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Immuno-Oncology and Immunotherapy

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1. Introduction

The route that mesothelial cells take on their way to becoming malignant is unknown and probably highly variable depending on several environmental and host factors, including polymorphisms and mutations in susceptibility genes, age and immunity. Links between cancer and inflammation were first noted by Rudolf Virchow in 1863, on the basis of observations that tumours often arose at sites of chronic inflammation and that inflammatory cells were present in biopsy samples from tumours (Balkwill & Mantovani 2001). In a SCID mouse xenograft model, it has recently been shown that inflammation precedes the development of human malignant mesothelioma (Hillegass e.a. 2010). Also, epidemiological studies have revealed that chronic inflammation caused by chemical and physical agents, autoimmune and by inflammatory reactions of uncertain aetiology, predisposes for certain forms of cancer (Coussens & Werb 2002). Increasing evidence indicates that the "inflammation-cancer" connection is not only restricted to the initiation of the cancer process, since all types of clinically manifested cancers appear to have an active inflammatory component in their microenvironment. These experimental findings and clinical observations have led to cancer–related inflammation being acknowledged as one of the hallmarks of cancer (Colotta e.a. 2009).

2. Cancer-related immunology

2.1 Tumour-immunosurveillance

By investigating murine tumour transplantation models, Llyod Old, George Klein, and others showed that the immune system of healthy recipient mice was able to differentiate transformed malignant cells from normal cells (Old & Boyse 1964; Klein e.a. 1966). Even preceding these publications, Frank MacFarlane Burnet and Lewis Thomas formulated their cancer immunosurveillance hypothesis: "It is by no means inconceivable that small accumulations of 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" (Burnet 1957). At that time this hypothesis was controversial, however, with the current knowledge and ongoing research, it's apparent their premise seems to be correct because there is strong evidence from animal studies that cells of the adaptive immune system carry out surveillance and can eliminate nascent tumours, a process called immuno-editing (Dunn e.a. 2004).

Tumour-associated antigens (TAA) are antigens acquired by tumour cells 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 over expression of these abnormal proteins. The immune system can discriminate between malignant cells and their normal counterparts through recognition of these TAA. It is known that several immunological cell types are involved in the recognition and destruction of tumours during early stages of development. These include cells and factors of the innate immune system, including macrophages, neutrophils, complement components, γδ T cells, natural killer (NK) cells, NKT cells and certain cytokines (IL-12, IFN-γ) and cells of the adaptive immune system, including B lymphocytes, helper T cells (Th cells) and cytotoxic T lymphocytes (CTLs).

TAA need to be presented to the cells of the adaptive immune system. Dendritic cells (DCs) are widely acknowledged for their potent antigen presenting capacity and play a key role in the initiation of this adaptive immune response by activation and modulation of lymphocyte subsets (Steinman e.a. 1983). DCs originate from bone marrow precursor cells and are found at low frequencies in peripheral tissues where they maintain an immature phenotype and search their surroundings for foreign substances. Immunogenic TAA are secreted or shed by tumour cells or released when tumour cells die. When TAA are taken up by DCs or other antigen presenting cells (APCs), cells mature and migrate to regional draining lymphoid organs. The captured antigen is processed and presented by major histocompatibility complex (MHC) class I and class II molecules on their cell membrane leading to the activation of antigen-specific lymphocytes. This results in antibody production by B lymphocytes and tumour-specific CTLs to assist the innate immune responses in the killing of tumour cells.

2.2 Tumour immune escape

Increasing evidence reveals that when tumour progress in time, tumour cells undergo changes to escape immune surveillance. 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 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. During this period, tumour cells undergo mutations caused by their genetic instability; potentially generating variants that can escape the immune system, by either evading the induction of an immune response or by inhibiting anti-tumour responses via a variety of mechanisms.

2.3 Immune suppressive mechanisms

The induction of an immune suppressive tumour microenvironment is an important escape mechanism how tumours can resist immune destruction. In this microenvironment, inflammatory cells and molecules have a major influence on cancer progress. Effective adaptive immune responses are suppressed through the activation of several pathways. For example, the differentiation and activation of dendritic cells, which are the key initiators of adaptive immune responses, are inhibited by signals (such as IL-10 and VEGF) present in the tumour microenvironment. In addition, tumours but also peripheral blood and lymph

nodes contain regulatory T cells (Tregs), which suppress both the adaptive and innate immune responses. Also, a heterogeneous population of myeloid-derived suppressor cells (MDSCs) are induced in tumour-bearing hosts; these cells, as well as conventional tumour-associated macrophages (TAMs), are potent suppressors of antitumour immunity. Not only do MDSCs and TAMs suppress the antitumour response, they also assist the malignant behaviour of tumour cells by secreting cytokines, growth factors, matrix-degrading enzymes and proteases, which promote tumour progression or enhance metastasis.

In conclusion, 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 (Mantovani e.a. 2008). In the following sections, the presence and functions of MDSCs, TAMs and Tregs are discussed.

2.3.1 Myeloid-derived suppressor cells

MDSCs are a heterogeneous population of bone marrow-derived myeloid cells, comprising of immature monocytes/macrophages, granulocytes, and DCs at different stages of differentiation (Gabrilovich e.a. 2007). A subset of MDSCs, mononuclear MDSCs (MO-MDSCs) is mainly found at the tumour site while polymorph nuclear MDSCs (PMN-MDSCs) subset is found in blood, lymphoid organs and at the tumour site. They express a number of surface markers, that are on themselves not unique but in combination can define MDSCs. MDSCs are increased in cancer patients and it is anticipated that MDSCs play a suppressive role during the innate and adaptive immune responses to cancer, but have also been described in the course of other pathologic processes such as thermal injury, various infectious diseases, sepsis, trauma, after bone marrow transplantation and in some autoimmune disorders.

Activation of MDSCs not only requires tumour-derived factors (e.g. tumour-derived prostaglandin E2 (PGE2)), 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). Activation of cytokine receptors on MDSCs leads to activation of STAT-signalling pathways, resulting in the production of immune suppressive substances (like TGF-β, ROS and NOS).

