effusion fluid (> 30% of the cases), a thoracoscopy is routinely performed in order to obtain an appropriate diagnosis (this is a standard procedure). With this technique, histological specimens from a visible tumor are collected for diagnosis. Patients will then be asked to agree on taking four additional tumor samples for the preparation of tumor lysate.
Patients will receive chemotherapy (4 cycles of Alimta/cisplatin). Then, ten patients who had stable disease or partial response after chemotherapy are asked to participate in adjuvant treatment with low dose of CTX in combination with DC-based immunotherapy.
After a resting period of 8 weeks, leukapheresis is performed to obtain monocytes for the differentiation to DCs in a cleanroom facility. The procedure to obtain DCs in vitro and pulse them with tumor lysate is performed according to our previous DC-immunotherapy protocol (CHAPTER 3). Three doses of pulsed autologous DCs are then re-injected every two weeks as shown in Figure 1. Quality control tests are performed before the cellular vaccine is released by an accredited Qualified Person (Pharmacist).

Patients will be treated with metronomic CTX (Endoxan) for seven day in a row the week before the 1st vaccination [week 22], in the weeks between the 2nd and 3rd [week 24 and 26], and for one week after the 3rd DC vaccination [week 28] (Figure 1). The dose of CTX is 100 mg (two [2] tablets)/day. The patient will take the medication 2 hours after breakfast and after diner and will be asked to increase their fluid intake by extra drinking water or other non-caffeinated beverages

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TABLE 1

INCLUSION CRITERIA

- Patients with clinically and histological or cytological confirmed newly diagnosed MM, that can be measured by modified RECIST criteria by a radiologic imaging study.
- Patients must be at least 18 years old and must be able to give written informed consent.
- Patients must be ambulatory (Karnofsky scale > 70, or WHO-ECOG performance status 0 or 1) and in stable medical condition. The expected survival must be at least 9 months at the time of diagnosis.
- Patients must have normal organ function and adequate bone marrow reserve: absolute neutrophil count > 1.5 x 10^9/l, platelet count > 100 x 10^9/l, and Hb > 6.0 mmol/l.
- Positive DTH skin test (induration > 5 mm after 48 hrs) against at least one positive control antigen tetanus toxoid after finishing of chemotherapy.
- Stable disease or any response after chemotherapy as determined with modified RECIST criteria
- Availability of sufficient tumor material of the patient to load dendritic cells, determined at the department of pulmonary disease of the Erasmus MC.
- Ability to return to the Erasmus MC for treatment related procedures and adequate follow-up as required by this protocol.
- Able to tolerate oral therapy with cyclophosphamide.
- No impairment of gastrointestinal (GI) function or GI disease that may affect or alter absorption of cyclophosphamide (e.g. mal-absorption syndrome, history of total gastrectomy/significant small bowel resection).
- No history of allergic reactions (> grade 3 or 4) to compounds of similar chemical or biologic composition to CTX (i.e., alkylating agents).
- No known intolerance or hypersensitivity reaction to CTX

EXCLUSION CRITERIA

- Conditions that make the patient unfit for chemotherapy or progressive disease after 4 cycles of chemotherapy.
- Pleurodesis at the affected side before the pleural fluid is obtained.
- Medical or psychological impediment to probable compliance with the protocol.
- Patients on steroid (or other immunosuppressive agents) are excluded on the basis of potential immune suppression. Patients must have had 6 weeks of discontinuation and must stop any such treatment during the time of the study.
- 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.
- Serious concomitant disease, no active infections. Patients with a history of autoimmune disease or organ allografts, or with active acute or chronic infection, including HIV (as determined by ELISA and confirmed by Western Blot) and viral hepatitis (as determined by HBsAg and Hepatitis C serology).
- Patients with serious intercurrent chronic or acute illness such as pulmonary (asthma or COPD) or cardiac (NYHA class III or IV) or hepatic disease or other illness considered by the study coordinator to constitute an unwarranted high risk for investigational DC treatment.
- Patients with a known allergy to shell fish (may contain KLH).
- Pregnant or lactating women.
- Patients with inadequate peripheral vein access to perform leukapheresis.
- Concomitant participation in another clinical trial.
- An organic brain syndrome or other significant psychiatric abnormality which would comprise the ability to give informed consent, and preclude participation in the full protocol and follow-up.
- Absence of assurance of compliance with the protocol. Lack of availability for follow-up assessment.

throughout the day.
Liver and kidney function is checked to prevent complications of medication and CT-contrast agents during therapy. Additionally, one week after cyclophosphamide treatment, urine of patients is routinely checked for signs of hematuria, to avoid complications caused by side effects of medication. During vaccination procedures patients are restricted to the department of Pulmonary Medicine at the Erasmus MC for a four hour period for observation. Blood pressure, body temperature and oxygen saturation are checked routinely until four hours after vaccination.

TREATMENT EVALUATION AND CLINICAL FOLLOW-UP

SAFETY AND TOXICITY
To define the safety and toxicity of the combination of metronomic CTX (orally) and tumor lysate-pulsed DCs injected intradermally and intravenously in patients with MM after chemotherapy. Serum and blood samples are regularly tested for liver and renal functioning and for the development of auto-immunity by analysis of ANA, ENA and rheumatoid factors.

IMMUNE RESPONSES
Serum and blood samples will be taken at numerous time points for leucocyte analysis (B cells, T cells [Tregs and CTLs]), IFN-gamma ELISPOT and other cytokines to reveal immune responses and tumor-specific responses. Humoral responses to the model antigen 'keyhole limpet hemocyanin (KLH)' are measured in the serum by ELISA. Tregs will be analyzed by polymerase chain reaction (PCR) for the presence of the transcription factor FoxP3, GITR and CTLA4 expression. Viable Tregs will be identified, isolated, and characterized from patient blood samples using CD4, CD25 and CD127 antibodies and sorted using a cytometry sorter FACSaria. Cells will be functionally analyzed *in vitro* in a suppression assay with responder lymphocytes at different ratios in the presence of a stimulus (Treg Suppression Inspector, Multinyi Biotec). In addition, anti-tumor activity is measured using 4 hour incubation of PBMC from specific time-points with radioactive (51Cr) labeled autologous tumor cells. Lysis of tumor cells in this period is determined by gamma-scintillation counting.

To determine if this immunization results in a detectable immune response, DTH tests will be performed twice (Skin DTH tests at week 17/18 and 29). Therefore, unpulsed DCs, DCs pulsed with tumor lysate, DCs pulsed with KLH and tumor lysate will intradermally be injected at the inner side of the forearm with approximately 1.000 DCs / site. Readings are carried out after 48 hours.
A positive reaction is characterized by an induration of at least 5 mm. Each positive reaction should be recorded as the sum of the perpendicular diameters divided by 2. Six millimeter punch biopsies might be taken from the positive reactions and tested for CD4, CD8, and NK cells using immunohistochemistry. All DTH results will be recorded in the patient's chart. The reading will be assessed preferably by the same study coordinator. Digital color photographs of the

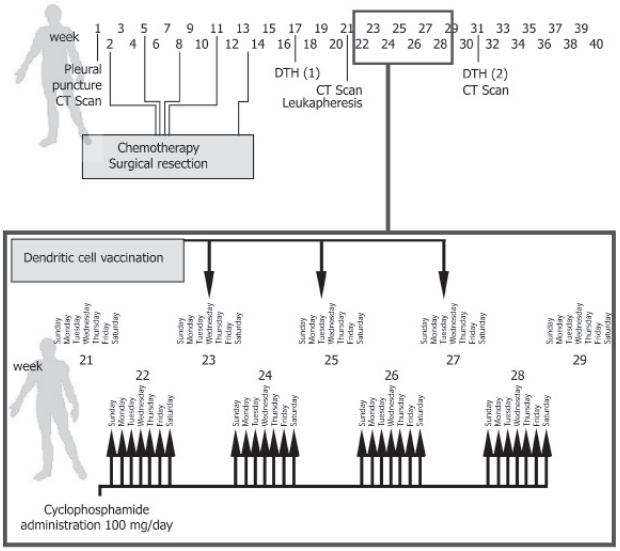


Figure 1. Synopsis of the study consisting of a combined treatment with chemotherapy followed by active immunotherapy using autologous tumour lysate-loaded dendritic cells (DCs) in combination with metronomic cyclophosphamide (CTX 100mg; administration: 50mg tables 2dd1). DTH = delayed type hypersensitivity.

skin DTH response will be taken and all results are stored confidentially according to Personal Data Protection Act (WBP: Wet bescherming persoonsgegevens).

CLINICAL RESPONSES
CT-scans (at least at week 1, 14, 21, 31), re-staging tests, and time to progression will be performed at scheduled intervals throughout the study. To observe and document the clinical response in MM patients who receive the complete treatment, as measured by the evolution of evaluable and measurable disease lesions in response to the treatment.

LIFELONG FOLLOW-UP OF PATIENTS
All patients will be seen at specified intervals during the study. After finishing the study protocol, patients are re-directed to the most appropriate outdoor hospital at regular intervals according to standard clinical practice. Study coordinator maintains lifelong contact with patient / general practitioner.

PROJECT EVALUATION (SUCCESS OR FAILURE)
Historical controls and the earlier performed study (CHAPTER 3) are used for the interpretation and evaluation of the results. Then, all patients had an induction of immune responses against KLH and an increase of the percentage of granzyme B CD8+ cells between pre-vaccination and post-vaccination. We anticipate this will be found in >90% (>9) of the participating patients in this study, indicating that the injected dendritic cells are capable of inducing stronger immune responses.

