In situ tumor destruction: towards *in vivo* modulation of immune responses by dendritic cells

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In situ tumor destruction: towards *in vivo* modulation of immune responses by dendritic cells

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CONTENTS

Chapter 1

General Introduction

The immune system

The human immune system is dedicated to protect the body against harmful pathogens like bacteria, parasites and viruses[1]. This complicated defence system comprises many different cell types, which all work together to serve immunity. The functioning of the immune system is based on two distinct types of responses, the innate and the adaptive immune response. The innate immune system, in cooperation with physical barriers like the skin, forms the first line of defence against invasion by pathogens. Cells that orchestrate the innate response are macrophages, monocytes, natural killer cells (NK cells), granulocytes, neutrophils and dendritic cells (DC). The adaptive immune response forms the second line of defence, which depends on the activity of effector T and B cells, and is notable for its specificity for selected proteins (antigens) derived from invading pathogens and the generation of memory for these antigens. This makes the response to recurring encounters with the pathogens increasingly efficient. The specificity is mediated by receptors that, alltogether, have a very large variety of antigen binding capacity. For instance, with their T cell receptor (TCR) T lymphocytes recognize small peptides derived from pathogens, which are presented to them via molecules of the major histo-compatibility complex (MHC). B lymphocytes have membrane immuno-globulins to bind specific antigens from the pathogen. Upon binding they turn into antibody-producing plasma cells. The excreted antibodies can in turn bind to the pathogen and make the invading structures more recognizable for the immune system.

In these processes antigen presenting cells (APCs) play a key role. Decisions on whether T or B cells should expand and eliminate specific targets are made by APCs. Antigen presenting B-cells, macrophages and DC all have the capacity to engulf foreign material and process this into peptide fragments, which can be presented on their MHC molecules. There are two types of MHC molecules, which have distinct functions. Whereas all cells of the body express MHC class I, the expression of MHC class II is a unique feature of APCs. In general, MHC class I molecules present peptides derived from intra-cellular structures and MHC class II molecules from extra-cellular structures. In this way, all cells can make themselves recognizable for the (own) immune system, whereas initiation of T or B cell expansion remains a task for dedicated APC.

However, exogenous antigens can also enter the MHC class I pathway of APCs by a process called 'cross presentation'[2]. Functionally, cross-presentation is thought to be crucial for priming CD8+ T cell dependent responses against tumor cells or cells infected by viruses. Usually APC do not initiate responses to antigens from these 'self' structures and the mechanism of cross-presentation therefore is of interest because it appears to involve overcoming a normally impenetrable barrier. Within the APC population, DCs are by far the most professional in cross-presentation. The DC's decision whether to cross-present an antigen or not is closely related to the contextual recognition of 'danger'. This 'danger' can present itself to the DC as conserved 'pathogen-associated molecular patterns' (PAMPs) derived from for instance viruses, but also as molecular 'flags' on the outside of infected or malafide cells. PAMPs and the molecular flags can trigger specialized molecules on the DC, like for instance the 'Toll-like receptors' (TLRs). Inducing cross-presentation of tumor antigens via DCs is a common strategy in tumor immunology.