MDSCs inhibit the immune response in several ways;

- MDSCs are capable of producing reactive oxygen species (ROS) and peroxynitrite, which is responsible for most of the adverse effects on T cells, linked to ROS. Changes caused by nitration of the T cell receptor makes T cells incapable of interacting with the MHC complex on antigen presenting cells, which is necessary to obtain T cell specific stimulation (Nagaraj & Gabrilovich 2007; Kusmartsev e.a. 2004).
- MDSCs can inhibit the anti-tumour response in an antigen non-specific manner by the high expression of the enzyme inducible nitric oxid synthetase (iNOS), leading to the generation of NO. NO can suppress T cell function though various mechanisms including the inhibition of the cell signalling pathways and inducing DNA-damage to T cells.
- Arginase-I activity by MDSCs depletes L-arginine from the environment, contributing to the induction of T cell tolerance by the downregulation of the CD3ζ-chain expression of the T cell receptor (Bronte e.a. 2003; Rodríguez & Ochoa 2008).

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- MDSCs block T-cell activation by sequestering cystine and thus limiting the availability of the essential amino acid cysteine (Srivastava e.a. 2010).
- MDSCs can inhibit T cell proliferation by producing IL-10 and TGF-β (Hequan Li e.a. 2009).
- Anti-tumour cells, like NK- and NKT-cells, can be inhibited by MDSCs via TGF-β1 depending mechanisms. MDSCs 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 (Hequan Li e.a. 2009).
- The plasma membrane expression of enzyme ADAM17 on MDSCs cleaves L-selectin on naïve T cells, decreasing their ability to home to sites where they could be activated (Hanson e.a. 2009).
- MDSCs can indirectly enhance immune suppression via the induction of Tregs (Huang e.a. 2006; Pan e.a. 2010; Kusmartsev & Gabrilovich 2006).
- MDSCs differentiate under certain biological conditions into mature functionally competent macrophages or to DCs influencing tumoural responses (Gabrilovich & Nagaraj 2009).

2.3.2 Tumour-associated macrophages

Macrophages are a major component of the leukocyte infiltrate in the tumour micro-environment (Mantovani e.a. 2002). Classically activated (M1) macrophages, following exposure to IFN-γ, have anti-tumour and tissue destructive activity. In response to IL-4 or IL-13, macrophages undergo alternative (M2) activation. M2 macrophages are oriented to tissue repair, tissue remodelling and immunoregulation. 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 (Sica e.a. 2006).

TAM recruitment in tumours is mediated by several cytokines including colony stimulating factor-1 (CSF-1), vascular endothelial growth factor (VEGF) and chemokines (like CCL2) (Mantovani & Sica 2010). It has been shown that MO-MDSCs are capable of differentiating towards TAMs. Therefore, similar recruitment factors are described that contribute to the infiltration of TAMs and MDSCs into tumour tissue (Mantovani & Sica 2010).

In addition, dynamic changes of the tumour microenvironment occur during the transition from early neoplastic events toward advanced tumour stages resulting in local hypoxia, low glucose level and low pH. These events drive the switch from a M1 macrophage toward the M2 type by profound changes occurring in the tumour microphysiology.

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

- TAMS are able to produce immune suppressive cytokines, like CCL17, CCL18, CCL22, IL-1β, IL-6, IL-10 and TGF-β. IL-10 in combination with IL-6 can lead to upregulation of molecules in TAMs, which are implicated in suppression of tumour-specific T cell immunity (Kryczek e.a. 2006).
- TAMs express the enzyme indoleamine 2,3-dioxygenase (IDO), a well-known suppressor of T cell activation. IDO catalyzes the catabolism of tryptophan, an essential amino acid acquired for T cell activation (Grohmann e.a. 2003).

- TAMs contribute to immune suppression via indirect ways. Secretion of CCL18 leads to recruitment of native T cells. Attraction of naive T cells into the tumour microenvironment is likely to induce T cell anergy (Balkwill 2004). Besides CCL18, CCL17 and CCL22 are abundantly expressed. These cytokines interact with CCR4 receptor, expressed by Tregs and induces T-helper 2 polarization (Bonecchi e.a. 1998). Via expression of VEGF, TAMs can block antigen uptake by APCs and attract MDSCs, which can function as TAM precursors but are also actively suppressing T cell function. MDSCs are depending on prostaglandin E2 (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 (Nagaraj & Gabrilovich 2008).
- In tumour stroma, TAMs produce matrix metalloproteases (MMPs) and other proteases, leading to degradation of the extracellular matrix. During this process several cytokines, chemokines and growth factors are released from the matrix that promotes and facilitates endothelial cell survival and migration and thereby enhances angiogenesis (Mantovani e.a. 2006).
- Besides indirect mechanisms, angiogenesis is also directly stimulated by TAMs. TAMs can produce proangiogenic factors like VEGF and platelet derived growth factors (PDGF). The release of these factors leads to the formation of (lymph)angiogenic structures and subsequent metastasis (Strieter e.a. 2004).

2.3.3 Regulatory T cells

Tregs are a population of CD4+ T cells with a central role in the prevention of autoimmunity and the promotion of tolerance via their suppressive function on a broad repertoire of cellular targets (Baecher-Allan & Hafler 2005). 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 (Larmonier e.a. 2007). 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 (Ormandy e.a. 2005). Adaptive Tregs arise from naive T cells and are triggered by suboptimal antigen stimulation and stimulation with TGF-β. Adaptive Tregs can be subdivided into IL-10 secreting Tregs type I (Tr1) 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 (Wei e.a. 2006).

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 (H Jonuleit e.a. 2000). Elevated levels of Tregs have been identified in blood of cancer patients compared with normal individuals and their presence predicts for poor survival (Apostolou e.a. 2008). In mesothelioma patients, elevated levels of Tregs have also been identified in pleural fluid, with a clear patient to patient variability (DeLong e.a. 2005).