Important factors to continue or impediment the strategy of modulating the suppressive environment during DC-based immunotherapy are : 1). a decrease of 25% in the percentages of Tregs within the CD4+ T cell population in peripheral blood (was 9.8%) by CTX intake in at least 70% of patients (at week 30/31), 2). at least an increase of 10% in tumor specific lysis (was 4.9%) in more than 60% of the patients (week 30/31). These results are combined with CT-scan findings (was 30% partial response), safety and toxicity findings, and clinical findings (time to progression, median survival (was 19 months)) for considering the effectiveness of the project

Acknowledgment*: We thank Eric Braakman and Anita Schelen from the Department of Hematology for clean room use. Meriam Grootes and others are thanked for apheresing the patients. Arnold Vulto and Anna de Goede are thanked for their expert-advices on pharmaceuticals and quality-control of the vaccines.*

CURRENT STATUS

6 of the 10 patients have been treated with adjuvant DC-based immunotherapy in combination with metronomic CTX. Preliminary data showed that DC-based immunotherapy combined with CTX is as safe and feasible as single treatment with DC-based immunotherapy. As described in our previous trial, self-limiting fever-like symptoms were observed after second and third vaccination; thus far no side effects were reported during treatment with the combination of DC-based immunotherapy with metronomic CTX. To avoid confirmation bias on interpretation of data, no analysis on Treg number and function can be performed in this ongoing trail. These analyses will be performed after completion of the study. We anticipate completing this clinical trial by the end of 2011.

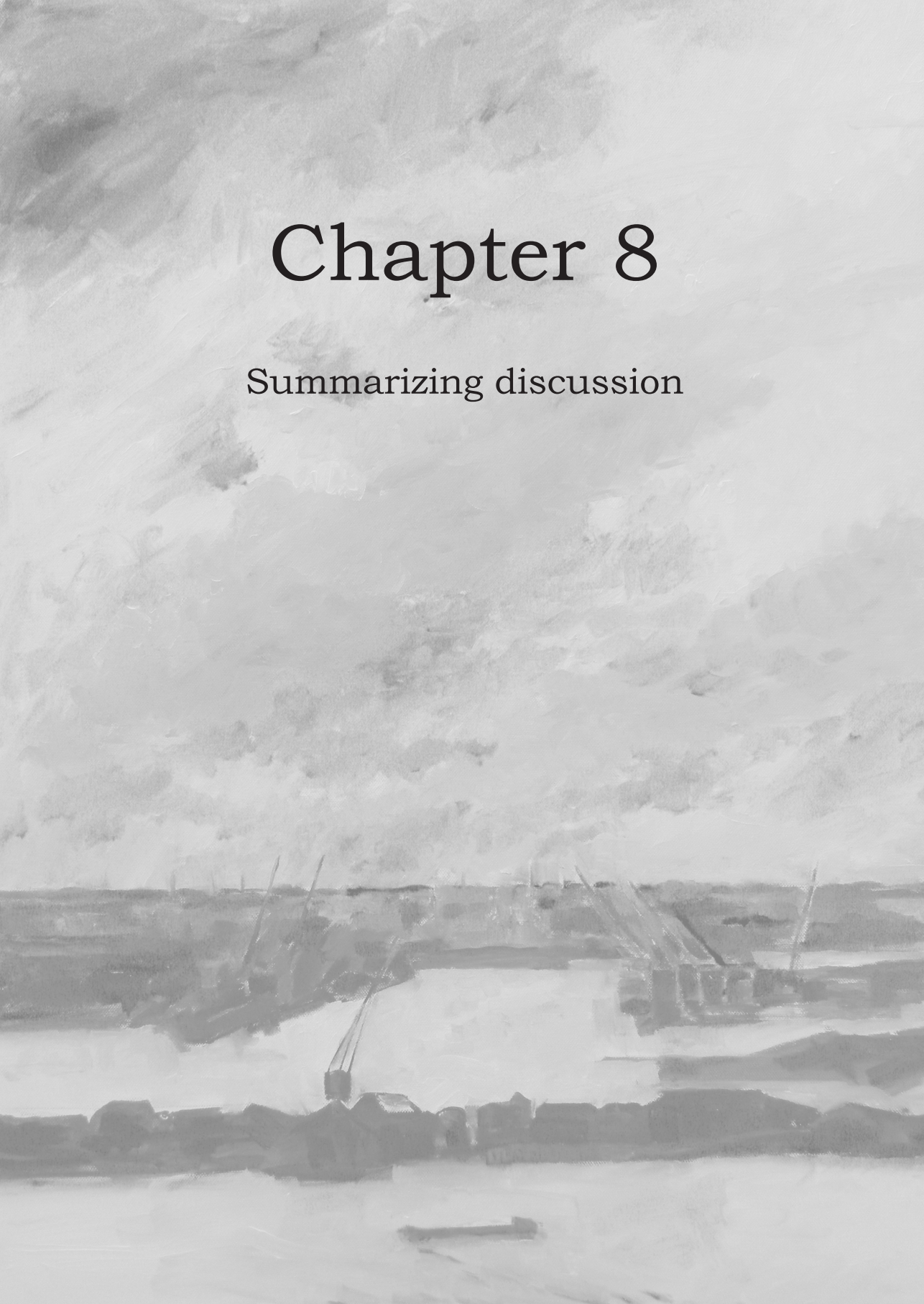
TABLE 2

PRE-STUDY SCREENING
All eligibility criteria should be assessed together with relevant baseline parameters within 21 days prior to initiation of the DC-based immunotherapy (week 17/18).
The following tests must be performed:

- Medical history and complete physical examination (performance status, including inclusion/exclusion criteria, blood pressure, weight, and body temperature).
- Clinical laboratory tests will include at least: complete blood count and differential count, creatine, urea and ionogram (including sodium, potassium, calcium), bilirubin, lactate dehydrogenase, creatine phosphokinase, transpeptidases and aminotransferases, serum proteins (total protein and albumin), ANA-titer.
- Radiological studies documenting tumor location.
- Viral serology: HTLV-I, -II and HIV antibodies, HBV antigen, and HCV antibodies.
- Bacteria serology: Syphilis
- DTH skin test with tetanus toxoid

Chapter 8

Summarizing discussion



Chaptier 8 Summarizing discussion

The studies presented in this thesis describe strategies to improve dendritic cell-based immunotherapy. To refine this approach we focused on targeting immune suppressive cells that are abundantly present in tumour-bearing hosts. In **CHAPTER 3**, the outcome of our clinical phase I trial is presented. In this chapter we showed that the administration of dendritic cells loaded with autologous tumour lysate is save and feasible, but more importantly, we were able to detect a specific anti-tumour response in the blood of patients (4 out of 6). However, optimization of this strategy was recommended to expand therapeutic potential.

The presence of immune suppressive cells has been proposed to be one of the main factors negatively effecting the induced immune response generated by immunotherapy. As described in chapter 1; MDSC, Tregs and TAMs are abundantly present in tumour environments. We aimed to determine the effectiveness of clinical agents in targeting of these immune suppressive cells in a murine model for mesthelioma. We selected registrated agents, to make research translational for future clinical immunotherapy studies. **CHAPTER 6** demonstrates that treatment with metronomic cyclophosphamide leads to a reduction on Tregs in our murine model. These findings have led to the initiation of a new clinical phase I/II trial investigating the effectiveness of the combination of DC-based immunotherapy and metronomic cyclophosphamide in mesothelioma patients (preliminary data **CHAPTER 7**). In this final chapter, strategies to refine immunotherapy by targeting immune suppressive cells will be discussed. Furthermore, suggestions for further refinement of this approach are given.

8. Overview of the experimental work that was performed

8.1. Dendritic cell-based immunotherapy in mesothelioma patients

Ten patients were treated with standard chemotherapy followed by three vaccinations of autologous tumour lysate-loaded monocyte-derived dendritic cells (DCs), each dose consisting of 50x106 clinical-grade cells. Chemotherapy was given prior to DC vaccination in order to reduce tumour load, thereby potentially augmenting the efficacy of the vaccination as was earlier found in mice (1).

Autologous tumour lysate priming strategies for DC loading was chosen to provide the full antigenic repertoire of the tumour and, particularly, unique individual tumour antigens, which will theoretically decrease the ability of tumours to evade the immune response by down-regulation of a single antigen. Lysates were prepared from patient's tumour material derived from pleural fluid or from tumour tissue obtained by thoracoscopy. Tumour cell lysates were generated by repeated freeze-thaw cycles and irradiation with 100 Gy. DCs could be cultured in sufficient amounts from monocytes isolated by elutriation of the leukapheresis product using GM-CSF and IL-4. After 5 days in culture, DCs were pulsed with autologous tumor lysate and the model antigen keyhole limpet hemocyanin (KLH). A cytokine cocktail of IL-1β, IL-6, TNF-α and PGE-2 induced the final maturation step (at day 8). The morphology (>95% veiled cells) and phenotype of cultured cells as assessed by flowcytometry was consistent with mature DC (**CHAPTER 3**).

Autologous tumour lysate-pulsed DCs were intradermally and intravenously administrated in MM patients after cytoreductive therapy with chemotherapy. All 10 patients received the three planned vaccinations. The results showed that injection of DCs was well-tolerated without major toxicity, with the exception of low-grade flu-like symptoms: fever, rigors (chills), and a temporarily local skin reaction after DC injection (mild grade 1 or 2). Local accumulation of CD4+ and CD8+ T cells were found at the vaccination sites. Body temperature was increased in 9 (of 10) patients but did not exceed 39 °C, mainly after the second and third vaccination. No participants experienced rash or lymphadenopathy

or developed any clinical evidence of autoimmunity. Delayed-type hypersensitivity (DTH) skintest and strong antibody immune responses (IgM and IgG) on the surrogate marker KLH were seen in all patients indicating that immune responses were generated. CT scans and X-rays from 3 patients revealed small to substantial regressions of the tumour during the DC treatment. Importantly, distinct anti-tumour activity against autologous tumour cells in the blood of 4 out of 6 patients was induced by the vaccinations. Although results are promising, further refinement of this therapeutic approach is recommended to improve clinical outcome. It has been suggested that tumour-related inflammation is one of the main reasons for immunotherapy failure. Since immune suppressive cells, recruited by tumour-derived factors inhibit the anti-tumour response and can induce T cell tolerance (2-5).