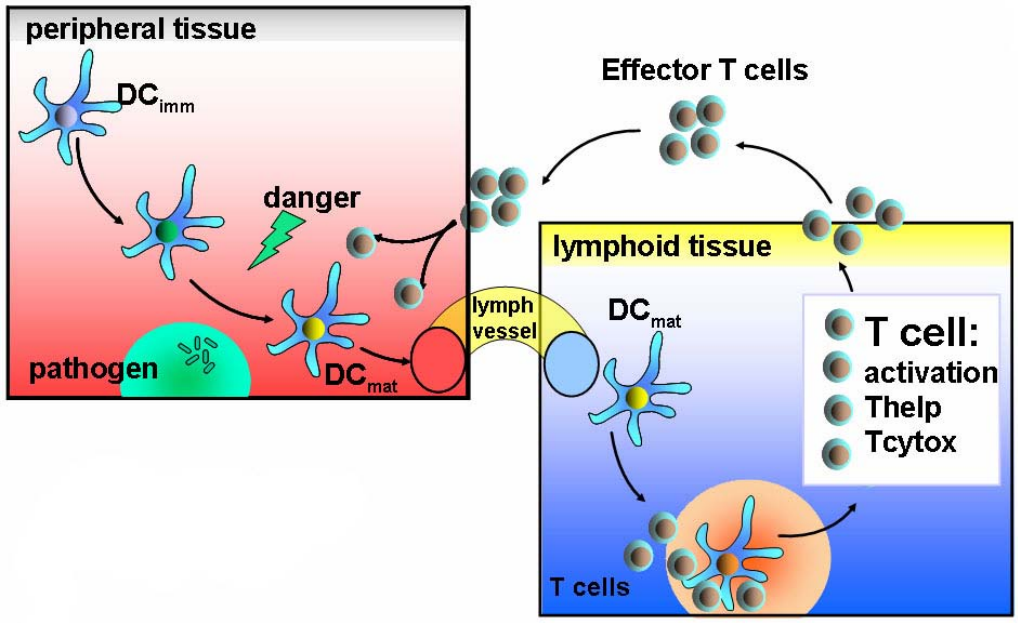

Fig. 1: Dendritic cell life-cycle. After DCs perceive antigen in the context of danger signals, they migrate into lymphoid tissues where they present the antigens to T and B cells. These effector cells play important roles in the subsequent clearing of the pathogen.

Dendritic cells

The past decade has led to an enormous progress in our understanding of dendritic cells and their control of the immune system[3,4]. DCs originate from bone marrow precursors and have the unique capacity to migrate to peripheral tissues, where they differentiate into immature DCs (Figure 1). In this stage, DCs are marked for their exquisite capacities to engulf material from both foreign and self origin. In case of inflammation or infection, the immature DCs undergo a process called maturation. Mature DCs are increasingly able to migrate into T cell rich areas of the secondary lymphoid organs, where they present antigen-derived peptides to T cells and B cells and activate NK cells. Once activated, these cells form the pool of effector cells that can effectively fight infections and serve immunity. In contrast, antigen capture in the absence of infection or inflammation, so in the absence of DC maturation stimuli, can lead to T cell tolerance rather than immunity. Activation of DCs therefore is a decisive factor in the development of efficient immunity.

In human blood, several populations of DCs and DC precursors are discerned. CD11c+CD123^{low} cells are termed “myeloid DCs” (mDCs), whereas CD11c-CD123^{high} DCs have morphological features similar to plasma cells and are therefore termed “plasmacytoid DCs” (pDCs). The differences between pDCs and mDCs include their tissue distribution, cytokine production and growth requirements. The mDC lineage exists of at least two subsets: Langerhans cells, which are found in stratified epithelia like the skin; and interstitial DCs that are found in all other tissues. pDCs are found primarily in the blood and lymphoid organs and are considered important cells in innate antiviral immunity and autoimmunity[5].

T cells in immunity and tolerance

Two main types of T cells can be distinguished: CD4+ T helper (Th) cells and CD8+ cytotoxic T cells (CTL). CTL recognize antigens presented by APC in the context of MHC class I, and are able to directly kill target cells. Th cells recognize antigens in the context of MHC class II and are involved in regulating the responses of other components of the immune system. Th cells can be divided into several subsets according to their functional phenotype[6]. The cells producing IL-2 and IFN-γ for the support of CTLs and NK cells are called Th1 cells. The cells producing IL-4 and IL-5 for the help in B cell responses are called Th2 cells.

Initiation of antigen-specific responses is a complex process, which needs tight regulation. The diversity of antigens that can be recognized by different TCRs is enormous. However, overlap between antigens from microbes and self-tissue exists. Several selection mechanisms and regulatory processes prevent the induction of autoimmune diseases induced by self-specific T cells. In the thymus, selection of T cells (based on their TCR) prevents highly self-specific T cells from entering the circulation[7]. The activation of T cells that do enter the circulation is subsequently controlled by DCs and Regulatory T cells (Treg). If T cells recognize an antigen presented by APC, but lack adequate co-stimulation by these APCs, they will either ignore the antigen or get eliminated. This process of regulated cell death is termed 'activation induced cell death' (AICD).