Natural Tregs are derived in the thymus and migrate into the periphery. 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). 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 (Strauss e.a. 2007). Although exact mechanisms are not fully explored, it has been shown that CCR4+ (receptor for CCL22) Tregs migrate toward tumour microenvironments expressing CCL22 (Sakaguchi e.a. 2009). Also CD62L and CCR7 have been described as important homing markers on Tregs (Gondek e.a. 2005). 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) (Nakamura e.a. 2001).

As MDCSs and TAMs, Tregs have several pathways that diminish immune responses to tumour tissue:

- Direct cell-cell interaction between Tregs and target cells is important for tolerance induction by Tregs (Thornton & Shevach 1998). These target cells include CD4+ and CD8+ effector cells, B cells, NK T cells, DCs and monocytes/macrophages. The cell-cell binding leads to apoptosis by activation of programmed cell death-ligands (PDL), the release of perforin (Boissonnas e.a. 2010) and granzyme-B (Nagaraj & Gabrilovich 2007) and by reducing the proliferation through upregulation of intracellular cyclic AMP (Fassbender e.a. 2010; Bopp e.a. 2007).
- Tregs produce themselves or induce other cells to secrete immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β to blunt immune responses (Hawrylowicz & O'Garra 2005), but also other molecules produced by Tregs like carbon monoxide (Lee e.a. 2007) and galectins (Garín e.a. 2007) are reported to play roles in suppression.
- Tregs can inhibit antitumour effector NK and NK T cells via membrane bound TGF-β (Ghiringhelli e.a. 2005). The binding of membrane-bound TGF-β on Tregs to the TGF-β-receptor on target cells leads to the activation of intracellular pathways, which eventually leads to the down regulation of the NKG2D- receptor on NK and NK T cells.
- Tregs are forming aggregates around DCs to prevent contact between DCs and T cells and in this way disturb the induction of the adaptive immune response by preventing proper antigen presentation (Onishi e.a. 2008; Tadokoro e.a. 2006).
- CTLA4+ Tregs induce the expression of indoleamine 2,3-dioxygenase (IDO) in APCs reducing the essential amino acid tryptophan to kynurenine, which is toxic to neighbouring T cells (Fallarino e.a. 2003).
- Treg aggregation leads to decreased upregulation of CD80 and CD86 on immature DCs and down regulate the expression of CD80 and CD86 on mature DCs (Oderup

e.a. 2006). These phenomena are antigen specific and dependent on lymphocyte function-associated antigen 1 (LFA-1) and CTL-associated protein 4 (CTLA-4) (Rooney e.a. 1998).

• Tregs induce B7-H4 expression by APCs, a member of the B7 family that negatively regulates T-cell responses (Kryczek e.a. 2006).

• Activated Tregs, which express higher affinity IL-2R than conventional T cells, may absorb IL-2 from the microenvironment (de la Rosa e.a. 2004).

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

2.4 Conclusion

In short, MDSCs, TAMs and Tregs are capable of suppressing the anti-tumour response with a variety of mechanisms and contribute to a complex interplay between cells that act on behalf or against the tumour. These cells have an essential role in tumor growth or destruction of tumour cells, as pictures in figure 1.
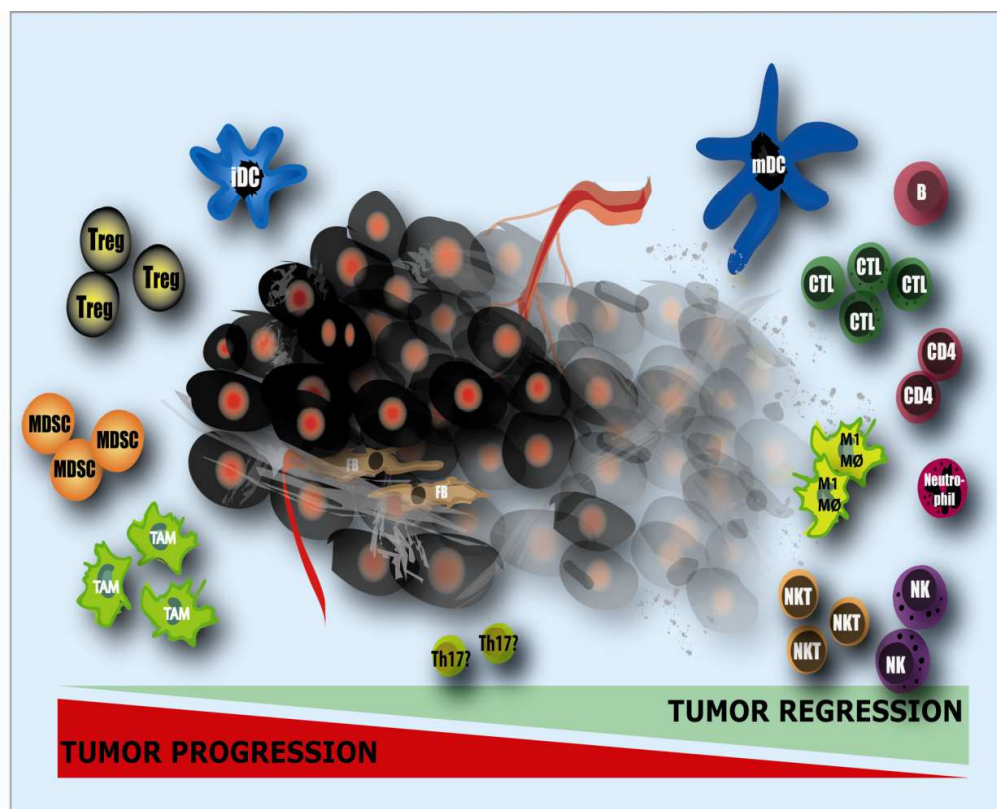

Fig. 1. Interplay between immunological cells that inhibit tumour growth on the right of the tumour and cells that aid in tumour progression on the left. (Tumour is depicted as black cells with a red nucleus in the middle). iDC = immature dendritic cell, mDC = mature dendritic cell, Th17 = helper T lymphocyte 17, M1 MØ= M1 macrophage, FB = fibroblast, B = B cell lymphocyte.