8.2. Targeting immune suppressive cells

Regulation of the immune system is under control of immune regulatory cells, including myeloid-derived suppressor cells (MDSC) and regulatory T cells (Tregs) (6-8). Besides, tumour-associated macrophages (TAMs) play a dominant role in tumour-microenvironments, facilitating angiogenesis, tumour-growth, metastasis and immune suppression (9). These cells are actively recruited and supported in their function by factors produced within the tumour microenvironment. In order to successfully influence these cells, it is important to determine which factors are critical for the recruitment and function of these cell types.

8.3. Myeloid-derived suppressor cells

MDSC consist of a heterogeneous group of myeloid cells. These cells are not present in the blood circulation under normal conditions. However, under pathogenic conditions these cells can accumulate rapidly, leading to elevated levels in the peripheral blood, lymphoid organs and the affected tissue (6, 10). In tumour-bearing mice, this increase can rise to a percentages of >30% of all splenocytes (**CHAPTER 4**). MDSC are characterized by the expression of immature myeloid markers and mature myeloid cell markers, but more importantly, they are defined by their high arginase-1 activity, their ability to produce nitric oxide (NO) and reactive oxygen species (ROS). Production of the radicals leads to the inhibition of T cell proliferation, T cell apoptosis and the downregulation of the ζ-chain of the T cell receptor (11). The downregulation of the T cell receptor mainly takes place in the lymphoid organs and has been described as the most important factor in the induction of T cell tolerance. This mechanism is unfavourable for immunotherapy approaches, because it causes disfunction of cytotoxic T cells in an antigen specific manner. In contrast, the inhibition of T cell proliferation and the induction of T cell apoptosis is an antigen non-specific way takes place at the tumour site (3, 6).

Investigations on MDSC function related to tumour-derived factors led to the understanding that in both inhibitory processes, ROS production and NO production, are depending on tumour-derived prostaglandin E2 (PGE2). The inducible enzyme cyclo-oxygenase 2 (COX-2) is required for the synthesis of PGE2 (12). Several investigations showed that PGE2 is produced by many tumour types and that the high levels of COX-2 and PGE2 correlate with decreased survival (13-16). The synthesis of PGE2 can be blocked by the inhibition of the COX-2 enzyme by using selective COX-2 inhibitors (e.g. Celecoxib (Celebrex®)). Via dietary administration of celecoxib in a murine mesothelioma model we were able to show that celecoxib blocks the production of tumour-derived PGE2, resulting in reduced activity of MDSC. Besides, our data provides evidence that celecoxib treatment not only reduced the function of MDSC but also their number in tumour bearing mice. Combining celecoxib treatment with DC-based immunotherapy improved the induced anti-tumour response. Since ROS production has been described to be the main factor in the induction of immune tolerance, we analyzed the effect of COX-2 treatment on ROS

production by MDSC. Analyzing different subsets of MDSC revealed that treatment with a selective COX-2 inhibitor reduced the ROS production by all subsets (**CHAPTER 4**).

Another approach to target MDSC might be via inhibition of recruitment factors. Vascular endothelial growth factor (VEGF) has been reported to be an important factor leading to MDSC expansion (3). Therefore the inhibition of VEGF by selective VEGF-inhibitors is an interesting approach to modulate MDSC. The selective VEGF-inhibitor Bevacizumab (Avastin) is an interesting candidate in combination with DC-based immunotherapy to reduce the number and / or modulate the function of MDSC.

8.4. Tumour-associated macrophages

Extensive research has been performed on the role of TAMs during tumour progression. TAMs play various roles in several processes facilitating tumour growth and tumour spread. Analyzing tumour tissues and pleural effusions of mesothelioma patients revealed that macrophages are present in high numbers. Besides, several clinical studies showed that there is a clear correlation between the number of tumour infiltrating macrophages and survival (18-21). To determine the effects of TAMs in relation to tumour growth, experiments were performed where macrophages were depleted using depleting liposomes (liposome-encaptured clodronate). Depletion of macrophages totally abolished tumour outgrowth. Therefore, targeting macrophages seems a promising tool to inhibit tumour progression; however liposome-encaptured clodronate is not applicable with DC-based immunotherapy. Liposomes are taken up by phagocytic cells and after the degradation of the liposome, clodronate is released inside the cell. Interference with intracellular cyclic AMP metabolism leads to apoptosis of these cells (22). Since DCs are also capable of phagocytosis, treatment with these liposome-encaptured clodronate also leads to the depletion of DCs.

Although, it is evident that TAMs play a crucial role during tumour progression, no commercial agents are available that specifically target macrophages. Since Non-N-containing bisphosphonates have shown to interact with cell metabolism these bisphosphonates have been proposed as clinical applicable agent to target cells. Several studies revealed that prophylactic treatment with bisphosphonates prevents metastasis. In addition, a recent study by Gnant et al. showed that treatment the N-containing bisphosphonate, zoledronic acid (Zometa), prolongs disease-free survival. Therefore, zoledronic acid (ZA) has been proposed as a new anti-cancer drug; however the exact mechanism by which ZA prevents tumour progression remains unclear (23). As treatment with ZA also influences cytokine, chemokine and growth-factor levels and reduces angiogenesis within tumours, it became more distinct that ZA may have an immune modulating effect on macrophages. For this reason we investigated if treatment with ZA affects the differentiation and function of TAMs in tumour bearing mice.

To determine the effect of ZA on macrophage function and differentiation, macrophages were investigated in vitro as well as in vivo. Although it was found that ZA had an inhibitory effect on macrophages differentiation and polarization, no significant improvement of survival was observed. Several explanations were proposed. One of the most striking observations during the experiments was that treatment with ZA led to an increase in immature myeloid cells because of inhibition of differentiation. These cells were characterized by an MDSC-like phenotype. As reported previously, these cells suppress anti-tumour activity and contribute to tumour progression. It was suggested that the decrease in TAMs was correlated with an increase in immature myeloid cells, since it was found that ZA inhibits myeloid differentiation in vitro resulting in a higher number of immature myeloid cells after 6 days of culture. Most studies on the effect of ZA on survival were investigated in tumour models with high metastatic rates. As TAMs play an important role in tumour metastasis, ZA may be beneficial in these tumour types where metastasis is the cause of death. Although ZA leads to a reduction in TAMs and affect the phenotype of macrophages, refining immunotherapy by the addition of single treatment

with ZA is believed to be insufficient (**CHAPTER 5**). Therefore, future studies will show if the combinating ZA with celecoxib holds more promise, since this approach might target both TAMs as well as MDSC.

Recently, Yondelis (Trabectedin) was reported as a potent agent to target macrophages, however severe side effects have been observed in a majority of patients and the cytotoxic character of the drug may interfere with the immunotherapy vaccine approach (24-25). Therefore, this is not an agent of first choice. Another approach to target TAMs may be via modulation of macrophage polarization or inhibition of factors linked to TAM recruitment. As hypoxia within tumours leads to the adaptation of TAMs, tumour-hypoxia has been proposed as a target to affect TAM accumulation. Hypoxia leads to the induction of hypoxia-inducible factor (HIF)-1α which plays a critical role in the polarization and homing of TAMs (26). Recent, studies have shown that targeting of HIF-1 activity may disrupt the HIF-1/CXCR4 pathway and therefore affect the accumulation of TAMs (27). Chemokines and chemokine receptors are also targets to control macrophage recruitment and infiltration. MCP-1/CCL2 has been proposed as most promising in this approach (28).

Furthermore, influencing the production of soluble factors by macrophages has been suggested as therapeutic targets. Since TAMs contribute to various processes during tumour development via production of soluble factors, specific inhibition of these products may also slow down tumour progression (4, 29). In this prospective, changing the balance from a M2 polarized macrophage to a M1 phenotype is most challenging (30). However, shifting this balance leads to certain difficulties, since the polarization is caused by defective NFκB activation. Restoration of the NFκB activation restored M1 inflammation and intra-tumoural cytotoxicity leading to the rejection of solid tumours in murine models (31). However, at this moment no clinical registered agents are available which specifically inhibit TAM recruitment or restore NFκB activation, making translation to the clinic more difficult.

8.5. Regulatory T cells

Tregs is a subset of CD4+CD25+ T cells that are characterized by the expression of FoxP3. In the normal situation Tregs are present in blood, bone marrow and in lymphoid organs. They play a crucial role in controlling the immune system. Especially in protection against auto reactive T cells by suppressing cytotoxic T cell function. However, during tumour development these cells expand by several factors and contribute to the induction of tolerance against tumour cells. Therefore, targeting Tregs has been proposed in the optimization of immunotherapy.

One proposed strategy is targeting CD25 via a CD25-specific depleting antibody (PC61). In 2006, Hegmans et al. showed that depletion of Tregs by PC61 (CD25 depleting antibody) led to prolongation of survival in a murine model for mesothelioma. Though, the combination of the human CD25 depleting antibodies; basiliximab and daclizumab; was not successful in cancer immunotherapy (32). An engineered recombinant fusion protein of IL-2 and diphtheria toxin (denileukin diftitox [Ontak]) was 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 tumour regression because it also depleted antitumor effector cells (33-36). The combination of CD25 depleting antibodies with DC-based immunotherapy seems inapplicable. We investigated the effectiveness of another agent in the combination with immunotherapy in depleting Tregs.