Furthermore, Tregs will inhibit the induction and effector phase of autoreactive T cells. Tregs carry the markers CD4 and CD25, and it is suggested that these cells are actively induced in the thymus. Different subsets of Treg seem to exist, which all have a different origin and mode of suppression[8]. The most commonly studied 'naturally occurring Treg', induced in the thymus, is present in the periphery of a naïve host and therefore can be reffered to as 'intrinsic Treg'. When stimulated via their TCR these cells are non-proliferative, but they are known to strongly inhibit the activation of effector T cells in a cell-contact dependent manner. Transfer of T cell populations from which the intrinsic Treg have been depleted into T cell deficient mice causes severe induction of autoimmune disorders, illustrating the importance of T cell regulation[9]. Recently, it has been shown that depletion of Treg can even actively boost tumor rejection in a variety of tumormodels[8,9]. A second group of Tregs is formed by the 'induced Tregs'. These cells, termed Th3 or Tr1 originate from naïve CD4 T cells and are induced in the periphery. It is generally believed that their induction is the result of antigen encounter in the presence of IL-10 and TGF-β. T cell clones stimulated by IL-10 such as derived from DC or tumors secrete different cytokines than conventional Th1 or Th2 cells. To discriminate Tregs from conventional T cells, expression of activation marker CD25 is commonly used. However, intermediate CD25 expression can also be found on highly activated conventional T cells, which complicates effective sorting. The forkhead-family transcription factor FoxP3, important in the development of Tregs and expressed in all Treg types, therefore seems to be a better alternative.

Immunotherapy of cancer and *in situ* tumor destruction

The ever-increasing knowledge of the immune system and its diverse components has created opportunities and ideas for new treatments of immune-related diseases. Immunotherapy has, for instance, become a promising strategy for the treatment of cancer. Vaccinations include injection of tumor antigens with adjuvans that activate the immune system, or tumor antigen-loaded DCs (discussed in chapter 2). In many mouse models, prophylactic DC vaccination was shown to protect from a subsequent tumor challenge. Therapeutic treatment, a more relevant therapy for cancer patients, proved to be more difficult. In many cases the tumor and the body's own regulatory mechanisms will actively dampen the responses induced by these vaccines. In order to break this state of tolerance, several new vaccination strategies are currently under investigation. In this thesis we investigated if *in situ* tumor destruction could be used for inducing long-lasting immunity by loading and activating DCs directly *in situ*. The results of this study would gain novel insights on DC behaviour *in vivo*, but also would have their impact on the application of *ex vivo*-generated DC vaccines.

For long it has been recognized that heating or freezing of tumors is an effective way of *in situ* tumor destruction. Today several local ablation techniques are in use, like cryo, radiofrequency, microwave, or laser-mediated tumor ablation. Of these methods, cryo and radiofrequency ablation have been used most frequently in clinical settings. Cryo ablation in patients freezes the local lesion, which results in direct tumor cell death and microvascular thrombosis. For radiofrequency treatment, a small electrode (15G) placed within the tumor delivers a radiofrequency current generating ionic agitation that is converted into frictional heat and subsequent breakdown of proteins and cell membranes. This leads to coagulation of most of the tumor and to a minor extent to cellular apoptosis in the distal parts of the tumor. The technical developments in the application equipment and monitoring devices strongly increased the applicability of *in situ* tumor ablation methods. Currently, tumor ablative techniques are not only applied to tumors located at superficial sites but also to tumors located within the body such as bone tumors, renal cancer and especially liver tumors. Application of RFA, either alone or in combination with resection (>4 cm), yielded 1 and 2 year survival rates of 80% and 60% respectively[11-14]. For cryo ablation median survival times vary from 26 to 32 months[10,15,16]. These data compare favourable to systemic chemotherapy, which results in a median overall survival of approximately 17 months. This difference may however be the result of patient selection.