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3. Immunotherapy

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

3.1 Passive immunotherapy

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

In this field, ipilimumab is an interesting newcomer, currently tested mainly in metastatic melanoma. Ipilimumab is a monoclonal antibody against cytotoxic T-lymphocyte antigen (CTLA)-4. It is normally expressed at low levels on the surface of naïve effector T cells, but is upregulated on the cell surface when there is a long-lasting and strong stimulus via the T cell receptor (TCR). CTLA-4 then competes with CD28 for CD80/CD86 on APCs, effectively shutting off TCR signalling and thereby serves as a physiologic "brake" on the activated immune system. Ipilimumab can thus prevent this feedback inhibition, resulting in an unabated immune response against the tumour. The side effects of this therapy, however, can be significant due to the downregulation of tolerance to patient's own normal tissue and colitis is often seen in patients.

Another method of passive immunotherapy uses adaptive transfer of antigen specific effector cells (like T cells and NK cells) that 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. This led to the concept that antigen specific T cell transfer can be used as an anti-tumour therapy to eradicate established tumours. The approach of adaptive T cell transfer to eradicate malignancies is challenging.

3.2 Active immunotherapy

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

Several cytokines are capable of activating and recruiting specific immune cells that can enhance anti-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.

Defined TAA epitopes have been used to vaccinate cancer patients; however this approach is limited by the relatively low number of identified specific epitopes and by the requirement of MHC typing. Nevertheless, some authors have reported the applicability of this approach. By using the whole TAA protein for immunization, the need of peptide identification can be circumvented. These proteins can be taken up by APCs and endogenously processed into epitopes for presentation to T cells. Adjuvants need to be added to induce APCs activation and avoid tolerance induction (Berger e.a. 2005).

DNA sequences coding for specific TAAs can be directly injected into the skin. DNA then needs to be taken up, transcribed into mRNA, translated into a protein and processed into peptides by APCs. An important restriction is the relatively inefficient delivery into APCs. Viruses engineered to express TAAs 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 TAAs, resulting in a strong antivirus response leading to virus eradication and attenuation of the anti-tumour immune response.

The ideal source of TAAs is the tumour itself, since it expresses all the TAAs 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.

Sipuleucel-T is an active cellular immunotherapy consisting of autologous peripheral-blood mononuclear cells (PBMCs), including APCs. Recently, Kantoff *et al.* published a phase III trail where they used *ex vivo* activated Sipuleucel-T with a recombinant fusion protein (PA2024). PA2024 consists of a prostate antigen, prostatic acid phosphatase, that is fused to granulocyte–macrophage colony-stimulating factor (GM-CSF), an immune-cell activator. Sipuleucel-T prolonged survival among men with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (Kantoff e.a. 2010).

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 DCs to therapeutic cancer vaccines has been prompted (Banchereau & Palucka 2005). DCs can be generated in large amounts *ex vivo*, and pulsed with tumour antigens under optimal conditions. Subsequently, the injection of matured tumour antigen-pulsed DCs led to the induction of an anti-tumour response in murine models as well as in patients (Hegmans e.a. 2005). Moreover, DC activation also induces the formation of antibodies against tumour components. 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 and clinical studies.

4. DC-based immunotherapy in mesothelioma

We previously investigated the effect of DC-based immunotherapy on the outgrowth of mesothelioma in a murine model (Hegmans e.a. 2005). Because the TAAs are not known for mesothelioma, we used tumour cell lysates as antigen source to pulse DCs. We established that DC-based immunotherapy induced strong tumour-specific CTLs responses leading to prolonged survival in mice. The efficacy of immunotherapy was dependent on the tumour load; most beneficial effects were established at early stages of tumour development.

On the basis of these preclinical animal studies, we have performed the first clinical trial in which autologous tumour lysate–pulsed DCs were administered in mesothelioma patients (Hegmans e.a. 2010). Patients were eligible for the study when sufficient tumour cells could be obtained from pleural effusion or tumour 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. Concentrated leukocyte fractions were generated through peripheral blood leukapheresis. Peripheral blood mononuclear cells were then enriched, cultured and matured to DCs. Patients received three immunizations with mature DCs, loaded with autologous tumour lysate and keyhole limpet hemocyanin (KLH) as positive control, in 2-week intervals. Each immunization, consisting of 50×10^6 cells, was administered intradermally and intravenously (figure 2).
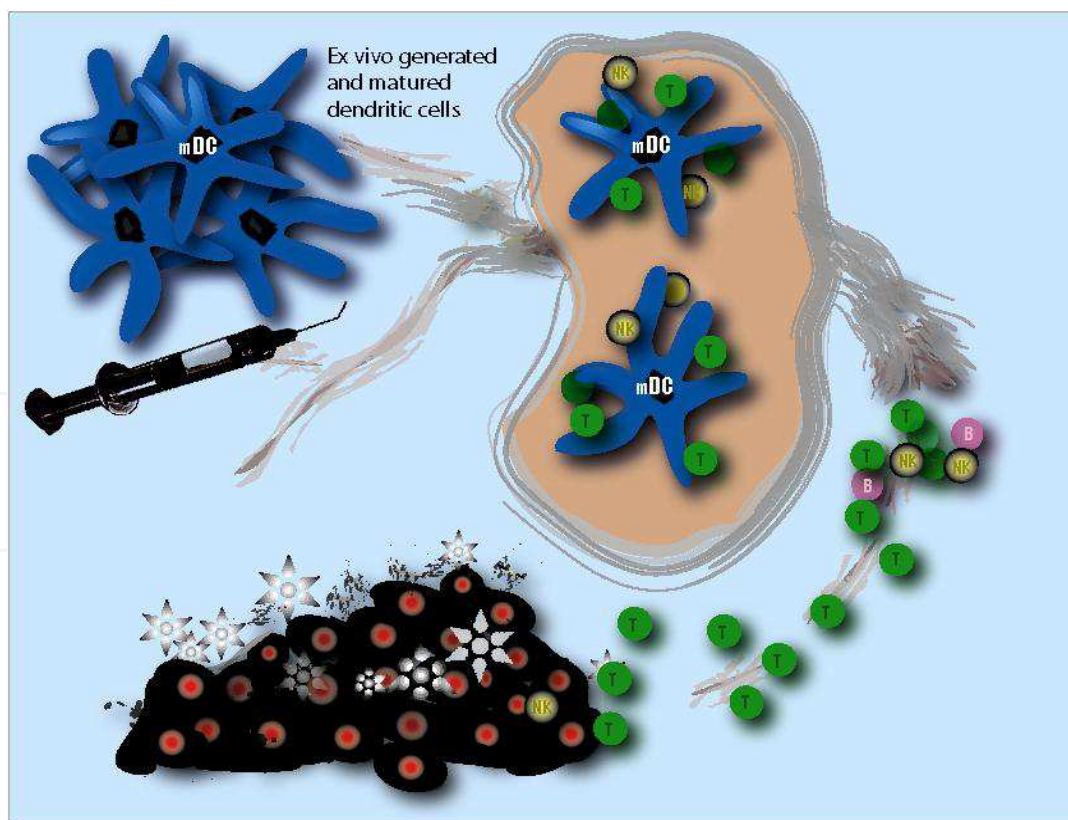