Cyclophosphamide (CTX [Endoxan®]) is a nitrogen mustard alkylating cytotoxic pro-drug that can be used as an immune modulating agent in a dose depending manner (37-46). Metronomic administration of CTX prevents the induction of Tregs and inhibits Treg function; nevertheless the mechanism is not fully understood. It has been proposed that Treg are more sensitive to apoptosis induced by CTX, since the levels of the enzyme activity of aldehyde dehydrogenases (ALDH) is very low in T cells (especially Treg) compared to other lymphocytes (47-48). CTX is metabolised in the liver to active forms. ALDH is

essential in converting the toxic metabolite of CTX into non-toxic isoforms. Besides, CTX decreases the expression of GITR and FoxP3 on Tregs, which is associated with reduction in suppressive function (49-53). We showed that the therapeutic combination of CTX with DC-based immunotherapy was effective in a murine model for mesothelioma. Metronomic administration of CTX led to a significant reduction of Tregs in the lymphoid organs and at the tumour site (**CHAPTER 6**).

Other possible approaches to reduce immunosuppression of Tregs is via CTLA-4 blockade (54-55), anti-GITR agonism (56), and vaccination against Foxp3 (57). IL-7 administration was shown to increase T cell numbers and decrease of the Treg fraction in humans (58), on the contrary, other reports have shown that IL-7 leads to the development of Tregs (59-60). Though, most of these approaches are still topic of research. Moreover, the results on the combination treatment of CTX and DC-based immunotherapy possess best translational opportunity, since low-doses CTX has been studied extensively and usage was found to be save and feasible in patients. Therefore, a new trial was initiated aiming at depleting Tregs using metronomic CTX in combination with DC-based immunotherapy as a multimodality treatment of residual tumour deposits following chemotherapy in mesothelioma patients (**CHAPTER 7 (preliminary results)**).

8.6. Main findings emerging from the experimental work

Administration of tumour lysate-pulsed dendritic cell vaccines in mesothelioma patients is safe and feasible (**CHAPTER 3**)

Dendritic cells pulsed with autologous tumour lysate are capable of inducing immunological response to tumour cells (**CHAPTER 3**)

Local tumour growth leads to systemic alteration in the immune system affecting multiple organs like the bone marrow and lymphoid organs (**CHAPTER 4,5,6**)

Tumour-derived factors induce expansion and activation of immune suppressive cells (**CHAPTER 4,5,6**)

MDSC can be divided in three subtypes; polymorph nuclear MDSC, mononuclear MDSC and Gr-1low MDSC in tumour bearing mice (**CHAPTER 4**)

Celecoxib reduces the number and suppressive function of MDSC, thereby improving immunotherapy (**CHAPTER 4**)

Zoledronic acid impairs myeloid differentiation towards TAMs in vitro and in vivo resulting in higher numbers of immature myeloid cells (**CHAPTER 5**)

Zoledronic acid affects the phenotype of macrophages leading to reduced TAM characteristics and lower expression of TAM-associated factors (**CHAPTER 5**)

Administration of metronomic cyclophosphamide reduces the number of Tregs in tumour-bearing mice (**CHAPTER 6**)

Reducing Tregs improves cytotoxic T cell function and improves dendritic cell-based immunotherapy (**CHAPTER 6**)

The action of immune suppressive cells like MDSC and Tregs takes place at the tumour site and lymphoid organs leading to the induction of T cell tolerance and T cell apoptosis (**CHAPTER 4,6**)

Single treatment with agents targeting immune suppressive cells can prolong survival, however, these agents potentially synergize in combination with treatment approaches leading to activation of the immune system against tumour cells (**CHAPTER 4,5,6**)

8.7. Composition of tumour environments in cancer patients

It is now thought that the effectiveness of immunotherapy is largely depending on suppressive environmental conditions of the patient. This environment is created by factors produced within the tumour microenvironment. These factors can either inhibit the anti-tumour response directly, but more importantly, induced hematopoiesis and recruitment of leukocytes capable of suppressing effector cells and enhancing tumour growth. As described in this thesis; MDSC, TAMs and Tregs are recruited by several factors and contribute to this tumour immune escape (2-5).

Several studies attempt to reduce immune suppression by reducing the number and function of these immune suppressive cells. In order to do so, they focus on cell function and identification of recruitment factors. Immune suppressive cells can be targeted in various ways (Figure 1). However, as the numbers of reports on tumour microenvironments increases, it's becoming more evident that the composition of the microenvironment differs between tumour types, but more strikingly may even differ between tumours from the same histological subtypes (61-63). Analyses of pleural effusions and tumour cell-lines of mesothelioma patients showed that they contain high levels of chemokines, growth factors and cytokines associated with recruitment and activation of several immune cells. In addition, it was shown that the expression of these factors reflects the presence of immunological cells within the tumour tissue (63). The expression level of these factors differed between patients. Same observations were done by Fukuyama et al. who determined the cytokine profile of 31 lung cancer cell-lines.and showed that high expression of GM-CSF and IL-8 is related to macrophages infiltration and enhanced angiogenesis in tumour tissue (61). Therefore, it can be hypothesized that variations in the composition of immune suppressive environments in cancer patients are largely depended on factors produced by tumour cells, and the composition of the environment within the tumour bearing host is a major factor influencing patient's prognosis.

For example, high VEGF and COX-2 expression in squamous cell carcinoma and lung cancer correlates with poor survival (13, 64). Both factors were described as important factors in MDSC recruitment and function. A correlation was demonstrated between COX-2 expression and arginase activity in MDSC and VEGF and the number of MDSC (3, 65-66). In this line, it can also be suggested that patients with tumours secreting low levels of GM-CSF, M-CSF and CCL-2 have decreased metastatic potential and better survival prospects, since these tumours are less sufficient in recruiting and activating TAMs (29, 67-68). Though, more investigations are required to test these hypotheses.

In other to test the hypotheses, we need to address various questions. For example, how can we identify and investigate immune suppressive cells in cancer patients and can patients be categorized based on these findings? And can we establish which factors and adjustments during tumourigenesis determine the difference in microenvironment composition?

In order to answer these questions, monitored of immune suppressive cells during treatment is required. By collecting blood and serum samples at different time points during follow up a base line level can be established. Variations in cell number should be correlated with cytokine- and chemokine profiles and may eventually show predictive value for disease progression. In addition, resection material and radiological findings

may contribute to our understanding on differences between local and systemic immune suppression and may hold predictive potential on tumour spread. Recent studies have shown that Tregs have value on predicting disease progression in several tumour types (69-70). However, no single molecule or cell examined thus far within the tumour environment can serve as an absolutely independent indicator of patient survival. Therefore, it will be necessary to examine multiple tumour-associated cell populations in tandem with well-defined pathological, clinical and genetic parameters to predict more accurately patient outcome (71). One of the challenges in these investigations will be the broad variety on therapeutic approaches, which eventually may interact with the immune system and therefore may cause difficulties in interpretation of the data. For example, recent studies have shown that certain types of chemotherapy affects MDSC more then other cells (72-73). Besides this, many patients use co-medication that might effect the immune system.

To investigate differences in tumour behaviour related to tumour induced inflammation, murine models might be helpful. As cell-lines may differ in cytokine, chemokine and growth factor production, these differences may be reflected in the composition of the microenvironment. Recently, micro array data on two murine mesothelioma cell lines revealed that there is an enormous difference in gene expression. For example, AB1 cells produce mainly VEGF and PGE synthase, whereas AC29 tumour cells hardly secreted these factors. In contrast, AC29 expressed a range of chemokines and cytokines (e.g. TGF-β, MCP-1/CCL-2, MMP's, IL-18), while production of these factors was diminished in AB1. Differences in secreted factors may influence tumour behaviour in vivo (preliminary data).

As our understanding about the interactions between the immune system and tumour cells increases, it's becoming more evident that differences in clinical presentation and prognosis may be closely related to variations in the constitution of tumour environments. At first instance, this new hallmark in tumour biology makes understanding of tumourigenesis and progression even more complex then previously believed. However, understanding these mechanisms is exceptionally important for refinement of novel treatment strategies, like immunotherapy. It can be assumed that those patients, with cancer-related inflammation enhancing MDSC expansion, will benefit from treatment with agents targeting MDSC (like celecoxib). Patients, with high numbers of Tregs, may benefit from treatment with metronomic cyclophosphamide.