Unfortunately, when radiofrequency ablation is applied for the destruction of larger sized lesions, local recurrence rates of over 35% have been reported[12]. However, survival is mostly limited by the appearance of new metastatic lesions. These data illustrate the requirement of a systemic anti-tumor therapy in addition to local tumor ablation.

Scope of this thesis

Dendritic cells are the most potent antigen-presenting cells of the immune system and represent a promising tool in the therapeutic vaccination against cancer. However, a better understanding of how DCs succeed to induce and modulate immunity is necessary to optimally exploit DCs in anti-cancer vaccines.

The scope of this thesis was to investigate if *in situ* tumor destruction could be used for inducing long-lasting immunity by loading and activating DCs directly *in situ*. The results of this study would gain novel insights on DC behaviour *in vivo*, but also would have their impact on the application of *ex vivo*-generated DC vaccines.

In Chapter 3, the induction of anti-tumor responses following *in situ* tumor ablation is described. Herein, we used a newly developed murine B16-OVA tumor model to explore: 1) the immunological consequences of *in situ* tumor destruction and 2) the efficacy of a combination approach of tumor destruction and immunostimulation. Applying this model system we demonstrate that following radiofrequency ablation, a weak but detectable immune response develops, which can be augmented by a blocking mAb against cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) at the time of tumor destruction.

In **Chapter 4** we show that the antigen specific immunity observed after local tumor destruction is mediated by DC. Furthermore, two types of tumor ablation are compared in this study. Upon tumor destruction by radiofrequency large numbers of draining lymph node DC acquire antigen. This number is, however, doubled when cryo ablation was used to destroy the tumor tissue. Compared to conventional DC vaccination, far more antigen positive DC could be discerned in the lymph node after ablation. Analysis of DC maturation revealed that both destruction methods were able to enhance DC maturation. This chapter also shows that *in situ* tumor ablation can be efficiently combined with immune modulation by depletion of regulatory T cells. This leads to increased numbers of tumor-specific T cells in tumor-bearing mice, protecting them from lethal tumor challenges.

Chapter 5 provides a more in-depth view on the DC inducing immunity upon tumor ablation. It illustrates that *in situ* tumor destruction can be seen as an '*in vivo* DC vaccine'. The tumor debris generated following ablation can provide antigens that are taken up by DC. An additional immunostimulatory PAMP like CpG-ODN matures DC, induces cross-presentation and enhances the anti-tumor response in a synergistic way.

Chapter 6 demonstrates that PAMPs are not only relevant *in vivo* for DC, but also for regulatory T cells. When TLR2 ligands are provided to regulatory T cells, they temporarily lose their suppressive abilities and acquire the capacity to proliferate. The relevance of TLR2 triggering in regulatory T cells is demonstrated in an *in vivo* infection model.

Finally, in **Chapter 7** the implications of these novel findings for DC/Treg function, and the use of DC in vaccination strategies are discussed.