Fig. 2. Schematic representation showing the administration of *ex vivo* maturated autologous dendritic cells into a patient, resulting in antigen presentation in the lymph node and a specific anti-tumour cytotoxic anti-tumour response.

Overall, the vaccination regimen with loaded DCs was well-tolerated in all patients and no CTC grade 3 or 4 toxicities were reported. A local skin rash occurred at the site of the intradermal injection after the first vaccination in 8 of the 10 patients. Subsequent vaccinations (second and third) gave a quicker and increased induration and erythema in all patients suggesting that some form of immunity was induced. Most patients developed mild to severe flu-like symptoms after the vaccination, particularly fever, muscle aches, chills, and tiredness, these symptoms normalized after one day. Since it was a phase I study, no conclusions can be drawn regarding improvement of the progression-free survival or overall survival. However, serum samples from all patients showed a significant increase of pre-vaccine versus post-vaccine antibodies reactive to KLH, both of the immunoglobulin (Ig)G and IgM isotype. 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 suggesting that several vaccinations were necessary to induce a more potent humoral response. Antibodies against KLH in serum could easily be detectable by ELISA in all patients after three vaccinations. The response remained at the same level for several months after the last DC injection and gradually decreased after 6 to 12 months. This proves that a successful immunoreaction was induced by the DC vaccinations. Furthermore chromium release assays were performed in 6 of 10 patients from whom pleural fluid was obtained. In 4 patients a clear inductions of cytotoxicity against autologous tumour cells were measured. The cytotoxicity levels of one patient increased after every vaccination; for the other three patients three vaccinations were necessary to induce cytotoxicity (figure 3).
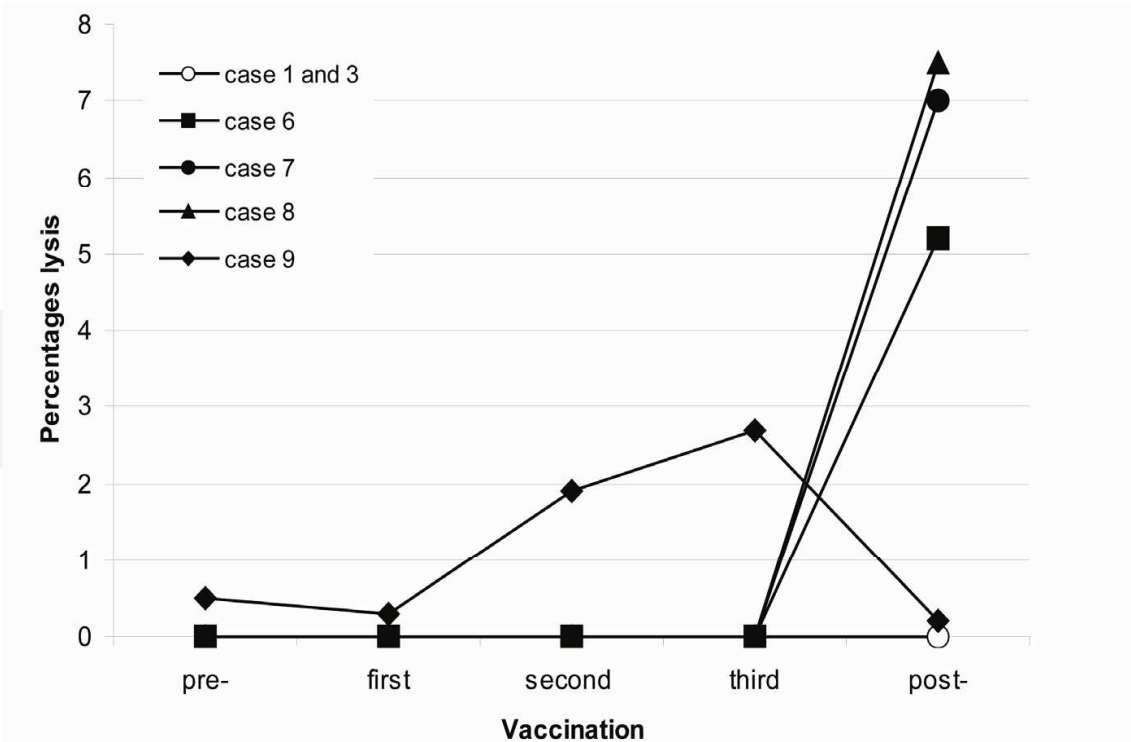

Fig. 3. Percentage of tumour lysis of 6 patients treated with autologous-loaded dendritic cell immunotherapy, showing a clear increase in tumour lysis in half of the patients after the third vaccination.