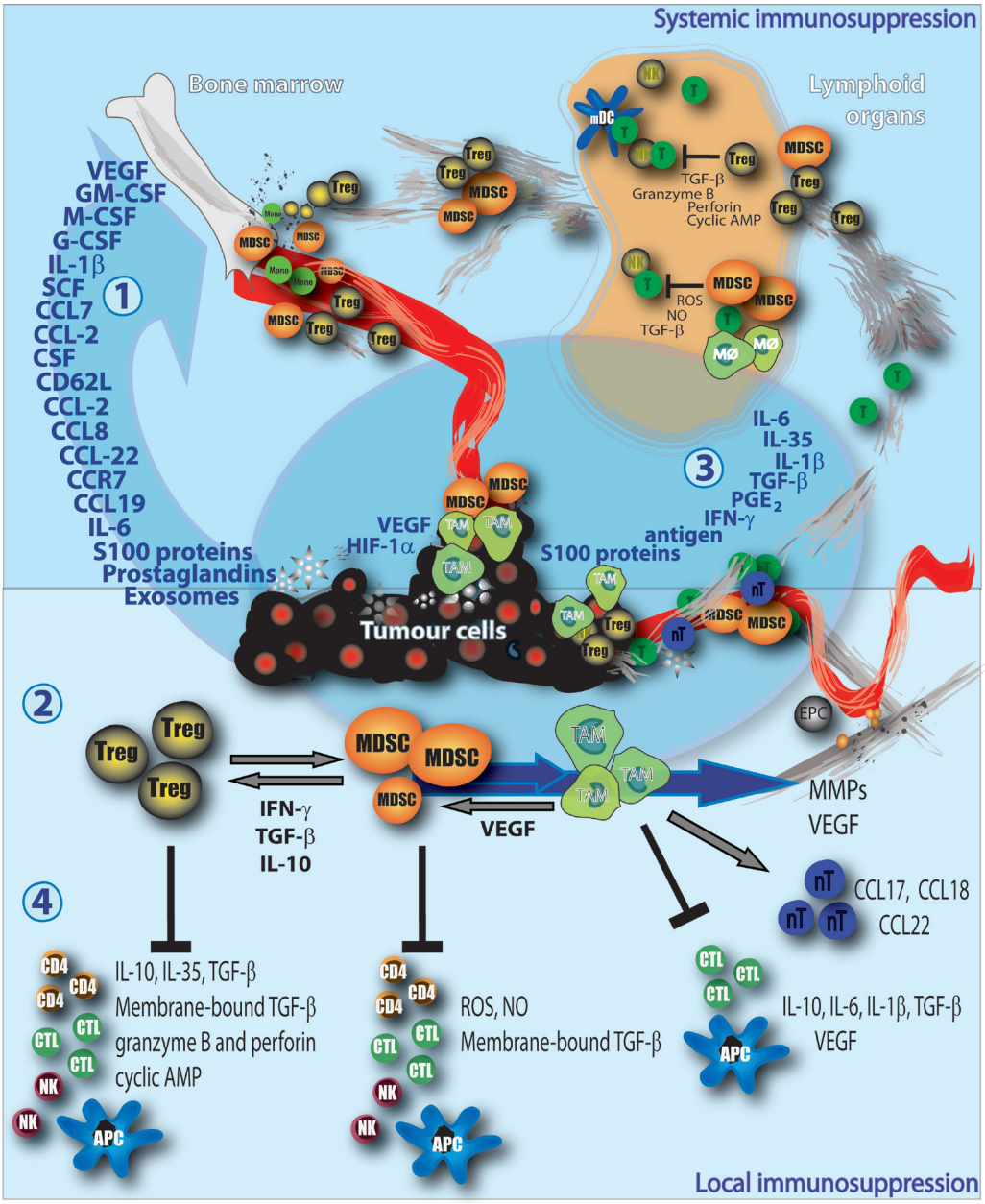


Figure 1. Targeting immune suppressive cells: Immune suppressive cells are localized in the tumour microenvironment as well as systemically, in tumour bearing hosts. These cells contribute to tumour progression and reduce the effectiveness of novel therapeutic approaches, like immunotherapy. Immune suppressive cells can be targeted in various ways. Firstly, targeting tumour cells by itself might eventually influence the composition of the microenvironment, as different therapeutic agents may selectively deplete tumour cells secreting certain factors (1). Secondly, neutralizing recruitment factors may also bring suppressor cell recruitment and differentiation to a hold (2). Other applicable approaches target immune suppressive cells directly by killing of these cells or inhibiting their cell function (3). Additionally, influencing suppressor cell function can be attempted by neutralization of suppressor cell stimulating factors (4). Finally, the effect of immune suppression can be abolished by eradicating of products produced by immune suppressive cells (5).

8.8. Clinical consequences and recommendation for refinement of dendritic-cell based immunotherapy

This thesis reports on DC-based immunotherapy in mesothelioma and investigates approaches for refinement. We showed that the administration of clinical-grade dendritic cell vaccines in mesothelioma patients is safe and feasible. In addition, we demonstrated that an anti-tumour response against mesothelioma can be measured using this approach. Though, to reach full therapeutic potential, refinement of the strategy was recommended. In this thesis we determined the efficacy of several strategies to target immune suppressive cells. These cells were targeted with clinical applicable agents. In our murine model for malignant mesothelioma targeting these cells improved the survival and clinical out-come of immunotherapy. Significant challenges exist in refining immune therapy by determining the optimal combination of agents to abolish the tumour-derived environment inhibiting anti tumour responses. In order to do so, we should not focus on tumour types (and subtypes), but focus on individual patient characteristics. Cytokine-, chemokine-, and growth factor-profiles need to be analyzed in large cohorts of patients in order to provide information on immune cells that are present in the tumour tissue and the lymphoid organs.

To make DC-based immunotherapy accessible for a larger group of mesothelioma patients, exclusion based on insufficiency of tumour material needs to be challenged. We demonstrated that pulsing DCs with autologious tumour material is a safe and feasible method to generate an immune response. However, this approach has a major disadvantage. The quality and quantity of the obtained autologous tumour material (for loading the dendritic cells) of most mesothelioma patients was insufficient for participating in the study. For ethical reasons, extra tumour material is not collected from patients without medical need. Also patients who had a mechanical or chemical pleurodesis or receiving chemotherapy are excluded because of the absence of tumour material for dendritic cell-loading. This major impediment introduced a substantial bias in patient selection and inclusion, making the interpretation of the performed study difficult. In such cases, preparations from other persons' tumours of the same type might be used to pulse the autologous DCs in vitro, hypothesizing that common mesothelioma antigens may be expressed between malignant mesothelioma individuals. The use of allogeneic mesothelioma cell lines as antigen source is advantageous in providing a simpler and more standardized method of delivering a optimized and broad antigenic repertoire of potential tumour associated antigens for dendritic cell pulsing. There are obvious many clinical, scientific and economic advantages to develop such an allogeneic approach in DC-based immunotherapy. In order to test if this approach is safe and leads to the generation of an anti-tumour response we will test this in a murine model. Autologous dendritic cells will be pulsed with different sources of allogeneic tumour cell derivatives generated from well-characterized mouse mesothelioma cell lines. The phenotype and activity of the dendritic cells after pulsing will be determined in vitro. Also the safety, immune responses and clinical activity in tumour-bearing mice will be studied following treatment with DC loaded with allogeneic tumour cell derivatives. Besides the optimal source of tumour cell derivatives, methods to increase the immunogenicity and further enrichment by protein fractionation will be investigated.

If shown safe and effective in animal models, we want to translate these findings to a clinical-trial with the intention to open up exciting new options to broaden the applicability for DC-based immunotherapy in the clinic. This would give a new prospective for the future to treat more mesothelioma patients with an optimized and standardized approach. It could make large multi centre trials possible that are urgently needed for the essential evaluation of the efficacy of dendritic cell-based immunotherapy.

Another intriguing future perspective for allogeneic loading of autologous DCs might be as prophylactic vaccine in individuals at risk of mesothelioma, in particular persons occupationally exposed to high amounts of asbestos fibres (e.g. workers at shipbuilding plants or industrial plants) or individuals expressing high levels of SMRP (MesoMark;

www.fdi.com/mesomark) without symptoms of disease. This cancer immunoprevention is currently of major interest in cancer research, however, it is far from clinic and several questions need to be answered through translational research before progressing into clinical trials (74-78). Nevertheless, if early therapeutic trials using DCs loaded with allogeneic material elicit significant immune responses with satisfying safety and toxicity results, first steps could be made in a preventive setting for mesothelioma. This is attractive approach because (I) there is clear group of individuals at risks, and (II) preneoplastic lesions caused by asbestos fibres are more sensitive to immune destruction than established tumours because they are smaller and less immunosuppressive. This was confirmed in mouse studies where DC-based immunotherapy was more effective in mice with smaller tumour loads (78), and (III) DC-based immunotherapy can induce long-term protective memory with no side effects (79). Prophylactic vaccination worked exceptionally well in case of infectious diseases. Also the success of the prophylactic cancer vaccine that immunizes against human papilloma virus and protects against cervical cancer has reinforced that prevention is better than therapy during disease (80-81). The prophylactic application of allogeneic approaches could be continuation on the long term.

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Ab = antibody
ALDH = aldehyde dehydrogenases
AP = alkaline phosphatase
APC = allophycocyanin
APC = antigen presenting cell
AWD = alive with disease

BSA = body surface area
BSA = bovine serum albumin

CO2 = carbon dioxide
COX = cyclo-oxygenase
CT = computed tomography
CTL = cytotoxic T lymphocyte
CTLA-4 = cytotoxic T lymphocyte-associated antigen 4
CTX = cyclophosphamide

DC = dendritic cell
DCFD = dichlorodihydrofluorescein diacetate
DOD = death of disease
DTH = delayed-type hypersensitivity

EBV = Epstein-Barr virus
EPC = endothelial progenitor cell

FBS = fetal bovine serum
FITC = fluorescein isothiocyanate
FoxP3 = forkhead box P3
FSC = forward scatter

GITR = glucocorticoid-induced TNF-receptor-related-protein
GM-CSF = granulocyte /macrophage colony stimulating factor
G-CSF = granulocyte colony stimulating factor

HGB = hemoglobin
HIF = hypoxia-inducible factor
HLA = human leukocyte antigen
HRP = horseradish peroxidase

IDO = indoleamine 2,3-dioxygenase
IFN = interferon
Ig = immunogloblin
IL = interleukin
iMC = immature myeloid cells

JAK = Janus kinase

KLH = keyhole limpet hemocyanin

LAG-3 = lymphocyte activation gene-3
LDH = lactate dehydrogenase
LFA-1 = lymphocyte function-associated antigen 1
LPS = lipopolysaccharide
M-CSF = macrophage colony stimulating factors
MDSC = myeloid-derived suppressor cells
MFI = mean fluorescence intensity
MHC = major histocompatibility complex
MM = malignant mesothelioma
MMP = matrix metallo-proteinase
MO = mononuclear