References

1. Janeway, C.A., Jr. How the immune system works to protect the host from infection: a personal view. *Proc Natl Acad Sci U S A* **98**, 7461-8 (2001).
2. Bevan, M.J. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**, 1283-8 (1976).
3. Adams, S., O'Neill, D.W. & Bhardwaj, N. Recent advances in dendritic cell biology. *J Clin Immunol* **25**, 87-98 (2005).
4. Banchereau, J. & Palucka, A.K. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* **5**, 296-306 (2005).
5. McKenna, K., Beignon, A.S. & Bhardwaj, N. Plasmacytoid dendritic cells: linking innate and adaptive immunity. *J Virol* **79**, 17-27 (2005).
6. O'Garra, A. & Murphy, K. Role of cytokines in determining T-lymphocyte function. *Curr Opin Immunol* **6**, 458-66 (1994).
7. Hoffmann, M.W., Heath, W.R., Ruschmeyer, D. & Miller, J.F. Deletion of high-avidity T cells by thymic epithelium. *Proc Natl Acad Sci U S A* **92**, 9851-5 (1995).
8. Sutmuller, R.P., Offringa, R. & Melief, C.J. Revival of the regulatory T cell: new targets for drug development. *Drug Discov Today* **9**, 310-6 (2004).
9. Sakaguchi, S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* **6**, 345-52 (2005).
10. Adam, R. et al. Place of cryosurgery in the treatment of malignant liver tumors. *Ann Surg* **225**, 39-8; discussion 48-50 (1997).
11. Bilchik, A.J., Wood, T.F. & Allegra, D.P. Radiofrequency ablation of unresectable hepatic malignancies: lessons learned. *Oncologist* 6, 24-33 (2001).
12. Solbiati, L. et al. Percutaneous radio-frequency ablation of hepatic metastases from colorectal cancer: long-term results in 117 patients. *Radiology* **221**, 159-66 (2001).
13. Curley, S.A. et al. Radiofrequency ablation of unresectable primary and metastatic hepatic malignancies: results in 123 patients. *Ann Surg* **230**, 1-8 (1999).
14. Bleicher, R.J. et al. Radiofrequency ablation in 447 complex unresectable liver tumors: lessons learned. *Ann Surg Oncol* 10, 52-8 (2003).
15. Joosten, J., Jager, G., Oyen, W., Wobbes, T. & Ruers, T. Cryosurgery and radiofrequency ablation for unresectable colorectal liver metastases. *Eur J Surg Oncol* **31**, 1152-9 (2005).
16. Ruers, T.J., Joosten, J., Jager, G.J. & Wobbes, T. Long-term results of treating hepatic colorectal metastases with cryosurgery. *Br J Surg* **88**, 844-9 (2001).

Chapter 2

Dendritic cells:
tools and targets for anti-tumor vaccination

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Dendritic cells in anti-tumor vaccination

The decisive role of DCs in the activation of immune responses has initiated the development of DC vaccines against cancer[8]. Numerous mouse studies applying bone-marrow-derived mDCs loaded with tumor antigens provided evidence that DC-based vaccination could be effective in fighting cancer (reviewed in [10] and [11]). The ability to generate large amounts of human DCs subsequently boosted research on DC vaccination in humans. Most human studies to date use monocytes to produce DCs *ex vivo*. When cultured in the presence of GM-CSF and IL-4 for a period of three to six days, monocytes develop into immature mDCs. Next to monocytes, CD34+ cells are also used to culture mDCs[8]. Clinical trials applying *ex vivo* generated monocyte-derived DCs indeed showed that DC vaccination is feasible and effective in some patients[12,13].

However, many questions regarding immunogenicity of tumor antigens and DC maturation and migration remain unanswered. In addition, *ex vivo* generation of DC vaccines for each patient is costly and labor intensive. In this chapter, we will review the developments in the field of *ex vivo* generated DC vaccines, and discuss novel approaches aiming to target DCs directly *in vivo*. We will do this in the context of three crucial processes: antigen loading of DCs, DC maturation and DC migration.

DC-based vaccines: The *ex vivo* generation

Ex vivo loading of dendritic cells

In studies applying *ex vivo* produced DC vaccines, several strategies have been explored to obtain presentation of antigenic peptides in the context of MHC class I and II molecules (See also Figure 1). Exogenous loading of DCs with synthetic HLA binding peptides has been the most commonly used approach[8,14]. A major advantage of this approach is that the immune response induced by the vaccine can be readily monitored using for example peptide-MHC tetramers. Although vaccination studies using *ex vivo* loaded DCs have given us important insights, the use of peptides also has its constraints. The limited knowledge regarding optimal tumor antigens and the restriction of a peptide to a certain HLA type limit the number of patients and types of cancer to be treated. Furthermore, the relatively short half-life (several hours) of the peptide-MHC complexes is likely to affect the potency of this type of vaccine. Strategies to prolong the presentation of peptide-MHC complexes include loading of DCs with peptide analogues with increased affinity for MHC class I molecules, the genetic modification of DCs by transfection with tumor antigen encoding cDNA or transduction with viruses encoding the selected target antigen. Furthermore, *in vitro* synthesized RNA encoding tumor antigens has shown to stimulate production of tumor proteins in the DCs themselves[15].