5. Improving DC-based immunotherapy

While DC-based immunotherapy was proven safe and feasible, it is not "prime time" yet for commencing a larger randomised trial. Because the applied therapy was technically challenging and the efficacy of this therapy was hampered by the presence of immunosuppressive cells in peripheral blood and within the tumour environment, DC-based immunotherapy can be further refined. Several strategies have been tested or are currently tested that target the immunosuppressive cells that diminish the immunoresponse aiming to improve the efficacy of the immunotherapy. In the following sections, we will focus on three populations of suppressive cells, the MDSCs, Tregs and TAMs, that are increased in most cancer patients. It is becoming increasingly clear that these populations contribute to the impaired anti-tumour responses frequently observed in cancer patients. Therefore, combating immunosuppression through modulation of these cell types will be an important key to increase the efficacy of DC-based immunotherapy, and should lead to better prognosis for cancer patients.

5.1 Targeting MDSCs

5.1.1 COX-2 inhibition by celecoxib improves DC-based immunotherapy and is associated with decreased numbers and function of myeloid-derived suppressor cells in mesothelioma

The production of reactive oxygen species (ROS), which is responsible for most of the adverse effects on T cells, by MDSCs is highly depending upon cyclooxygenase-2 (COX-2) enzyme activity (Sinha e.a. 2007). 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 (A Baldi e.a. 2004). In addition, several studies showed the relevance of COX-2 inhibition in cancer progression (Edelman e.a. 2008). Although the relation between COX-2 over-expression and prostaglandin E2 (PGE2) synthesis in cancer has been studied extensively, the impact on the tumour microenvironment is still under investigation (Zha e.a. 2004). Selective inhibition of COX-2 could therefore be a possible strategy for improvement of DC-based immunotherapy. Since celecoxib is a selective COX-2 inhibitor, we investigated the effect of celecoxib treatment on the four MDSC subsets that were identified in the spleen of tumour-bearing mice (Veltman e.a. 2010). 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 inoculation, the absolute number of MDSCs was significantly lower in mice receiving celecoxib diet compared with mice receiving control diet. This difference was even more pronounced at day 22 after tumour injection. Also, dietary celecoxib treatment reduced ROS production in all MDSC subtypes but was most effective in the MO-MDSC and Gr-1low MDSC subset 2 both in percentage as well as the median fluorescence intensity (MFI). Anti-tumour responses induced by DC-treatment were affected by suppressive cells in the spleen of tumour-bearing mice. However, the anti-tumour activity as indicated by AB1 lysis and IFN-g/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.

When combining DC-based immunotherapy and celecoxib treatment, a significant improvement of the immunotherapy was seen in comparison to no or single modality treatment (figure 4). 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 CTLs with superior immunostimulatory potency and anti-tumour activity because of the reduced MDSCs expansion.
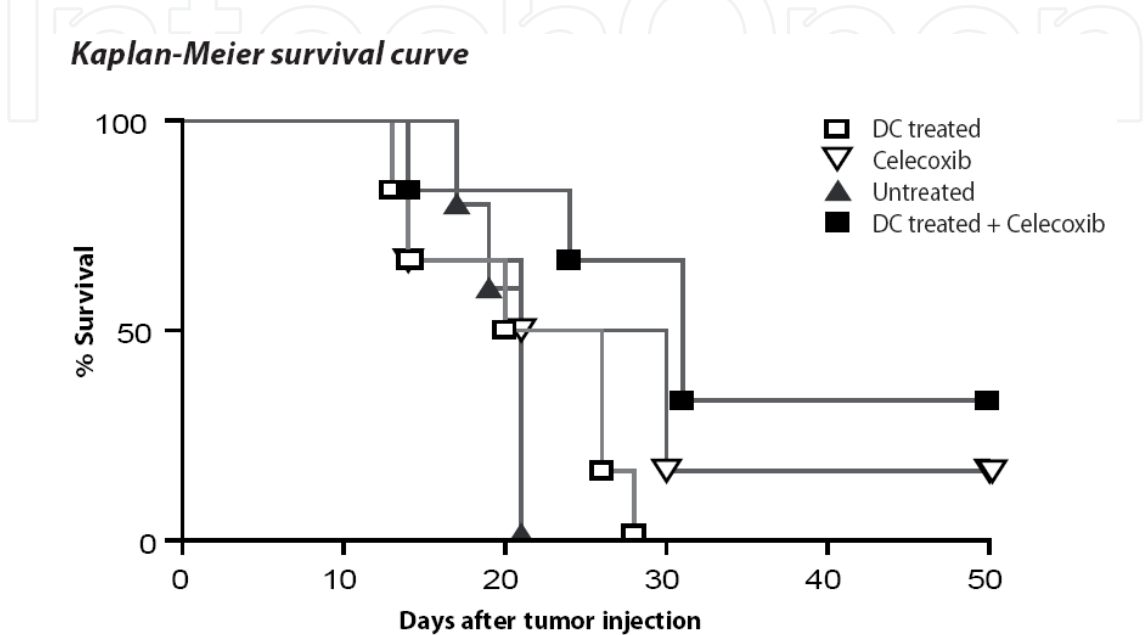
Fig. 4. Improved survival of tumor bearing mice that were treated by dendritic cell-based immunotherapy combined with a diet containing the selective COX-2 inhibitor celecoxib.

5.2 Targeting TAMs

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

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 (Veltman, e.a. 2010). 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). Treatment with liposome-encapsulated clodronate significantly reduced the number of macrophages in the peritoneal cavity of tumour inoculated mice. All mice (n=5) treated with control liposome-encapsulated phosphate buffered saline 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 developed tumours, tumour growth was less profound. Macrophages (M1/M2) were found scattered throughout the tumour of control mice.

These data confirmed that macrophages have a significant role in the onset and progression of tumour in our murine mesothelioma model. We observed an inhibition of myeloid differentiation to macrophages when zoledronic acid (ZA) was added to the culture *in vitro*,

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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.