NK = natural killer
NOS = nitrogen species
NO = nitric oxide

OCT = optimum cutting temperature

PBS = phosphate-buffered saline
PD = progressive disease
PDGF = platelet derived growth factor
PD-L1 = programmed death ligand-1
PE = phycoerythrin
PF = pleural fluid
PGE2 = prostaglandin E2
PLT = platelet count
PMN = poly-morph nuclear
PR = partial response

RECIST = response evaluation criteria in solid tumors
RPMI = Roswell Park Memorial Institute
ROS = reactive oxygen species

SD = stable disease
STAT = signal transducer and activator of transcription
SSC = side scatter

TAA = tumour-associated antigen
TAM = tumour-associated macrophage
TGF = transforming growth factor
Th = T helper
TNF = tumour necrosis factor
Treg = regulatory T cell
TT = tumour tissue

VEGF = vascular endothelial growth factor

WBC = white blood cell count
WT = Wilms tumor

ZA = zoledronic acid

Nederlandse samenvatting

Mesothelioom

Mesothelioom; beter bekent als asbestkanker of borstvlieskanker; is een ernstige vorm van kanker. 80% van de patiënten overlijdt binnen een jaar na vaststellen van de ziekte. De oorzaak van mesothelioom is in veel gevallen te wijten aan asbestblootstelling. Asbest vezels kunnen diep in de longen terecht komen en daar na jaren van irritatie kanker veroorzaken. Mensen die aan mesothelioom lijden presenteren zich vaak bij de arts met klachten van kortademigheid, gewichtsverlies en/of pijn aan de borstkas. De kortademigheid wordt in veel gevallen veroorzaakt door een ophoping van vocht in de borstholte (tussen de longvliezen), die ontstaat als reactie op irritatie door de groeiende kanker. Dit vocht wordt in de meeste gevallen weg gehaald en onderzocht. De aanwezigheid van kankercellen in het vocht (en eventueel biopt-materiaal) in combinatie met asbst blootstelling in het verleden leidt in veel gevallen tot de diagnose mesothelioom.
Hoewel het reeds lange tijd bekent is dat mesothelioom wordt veroorzaakt door de inademing van asbest vezels, heeft het jaren geduurd voordat het gebruik van asbest verboden werd. In Nederland is dit verbod sinds 1993 van kracht, maar toch zijn er nog veel landen waar het gebruik van asbest niet verboden is. In China, Rusland en India en Thailand wordt asbest nog op grote schaal gebruikt. Ook de productie van asbest-bevattende materialen is nog altijd niet gestopt. Canada is een van de landen waar nog actief asbest wordt gewonnen uit asbestmijnen. Vanwege het economisch belang dat Canada hierbij heeft is het delven en het gebruik van asbest daar niet verboden. Dit asbest wordt vervolgens geëxporteerd naar derde wereld landen waar het materiaal gebruikt wordt voor de vervaardiging van asbestcement en golfplaten om huizen mee te bouwen. Ook worden er nog regelmatig grote asbestontdekkingen gedaan in gebouwen en schepen en komt asbest nog geregeld vrij bij brand en sloopwerkzaamheden. Daarnaast is recentelijk gebleken dat de blootstelling aan asbest gevaarlijker is dan voorheen werd verondersteld en dat de regelgeving rondom asbestverwijdering niet strikt nageleefd wordt. De verwachting is dat de incidentie van mesothelioom de komende decennia verder zal stijgen en zijn piek pas na 2025 zal bereiken.
Momenteel is er geen goede behandeling voor mesothelioom. In de meeste gevallen worden patiënten behandeld met chemotherapie en/of operatie, maar beide behandelingen worden slechts gegeven om ziektesymptomen te verlichten en leiden niet tot genezing. Om deze reden is het van groot belang dat er nieuw behandelmethodes ontwikkeld worden voor deze vorm van kanker. Een van de behandelstrategieën waar momenteel veel belangstelling voor is, is immunotherapie.

Dendritische cel-immunotherapie

Immunotherapie heeft als doel de natuurlijke afweerreactie weer op gang te brengen. Dit kan op verschillende manieren, maar een van de methodes die momenteel veel belangstelling trekt is de dendritische cel-immunotherapie.
Dendritische cellen zijn heel goed in staat het afweersysteem op gang te brengen tegen "vreemde stoffen", zoals bacteriën en virussen, maar daarnaast spelen ze ook een belangrijke rol in de vroege detectie van kankercellen. Door fragmenten van kankercellen aan lymfocyten te presenteren, kunnen deze cellen kankercellen herkennen als "vreemde" cellen en ze in een vroeg stadium opruimen. In veel gevallen zal dit er toe leiden dat kankercellen nooit volledig tot een klinisch observeerbare vorm uitgroeien en zullen mensen hier nooit klachten van ondervinden. Maar in sommige gevallen krijgt het lichaam niet alle kankercellen geëlimineerd en kunnen tumoren uitgroeien tot kanker. Dendritische cellen kunnen dan gebruikt worden om de afweerreactie opnieuw op gang te helpen.
Dendritische cellen kunnen in grote hoeveelheden buiten het lichaam gekweekt worden uit witte bloedcellen (monocyten). Deze cellen kunnen onder optimale omstandigheden in kontakt gebracht worden met gefragmenteerde kankercellen (lysaat). Dit lysaat kan door dendritische cellen opgenomen worden, wat dendritische cellen in staat stelt een

specifieke afweer reactie te veroorzaken.

Dendritische cel-immunotherapie in mesothelioom patiënten

Nadat gebleken was dat immunotherapie met dendritische cellen tot goede resultaten leidde in een muismodel voor mesothelioom, zijn we in 2005 begonnen met een klinische studie waarin tien mesothelioom patiënten behandeld zijn met dendritische cellen opgeladen met autoloog tumor lysaat na chemotherapie (8-10 weken na de laatste chemotherapie). Uit deze studie is gebleken dat het veilig is patiënten op deze manier te behandelen. Er traden geen onverwachte bijwerkingen op en er waren geen tekenen van auto-immuniteit. Daarnaast was er na drie vaccinaties in alle patiënten een afweerreactie meetbaar in het bloed tegen het model-antigeen keyhole limpet hemocyanin (KLH). KLH is een antigeen waar mensen van nature niet mee in aanraking komen en daarom hebben mensen geen antilichamen tegen dit antigen in hun bloed. KLH wordt tijdens de dendritische cel kweek samen met het lysaat aan de dendritische cellen toegevoegd. Op deze manier kunnen dendritische cellen dus een afweerreactie teweeg brengen tegen KLH. Hieruit is af te leiden dat de dendritische cellen functioneel waren en na teruggave instaat zijn gebleken lymfocyten te activeren. Daarnaast werd er in 4 van de 6 geteste patiënten een directe afweerreactie tegen kankercellen gevonden en was er bij enkele patiënten een verkleining van de tumormassa zichtbaar op CT-scans.
Ondanks dat deze resultaten erg hoopgevend waren, waren er aanwijzingen dat het noodzakelijk was de therapie te verfijnen. Een van de belangrijkste oorzaken die genoemd worden voor de tegenvallende therapeutische effecten van immunotherapie is de aanwezigheid van immuunsuppressieve cellen in patiënten met uitgebreide kwaadaardige ziekten. Om immunotherapie in een klinische studie te kunnen verbeteren is er gezocht naar medicatie die te combineren is met immunotherapie en tegelijkertijd het afweersysteem zo kan beïnvloeden dat de functie en het aantal immuun suppressieve cellen afneemt. De effectiviteit van deze medicijnen in combinatie met immunotherapie werd eerst onderzocht in een preklinisch model.

Er spelen drie celtypes een belangrijke rol in het immuunsuppressieve milieu rond kanker; myeloide suppressor cellen (Myeloid-derived suppressor cells; MDSC), tumor-geassocieerde macrofagen (TAMs) en regulatoire T lymfocyten (Tregs).

Myeloide suppressor cellen

Myeloide suppressor cellen (MDSC) komen onder normale omstandigheden niet in het lichaam voor, maar in het geval van stress (infectie, inflammatie, letsel of kankergroei) zijn ze in de lichaamscirculatie te vinden. Deze cellen komen uit de myeloide bloed lijn, dat wil zeggen dat deze witte bloedcellen ontstaan uit een voorlopercel die myeloblast heet. Uit dezelfde lijn komen ook de macrofagen en dendritische cellen voort. In het geval van stress komen deze myeloide cellen in de lichaamscirculatie. Hier kunnen ze de afweerreactie onderdrukken. Vanwege deze eigenschap zijn deze cellen beter bekend als MDSC.
MDSC kunnen de afweerreactie tegen kankercellen op verschillende manieren remmen. Ze kunnen dit doen rond de tumor en in de lymfeklier. Hier produceren ze een zuurstof radicaal dat er voor zorgt dat de afweercellen die in actie gekomen zijn tegen de kankercellen beschadigd raken en dood gaan. In de lymfeklieren kunnen MDSC de afweerreactie belemmeren door een zuurstof radicaal te maken die er voor zorgt dat de witte bloedcellen; die geprogrammeerd zijn om kankercellen aan te vallen; hun programmering verliezen. Hierdoor worden kankercellen niet meer door het afweersysteem herkend en dooft de afweerreactie tegen kankercellen uit. Naast hun suppressieve rol hebben MDSC ook het vermogen om; vanuit hun onrijpe karakter; verder uit te rijpen naar macrofagen die een belangrijke rol hebben in de progressie van kanker.