Alternatively, DCs can be loaded *ex vivo* with tumor lysate, dead tumor cells, or total tumor-derived RNA. Studies applying for example vaccination of metastatic melanoma patients with monocyte-derived DCs loaded with tumor lysate have indeed led to the induction of melanoma specific T cell immunity[16]. All these approaches circumvent the need to define the appropriate tumor antigens and thus rely on the capacity of the DCs to select,

process and present the relevant antigens. Since auto-antigens will be co-loaded in parallel with the desired tumor antigens, the occurrence of potential auto-immune phenomena should be carefully monitored. Other strategies for loading DCs *ex vivo* include loading with recombinant proteins[17], protein-liposome complexes[18], exosomes[19], or immune complexes[20].

Recent mouse studies explored the injection of *ex vivo* generated DCs directly into the tumor. For example, intra-tumoral injection of syngeneic DCs into malignant murine gliomas led to infiltration of the tumor by T cells and the concomitant occurrence of tumor regression. These studies formed the basis for early phase I clinical trials like the one performed by Mazzolini and co-workers. In this study, patients with metastatic gastrointestinal carcinomas were treated by intra-tumoral injections of autologous IL-12-transfected monocyte-derived DCs[21]. The treatment resulted in increased levels of IFN-γ and IL-6 in the majority of the patients as well as peripheral blood natural killer activity in some patients. Further studies optimizing current protocols are, however, necessary to improve clinical efficacy.

Irrespective of the strategy selected to load the DCs, it is now evident that activation of both CD4+ and CD8+ T cells is highly beneficial for the induction of anti-tumor immunity. Therefore, loading of both MHC class I *and* MHC class II molecules on the DC is important to boost a strong immune response. Whereas pulsing of DCs with a nominal peptide epitope results in direct loading of MHC class I molecules, efficient presentation of intact tumor antigens or tumor cells relies on the unique capacity of DCs to cross-present exogenous antigens in MHC class I[2]. Although the molecular pathways involved and the factors that trigger cross-presentation are still poorly understood, recent studies in mice suggested that cross-presentation is dependent on the intracellular stability of the antigens[22] and is tightly controlled by DC maturation[23,24]. These novel insights demonstrate that antigen uptake, cross-presentation, and DC maturation are interlinked, and imply that timing of each of these processes is important for the optimal induction of immunity.

Maturation of ex vivo generated dendritic cells

Upon infection or inflammation, immature DCs are activated and differentiate into mature APC that are well equipped to prime effector T cell responses. DC maturation is associated with several coordinated events, including loss of endocytic and phagocytic receptors, upregulation of expression of co-stimulatory molecules such as CD58, CD80 and CD86, changes in morphology and re-organization of the DC's lysosomal and MHC class II compartments. DC maturation is highly complex and should be regarded as a flexible process of which the outcome is depending on the type of signals a DC receives in the periphery. For example, depending on the maturation signals received, a mature DC is able to produce large amounts of the pro-inflammatory cytokine IL-12, a key Th1-promoting cytokine[25].

Maturation of DCs can be accomplished by several distinct signals that alert the resting DCs to the presence of pathogens or tissue injury[26,27]. These triggers originate from either pathogen associated molecular patterns (PAMPs) or molecules released from damaged host tissue, both of which have been reported to activate Toll-like receptors

(TLRs)[28]. In addition, inflammatory cytokines such as IL-1, TNF-α and IL-6 can induce DC maturation. Mature DCs can be further activated by cell-cell contact and subsequent signaling via members of the TNF/TNF-receptor family, like CD40/CD40L. CD40 engagement on DCs by CD154 present on activated T cells, triggers full DC activation and enhances DC migration from the periphery to the draining lymph nodes. In the lymph node, these activated DCs are optimally suited to induce powerful effector CTL. CD40L has frequently been used for *in vitro* activation of murine DCs. When used on DCs derived from MHC class II-deficient mice, soluble CD40L was shown to generate DCs with the capacity to elicit primary and memory CD8+ T cells upon injection *in vivo*, so even in the absence of help from CD4+ T cells[29]. For this reason, triggering CD40 is often pictured as a therapeutic compound for cancer, especially when combined with TLR-triggering.