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. 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-MDSCs 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 macrophage 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 and IL-12.

5.3 Targeting Tregs

5.3.1 Targeting regulatory T cells in clinical studies

Owing to the significant role of Tregs in the failure of immune surveillance and immunotherapy, many attempts to deplete or inhibit Tregs in cancer patients have been made. Many of the strategies to reduce Tregs target CD25, which makes up the alpha-subunit of the IL-2R, that is present on the surface of Tregs and activated cells. An engineered recombinant fusion protein of IL-2 and diphtheria toxin (denileukin diftitox [Ontak]) and other CD25-directed immunotoxins (daclizumab, LMB-2, RFT5-SMPT-dgA) have been investigated for Treg depletion, which seems to kill selectively lymphocytes expressing the IL-2 receptor. However, early human trials have not proven that this approach results in tumor regression and have shown that these strategies may not adequately deplete Foxp3+ Tregs, and may also deplete antitumor effector cells (Attia e.a. 2005; Ruddle e.a. 2006; Attia e.a. 2006; Powell e.a. 2007). Other possible approaches to reduce immunosuppression of Tregs is via CTLA-4 blockade (e.g. ipilimumab)(Fecci e.a. 2007; Phan e.a. 2003), anti GITR agonism (Ko e.a. 2005), and vaccination against Foxp3 (Nair e.a. 2007) and some other suggested approaches, such as the inhibition of IDO, TGF-β, ectonucleotidase (expressed by Tregs and generates immunosuppressive adenosine), or the activation of other agents such as OX40 or Toll-like receptor 8 have not yet proven to be beneficial. IL-7 administration was shown to increase T cell numbers and decrease of the Treg fraction in humans (Rosenberg e.a. 2006), on the contrary, other reports have shown that IL-7 leads to the development of Tregs (Cattaruzza e.a. 2009; Mazzucchelli e.a. 2008). In conclusion, there are many conflicting results in abrogating the action of Tregs, and thus it is unclear which approach holds promise for cancer treatment.

5.3.2 Targeting Tregs with metronomic cyclophosphamide

Low-dose cyclophosphamide (CTX) prevents the development and functionality of the Tregs (Ghiringhelli e.a. 2007), the mechanism behind this effect, however, is not completely understood. We investigated the effect of CTX on immuno-suppression and the combination

of CTX and DC-based immunotherapy was studied in a murine MM model (Veltman e.a. 2010). Our data showed 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 the drinking water of tumor-bearing 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 was reduced. 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 or CTX administration alone.

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.

Following the murine model trial, we commenced a clinical trial in mesothelioma patients. As in our previous clinical trial, patients received three immunizations with mature DCs, loaded with autologous tumour lysate and KLH (as positive control), in 2-week intervals. Each immunization, consisting of 50×10^6 cells, is administered intradermally and intravenously. Metronomic cyclophosphamide is added in a dosage of 100 mg/day as pictured in figure 5.
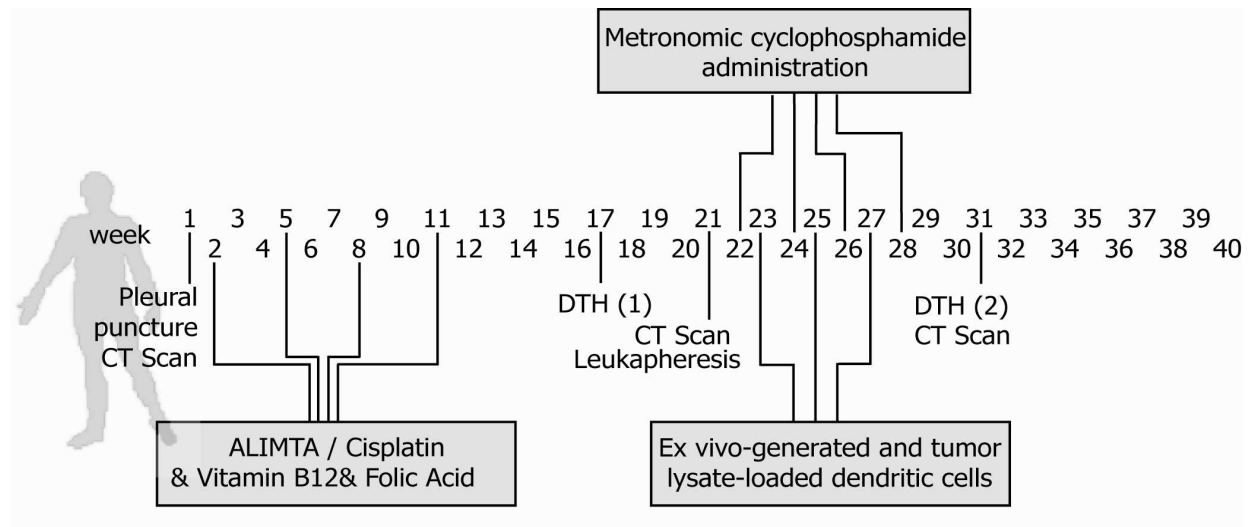

Fig. 5. Treatment plan of the clinical study, starting with diagnosis, followed by chemotherapy treatment. After leukapheresis, dendritic cell therapy with metronomic cyclophosphamide treatment is commenced. DTH = delayed type hypersensitivity, which was performed to test the response against KLH and autologous tumour-lysate.