Voor de productie van radicalen zijn MDSC afhankelijk van prostaglandinen (prostaglandinen zijn hormoonachtige stoffen die op lokaal niveau betrokken zijn bij het reguleren van fysiologische processen). Prostaglandine E2 dat door kankercellen gemaakt kan worden, beïnvloedt de functie van MDSC het sterkst. In de productie van prostaglandinen is het enzym cyclo-oxygenase-2 (COX-2) van cruciaal belang. De activiteit van dit enzym is te remmen met COX-2 remmers. Om te onderzoeken of behandeling met de selectieve COX-2-remmer; celecoxib; het aantal en de functie van MDSC beïnvloed, werden muizen in het mesothelioom model behandeld met celecoxib in combinatie met immunotherapie. Hieruit blijkt dat de muizen die met de combinatie van celecoxib en immunotherapie behandeld werden, significant langer leefden en dat deze overlevingswinst gepaard ging met een aanhoudende afweerreactie tegen kankercellen. Daarnaast nam het aantal en de functie van MDSC af. MDSC van muizen die met celecoxib behandeld werden, waren minder goed in staat zuurstof radicalen te produceren. De combinatie van celecoxib en immunotherapie lijkt een effectieve manier om immuunsuppressie die veroorzaakt wordt door MDSC is verminderen.

Tumor-geassocieerde macrofagen

Macrofagen hebben een belangrijke taak in het lichaam. In de normale situatie zijn ze betrokken bij de aangeboren afweer tegen ziekteverwekkers (inflammatoire macrofagen). Maar daarnaast zijn er ook macrofagen die betrokken zijn bij "herstel werkzaamheden" (alternatief geactiveerde macrofagen). Deze macrofagen zijn betrokken bij het ombouwen van weefsels. Zij zorgen er voor dat er nieuwe bloedvaten aangelegd worden, ruimen oude cellen (celfragmenten) op en produceren stoffen die andere immuuncellen aantrekken die nodig zijn bij de weefselombouw.
Kankercellen maken van deze eigenschappen van macrofagen gebruik om bloedvaten te generen en weefsel beter toegankelijk te maken voor verdere doorgroei. Daarnaast zorgen de stoffen die macrofagen maken ook voor de aantrekking van MDSC, die vervolgens als bron kunnen dienen voor nieuwe macrofagen. Maar macrofagen kunnen ook de afweer reactie negatief beïnvloeden. Net als MDSC kunnen macrofagen zuurstof radicalen maken die celdood van immuun cellen tot gevolg heeft. Daarnaast produceren ze stoffen die direct een negatieve invloed hebben op de functie van de immuun cellen.
Het is al geruime tijd bekend dat macrofagen een belangrijke rol spelen in het faciliteren van kankergroei. Toch zijn er weinig middelen beschreven die de aantrekking en de functie van tumor-geassocieerde macrofagen remt. Recentelijk is gebleken dan bisfosfonaten de potentie hebben het afweersysteem te modificeren. Bisfosfonaten worden in de dagelijkse praktijk gebruikt ter preventie van botontkalking (osteoporose) en ter preventie van breuken door uitzaaiingen van kwaadaardige ziekten naar het bot. Preventieve behandeling van kanker patiënten met deze middelen leverde nieuwe inzichten op. Uit grote studies bleek dat vroegtijdig starten met bisfosfonaten niet alleen het aantal botbreuken door uitzaaiingen verminderde, maar dat ook het aantal uitzaaiingen afnam. Dit onverwachte effect is moeilijk te verklaren en heeft tot veel discussie geleid. Uit deze discussies kwam als mogelijke verklaring naar voren, dat bisfosfonaten mogelijk een gunstig effect kunnen hebben op het immuunsysteem. In deze discussie is er veel aandacht voor een nieuwe bisfosfonaat; zoledroninezuur (Zometa).
Om het immuun modulerende effect van zoledroninezuur op macrofagen te onderzoeken werden macrofagen gekweekt in het laboratorium. De toevoeging van zoledroninezuur aan deze kweek had een vermindering van het aantal macrofagen tot gevolg, als een consequentie hiervan blijf het aantal immature myeloide cellen hoog. Daarnaast hadden deze macrofagen minder tumor-geassocieerde karakteristieken. Toch leverde de behandeling van muizen in het mesothelioom model met zoledroninezuur niet de gewenste resultaten. Hoewel er ook een afname van het aantal macrofagen detecteerbaar was in muizen die met zoledroninezuur behandeld werden leverde de behandeling geen overlevingswinst op. Er zijn verschillende verklaringen voor deze bevinding. Zo kan het zijn dat de afname in

macrofagen niet opweegt tegen de stijging van de immature myeloide cellen met MDSC kenmerken. Daarnaast kan het ook zo zijn dat de effectiviteit van zoledroninezuur in een muis model minder effectief is vanwege kinetische eigenschappen van het medicament. Uit de experimenten hebben we geconcludeerd dat single therapie met zoledroninezuur geen goede manier is om tumor-geassocieerde macrofagen te beïnvloeden in mesothelioom. Mogelijk is een gecombineerde behandeling van zoledroninezuur met andere medicijnen (zoals bijv. celecoxib) zinvoller. Meer studies zijn nodig om hier duidelijkheid over te verschaffen. Momenteel zijn er weinig alternatieven, om macrofagen te beïnvloeden. Yondelis (Trabectedin) wordt onderzocht maar lijkt geen geschikt middel voor de combinatie met immunotherapie vanwege ernstige bijwerken. Daarnaast wordt er onderzocht of de aantrekking van macrofagen voorkomen kan worden door stoffen weg te vangen die een rol spelen in de stijging van macrofagen.

Regulatoire T cellen

Het derde celtype die een belangrijke functie vervuld in de immuun escape zijn regulatoire T cellen (Tregs). Tregs komen normaal gesproken in een beperkte hoeveelheid in het lichaam voor. Ze zorgen er voor dat het afweersysteem niet opgang komt tegen lichaamseigen antigenen. Zo voorkomen ze auto-immuun ziekten en reguleren ze de immuunresponse.
Tregs komen verhoogd voor in het bloed, de lymfklieren en rond de tumor bij mesothelioom patiënten. Deze verhoging heeft een ongunstig effect op de afweerreactie en daarom lijkt het reduceren van deze cellen een goede strategie om immunotherapie te verbeteren. In 2006 heeft Hegmans *et al.* beschreven dat de behandeling met PC61 een verlaging van het aantal Tregs gaf en dat deze verlaging een verbetering van de overleving tot gevolg had in een muis model voor mesothelioom. PC61 is een antilichaam dat bindt aan CD25, wat onderdeel is van de interleukine 2 receptor. Deze receptor komt voor op Tregs, maar daarnaast ook op geactiveerde B en T lymfocyten. De behandeling met dit depleterend antilichaam leverde een vermindering van het aantal Tregs op en een verbetering van de overleving. Maar omdat PC61 ook geactiveerde T en B lymfocyten kan verminderen en deze cellen juist nodig zijn om de kanker te bevechten, leek de combinatie van PC61 en immunotherapie niet geschikt. Daarnaast zijn de studies die met de humane variant van PC61 gedaan zijn niet zinvol gebleken. Daarom hebben we gezocht naar een middel dat Tregs kan verminderen en gebruikt kan worden voor klinische doeleinden (om de stap naar een eventuele klinische studie mogelijk te maken). Een middel wat hiervoor geschikt lijkt is cyclofosfamide (CTX [Endoxan®]).
Cyclofosfamide is een "pro-drug" met een alkalyserende werking. Het middel heeft een therapeutische toepassing als chemotherapeuticum in de behandeling van lymfomen, maar daarnaast wordt het tegenwoordig ook in lage dosering toegepast bij de behandeling van auto-immuunziekten. Hoewel het werkingsmechanisme niet goed bekent is, lijkt het middel een immuunmodulerende werking te hebben en in een lage dosis specifiek Tregs te kunnen verlagen. Om deze reden hebben we onderzocht of de behandeling van muizen met CTX in combinatie met immunotherapie zinvol was. Muizen werden behandeld met CTX in verschillende doseringsschema's. Hieruit bleek dat continue behandeling met CTX in een lage dosis in combinatie met immunotherapie de beste resultaten liet zien. Het aantal Tregs nam significant af en de overleving verbeterde.

Dit heeft er toe geleid dat we begin 2010 gestart zijn met een nieuwe klinische studie waarin mesothelioom patiënten nu behandeld worden met de combinatie van immunotherapie en een lage continue dosis van cyclofosfamide. We hopen deze studie eind 2011 af te ronden. In de tussen tijd zullen we onderzoeken of het mogelijk is om patiënten beter te karakteriseren op basis van immuunparameters om te bepalen welke combinatie van medicijnen in de toekomst het beste gegeven kunnen worden in combinatie met immunotherapie voor de behandeling van individuele patiënten. Daarnaast zullen we onderzoeken of het mogelijk is patiënt eigen dendritische cellen op te laden met lysaat van

mesothelioom cellijnen (mesothelioom cellen die in het laboratorium gekweekt kunnen worden), om gerandomiseerde klinische studies met grotere groepen patiënten mogelijk te maken. Op deze manier kan de werkzaamheid beter onderzocht worden en zullen in de verre toekomst mogelijk risicogroepen preventief ingeënt kunnen worden tegen deze vorm van kanker.

About the author

Curriculum Vitae

The author of this thesis was born on March 21st, 1982 in Dordrecht (the Netherlands). In 2001 he finished high school (VWO) at the Stedelijk Dalton Lyceum in Dordrecht. The same year he started his study at the Erasmus University Faculty of Medicine in Rotterdam (the Netherlands). During his study he participated in research at the laboratory of experimental pathology at Josephine Nefkens Institute, Erasmus MC (supervised by dr. H. Stoop and Prof.dr. L.H.J. Looijenga). After having obtained his medical degree in 2007, he started his PhD research at the department of Pulmonary Medicine at the Erasmus MC (supervised by dr. J.P.J.J. Hegmans, dr. J.G.V.G. Aerts), resulting in this thesis. In January 2011 he will start his medical specialist training in Pulmonary Medicine at the Erasmus MC in Rotterdam (Head of department; Prof.dr. H.C. Hoogsteden).