Until today, 11 TLRs have been described for which many specific ligands have been identified (reviewed in [28,30]). TLR1, TLR2, and TLR6 interact with various lipopeptides, TLR3 with double-stranded RNA (dsRNA), TLR4 with lipopolysaccharide (LPS), TLR5 with flagellin, TLR7 and TLR8 with GU-rich ssRNA and synthetic imidazoquinolines, TLR9 with unmethylated CpG DNA and TLR11 with a profolin-like molecule isolated from Toxoplasma gondii[31]. TLR10, which has not yet been detected in mice, is expressed on human B cells and pDCs[32], but so far its ligand has not been identified. The signaling pathways associated with ligation to each of these TLRs are not identical and therefore distinct biological responses are initiated upon ligation. Human monocytes and mDCs express all TLRs except TLR7 and TLR9, whereas pDCs selectively express receptors 1, 7 and 9[33,34]. The differential expression of TLRs by distinct DC subsets is thus indicative for the difference in function of these DC-subsets within the immune system.

The necessity of DC maturation to induce potent immune responses has now been well established. Whereas immature DCs have proven to be weak immune stimulators and even can be tolerogenic, mature DCs induce functionally superior CD8+ T cells and concomitant anti-tumor immunity[35]. Accordingly, injection of monocyte-derived immature DCs in cancer patients yielded little T cell-mediated immune responses, whereas following injection of mature DCs these responses were readily detected[14,36].

The most commonly used method to mature DC in the clinic consists of a cocktail of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and prostaglandin E2, a hormone-like structure, which is secreted upon inflammation[8]. Currently, other stimuli including mixtures of TLR-ligands and inflammatory cytokines are explored to improve DC maturation and function. For example, human DCs cultured in a combination of IL-1β/TNF-α with type I and II interferons (IFN-α and IFN-γ) and the TLR-ligand poly I:C were very potent IL-12 producers and better inducers of CTLs *in vitro*[37]. Moreover, it was demonstrated that activation of DCs by solely pro-inflammatory cytokines yielded DCs that supported CD4+ T cell clonal expansion, but failed to efficiently direct helper T cell differentiation. In contrast, exposure of these cells to pathogen components generated DCs that did promote T cell help[37,38]. Recent studies suggest that the interplay of multiple TLRs and other pathogen receptors like C-type lectins and nucleotide-binding oligomerization domains (NODs, intracytoplasmic microbial-recognition proteins sensing for specific peptidoglycan muropeptides) may work synergistically (reviewed in[39]). Simultaneous stimulation of these different pathogen receptors might indeed more closely resemble the natural situation

where pathogens containing several molecular triggers will initiate multiple defense mechanisms. A better understanding of DC maturation and the potency of the differently matured DCs to induce and modulate immune responses will be highly beneficial for the rational design of DC-based vaccination strategies.

Migration of ex vivo produced dendritic cells

The migration of DCs from the sites where they encounter antigen to the draining lymph nodes is a fundamental part of their life cycle and is closely linked to their maturation[40-42]. Chemokines and their receptors, matrix molecules and adhesion molecules, on the DCs as well as on the surrounding tissues are coordinating this important journey. DC migration into lymphatic vessels and their subsequent localization within the lymph nodes is found to be controlled by the chemokine receptor CCR7 and its ligands CCL19 and CCL21[43]. Studies in CCR7 deficient mice demonstrated that CCR7 is crucial for migration of dermal and epidermal DCs, but not for Langerhans cells[44]. Maturation of DCs results in reduced tissue retention by the downregulation of chemokine receptors, whereas CCR7 and CD62L are upregulated[42].