Primary endpoint of the study is to determine the efficacy of metronomic cyclophosphamide on the modulation of Tregs numbers during DC-based immunotherapy in peripheral blood. Secondary endpoints are the effect on specific anti-tumour activity and clinical and radiological responses. At the moment of publication of this chapter, 8 out of 10 patients have fully completed the immunotherapy treatment. The secondary endpoints are not available yet, but Treg depletion has been confirmed in our first patient (figure 6).
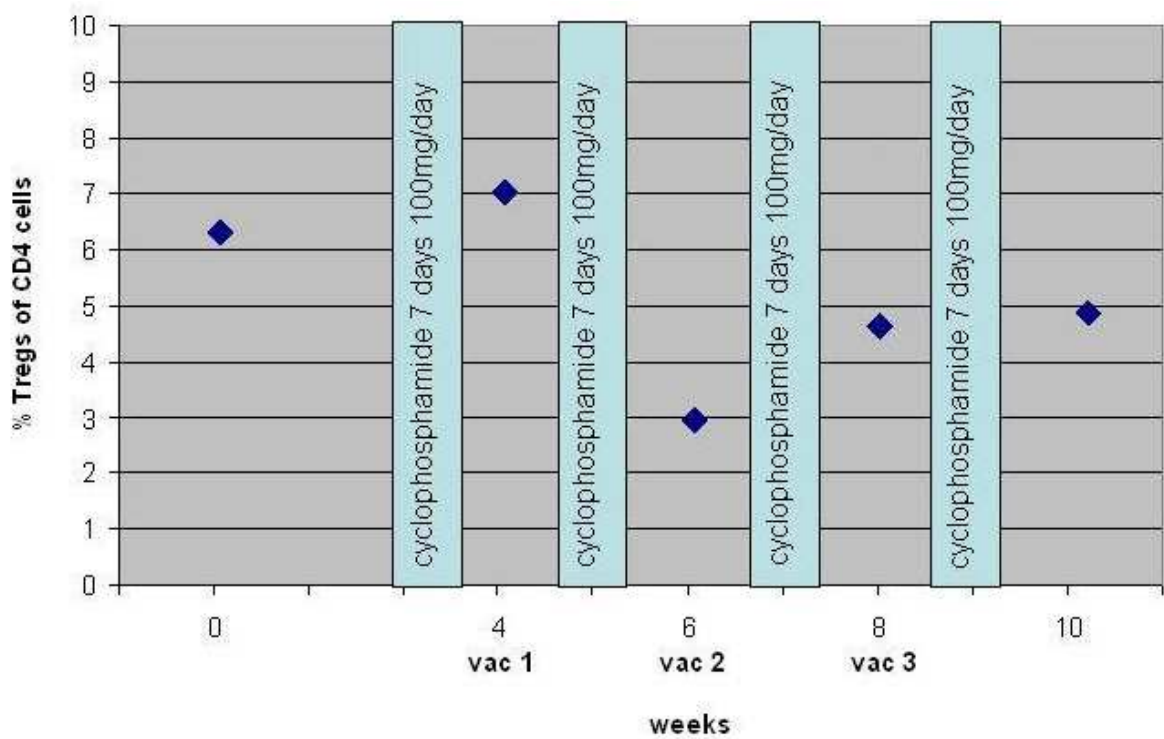
Fig. 6. The first patient in which Tregs were measured during dendritic cell-based therapy treatment combined with metronomic cyclophosphamide, showing a decrease in Treg number in peripheral blood (preliminary data).

6. Future research

6.1 Allogeneic DC-based immunotherapy

In mice, we've observed a distinctive immune response when autologous tumour lysate loaded DC therapy was given after injection of tumour cells. This response was also effective when allogeneic (DCs loaded with mesothelioma cell line lysate derived from other mouse strain) tumour loaded DC therapy was given. This provides opportunities because current clinical trail using autologous lysate loaded DCs are hampered by the amount and quality of tumour lysate available. Most patients already have been diagnosed with mesothelioma before being referred for experimental therapy; often it's not possible to have a patient-friendly way of gathering useful tumour cells. Also, in the future, dendritic cell therapy as a mature anti-tumour therapy would be far more practical if an effective allogeneic tumour cell suspension would be available.

6.2 Response evaluation

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

Commonly accepted treatment paradigm, however, suggests that treatments should initially decrease tumor volume, which can be measured using CT-scan. Also, progression-free survival increasingly is being used as an alternative end-point of studies. This seems to be unfortunate for immunotherapy, which may initiate an immunoresponse that ultimately slows the tumor growth rate, resulting in longer survival, but not a decrease in tumour volume on CT or an increased progression free survival. Future trials are currently planned to investigate these hypotheses, however, clinicians at this moment may need to reconsider how they measure success of their immunotherapy (Madan e.a. 2010).

7. Summary

In conclusion, the role of the immune system in mesothelioma is vast. The tumour uses villainous tricks to evade the immune system and to survive and even abuses immunological cells to harness itself against attack from the immune system. Immunotherapy tries to modulate this immune system to strengthen the anti-tumour effect, this battle however is not yet won and much research lies ahead of us.

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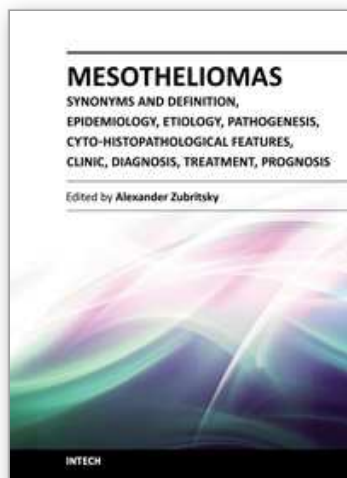
Mesotheliomas - Synonyms and Definition, Epidemiology, Etiology, Pathogenesis, Cyto-Histopathological Features, Clinic, Diagnosis, Treatment, Prognosis
Edited by Dr Alexander Zubritsky

Mesotheliomas are mysterious mesothelial tumors in that they are relatively rare, difficult to diagnose, with a large number of synonyms, and the etiology and pathogenesis of the disease are still not fully disclosed. This problem attracts the attention of various specialists in the field of medicine and biology every year. In recent years there has been a significant increase of mesothelioma morbidity in most of the countries, due to the further industrialization of society. In this regard, this book has been published with the participation of an international group of experts with rich experience from around the world . The book consists of 14 chapters containing the most advanced achievements of all aspects of the various types of mesotheliomas, both in humans and domestic animals, at a high methodological level. This book is intended for biologists and all health care workers, mostly oncologists of different profiles, as well as students of medical educational institutions engaged or even just interested in the problems of mesotheliomas.

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