List of publications

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Lambers ME, **Veltman JD**, Hendriks RW, Hoogsteden HC, Aerts JG, Hegmans JP.
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Hegmans JPJJ, **Veltman JD**, Lambers ME, de Vries IJ, Figdor CG, Hendriks RW, Hoogsteden HC, Lambrecht BN, Aerts JGJV.
Dendritic cell-based immunotherapy elicits cytotoxicity in malignant mesothelioma patiens
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PhD Portfolio

Johannes Dirk Veltman
Department of Pulmonary Medicine
Research School : Molecular Medicine Post-graduate School
PhD period : 2007-2010
Promotor : Prof.dr. H.C. Hoogsteden

In-depth courses

2009	Biomedical English Writing and Communication
2008	Molecular immunology
2008	Laboratory animal science (art. 12)
2007	Good Clinical Practice – Consultatiecentrum Patiëntgebonden onderzoek (CPO)
2007	Clean-room-behaviour course – Vereniging Contamination Control Nederland (VCCN)
2007-present	International seminar series in Immunology, Cell Biology and Molecular Medicine, provided by the Post-Graduate School Molecular Medicine, Erasmus MC
2007-present	Weekly internal and external presentations at the department of Pulmonary Medicine

(Inter)national scientific presentations

2010	Asbestslachtoffers Vereniging Nederland; najaars-meeting (oral)
2010	Heineken-Masterclass door prof. dr. Ralph Steinman: Ontwikkelingen in biologie en medische toepassing van dendritische cellen
2010	Rotterdam-Gent pulmonology meeting (oral)
2010	Asbestslachtoffers Vereniging Nederland; voorjaars-meeting (oral)
2010	NRS – 2nd Springmeeting (poster)
2009	Pulmonary Pathology Club 57th Meeting (oral)
2009	Art of Pulmonary Medicine – A common sense to lung disease
2009	NVVI – Dutch association of Immunology, Noordwijkerhout, the Netherlands (poster)
2009	Rotary Club Dordrecht (oral)
2009	Molecular Medicine Day Erasmus MC Rotterdam, the Netherlands (poster)
2008	The 9th International Conference of The International Mesothelioma Interest Group (poster)
2008	NVVI – Dutch association of Immunology, Noordwijkerhout, the Netherlands (poster)
2008	Molecular Medicine Day Erasmus MC Rotterdam, the Netherlands (poster)
2007	NVVI – Dutch association of Immunology, Noordwijkerhout, the Netherlands (poster)

Student coaching / Teaching

2010	Pulmonary medicine research course; first-grade medical students Erasmus faculty of medicine
2010	Journal Club courses; first-grade medical students Erasmus faculty of

	medicine
2010	"Try-out" study courses; fifth- and sixth-grade VWO-students
2010	Assisting Molecular medicine student; University Erasmus MC
2009	Supervising Masters-thesis; D.C. de Jong & J. Lammering; Pharmacology University of Utrecht
2009	Journal Club courses; first-grade medical students Erasmus faculty of medicine
2009	"Try-out" study courses; fifth- and sixth-grade VWO-students

Media Events

2010	Monitor; 01-09: Trainingskamp tegen asbestkanker (Magazine)
2010	Algemeen Dagblad; 13-04: Artsen geven patiënten met asbestkanker hoop (Newspaper)
2010	Spits; 13-04: Veelbelovende behandeling asbestkanker (Newspaper)
2010	BNdeStem; 13-04: Bredase arts ontwikkelt vaccin tegen asbestkanker (Newspaper)
2010	RTVrijnmond; 12-04: Vaccin tegen asbestkanker in de maak (Television/Radio)
2010	Telegraaf/Volkskrant/Algemeen Dagblad/Parool/Trouw; 12-04: Vaccin in de maak tegen asbestkanker (Newspapers)
2010	Clydebank post 07-04: Asbestos cancer destroyer; drug trail success gives hope for cure (Newspaper)
2010	Weekblad Party; 02-03: Rel rond asbest-Aak Beatrix (Tabloid)
2008	RTV Oost; 27-08: Nico Heijmer, asbestkankerpatiënt roept overheid ter verantwoording (Television/Radio)
2008	Telegraaf; 01-04: Opgegeven kankerpatient op 'bedevaart' voor medisch onderzoek (Newspaper)

Collaborations

2010-present	Department of Pathology; Prof.dr. M. den Bakker; The role of immune suppressive cells in tumour negative lymph nodes in lung cancer
2009-present	Department of Pharmacology; Prof.dr. A. Vulto and drs. A. de Goede; Preparation of CCMO-request and release permission of clinical grade vaccines (Phase I/II clinical trail NL24050.000.08)
2009-present	Department of Cell biology; G.J. Schaaf; determination of CD24-function on tumour cells

Dankwoord

Na een periode van 3 jaar sluit ik mijn onderzoek af met dit proefschrift. De afgelopen jaren heb ik met veel plezier gewerkt aan dit proefschrift, maar zonder de inzet en hulp van vele mensen was dit niet tot stand gekomen. Ik wil dan ook van de gelegenheid gebruik maken om een aantal mensen te bedanken voor al hun steun, hulp en aanmoediging.

Prof. dr. H.C. Hoogsteden: beste Henk, ons contact gaat verder terug dan 3 jaar. Toen ik startte met mijn coschappen was de afdeling longziekten van het Erasmus MC mijn eerste contact met het ziekenhuis. De sfeer op de afdeling sprak me direct aan en ik ben vanaf dat moment contact met de afdeling blijven houden. Ik wil je graag bedanken voor de mogelijkheid die je mij geboden hebt om te promoveren op dit boeiende onderwerp. Je opbouwende commentaren zijn van grote waarde geweest voor mij persoonlijk, en niet in de laatste plaats voor het uiteindelijke wetenschappelijke resultaat. Ook in de toekomst zie ik er naar uit om een deel van mijn opleiding binnen jouw afdeling te kunnen volgen.

Dr. R. Hendriks: beste Rudi, ik was nog maar net met mijn onderzoek gestart toen jij als opvolger van Bart aangesteld werd. Onder jouw leiding zijn er veel nieuwe contacten ontstaan tussen collega's en is de sfeer op de afdeling steeds beter geworden. Ook waardeer ik je motivatie om nieuwe onderzoeksgebieden te verkennen. Hierin ben je erg veelzijdig gebleken. Daarnaast wil ik je bedanken voor alle steun die je mij geboden hebt bij het schrijven van dit proefschrift.

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Dr. J.G.J.V. Aerts: beste Joachim, ik kan mij onze eerste ontmoeting nog goed herinneren; ik was als co-assistent aanwezig op een van de regionale refereeravonden en er werd mij verteld dat er een gedreven longarts uit het Sint Franciscus Gasthuis zijn "baanbrekende" onderzoeksresultaten kwam tonen; dat was jij dus. Niets is minder waar gebleken. De manier waarop jij onderzoek combineert met klinisch werk is ongeëvenaard. Ook het tempo dat je in het onderzoek weet te houden is bewonderenswaardig. Dit, in combinatie met je motiverende kritieken, maakt je tot een co-promotor die veel promovendi zich zouden wensen.

De overige leden van de kleine en grote commissie: Prof. dr. B.N.M. Lambrecht, Prof. dr. L.H.J. Looijenga, Prof. dr. M. den Bakker, Prof. dr. C. Figdor en Prof. dr. J. van Meerbeeck wil ik bedanken voor het beoordelen van mijn proefschrift en het plaatsnemen in de commissie.

Mijn paranimfen: best Menno, hoewel we weinig experimenten samen gedaan hebben, ben je toch ook van doorslaggevend belang geweest bij de voltooiing van dit proefschrift. Promoveren op een lab valt niet altijd mee en daarom is steun vaak hard nodig. Als je die kan vinden bij iemand die op alle fronten eenzelfde kijk op de dingen lijkt te hebben, is dat een geluk dat je niet vaak tegen komt. Ik ben zelden iemand tegen gekomen die zo veel interesses heeft op allerlei gebieden en daar ook nog iets zinnigs over kan zeggen. Naast een goede collega ben je ook een goede vriend geworden.
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Rotterdam 8 December

Thierry Veltman
Harbour of Rotterdam 2010

Het schilderij op de omslag van dit proefschrift is gemaakt door Thierry Veltman. Het werk verbeeldt het uitzicht op de Rotterdamse haven vanaf de 22ste verdieping van het faculteitsgebouw van het Erasmus MC, waar het laboratorium Longziekten gehuisvest is. Voor dit beeld is gekozen, omdat de haven in belangrijke rol heeft gespeeld bij de initiatie van het onderzoek naar asbestkanker in het Erasmus MC. In de jaren 60/70 is er veel scheepsbouw activiteit geweest in dit havengebied. Bij het bouwen van deze schepen is veel asbest gebruikt en zijn veel mensen aan deze kankerverwekkende stof blootgesteld gweest. Dit heeft er toe geleid dat de incidentie van het aantal mesothelioom patiënten in Rotterdam ver boven het landelijk gemiddelde ligt. Daarom is er in het verleden het initiatief genomen om een onderzoeksgroep op te richten die hun aandacht richten op het bevordering van de diagnostiek en behandeling van mesothelioom. Daartoe is in 1998 de Stichting Asbestkanker opgericht. De Stichting Asbestkanker werft fondsen die ten goede komen aan onderzoek waarmee versneld therapieën beschikbaar komen in de kliniek voor een betere behandeling van asbestkanker. Zonder hun financiële steun was het onderzoek niet mogelijk geweest. **www.asbestkanker.com**