DC migration is not only dependent on chemokine patterns, but also involves a complex set of other mediators. Eicosanoids like leukotrienes and prostaglandins are involved in DC migration from the skin. These lipid mediators, in cooperation with the total lipid profile (LDL vs. HDL, oxidized vs. non-oxidized) can influence migration either positively or negatively[45]. Another factor of basic importance for DC migration is the balance between matrix metalloproteinases and their inhibitors. This has shown to influence migration independent of their maturation status[46].

Optimal migration of administered DC vaccines will require a dynamic regulation of these various mediators, which will have to be adapted to the maturation status of the DC. Nevertheless, in current vaccination with *ex vivo* produced DCs, in general less than 5% of intra-dermally injected mature DCs reach the draining lymph nodes. This was for instance demonstrated in a patient trial, which used radioactive labeled monocyte-derived DCs to monitor migration[40]. Data from mouse models employing bone marrow DCs confirmed this efficiency range[47,48]. On the other hand, it needs to be studied in more detail if increasing migrating DC numbers actually also supplies *optimal* numbers for immune response induction. Finally, the route of administration of the vaccine also leads to different distribution of DCs in lymphoid tissues. It was shown that this distribution not only determines the location of the primary immune response, but also the tissue-specific homing of memory cells and the ability to control outgrowth of tumors at different sites in the body[49]. Optimizing migration of *ex vivo* produced DC vaccines would not only enhance efficacy, but would also allow us to use other sources or types of DCs (normally present in small numbers), with less massive culturing.

DC-based vaccines: The *in situ* generation

Taking into consideration the complex mechanisms that control and regulate crucial DC functions, like antigen processing, DC maturation and migration, as well as cytokine production, it will be a major challenge to optimize all these parameters for *ex vivo* DC vaccines. In addition, generation and *ex vivo* manipulation of DCs is laborious and costly. Therefore, the ability to load and mature DCs directly *in vivo* would greatly facilitate the application of DC-based vaccines and may well improve vaccine efficacy. A variety of strategies has been explored to create such '*in vivo* DC vaccines', which will be reviewed in the next section.

In vivo loading of dendritic cells

The first step in the design of any '*in vivo'* DC vaccine is to load directly *in vivo* with tumor antigens. Several studies have explored targeting of tumor antigens to endogenous DCs using either proteins or mAbs against cell-surface antigens that preferentially bind to DCs (Fig. 1). Several mouse models demonstrated that uptake of antigens by DCs can be improved dramatically by conjugating them to molecules that direct towards antigen presenting cells, including DCs. For instance, APCs are able to take up complexes of heat shock proteins (HSP) and peptides via surface receptors like CD91. These HSP-peptide complexes have shown to elicit peptide-specific CTL responses and tumor immunity[50]. Also bacterial or viral proteins, chemically coupled to target antigens have been used successfully to promote processing and cross-presentation in DCs. Antigen specific T cell responses could be obtained for instance with cholera toxin, binding to the GM1 receptor[51], or with parvovirus-like particles[52].

Antigen targeting of endogenous DCs, mediated by monoclonal antibodies (mAbs), has focused on specific lectin-like receptors that are expressed by DCs, as many of these receptors are involved in the uptake of pathogens. For instance, infusion of a mAb directed against DEC-205 resulted in the rapid accumulation of the mAb plus a coupled antigen in DCs[53]. Interestingly, this type of targeting not only resulted in MHC class II presentation, but also in MHC class I presentation, enabling the induction of CD8+ T cell responses. Anti-DEC-205 mAbs covalently attached to hen egg lysozyme $(HEL)_{46-61}$ peptides or genetically engineered to contain T cell epitopes were also efficiently targeted to DCs and resulted in activation of MHC class II-restricted CD4+ T cells directed against the target antigen[54,55]. In a recent *in vitro* study, antibodies against the human C-type lectin DC-SIGN were also shown to strongly enhance antigen presentation by human monocyte derived DCs[56,57]. Likewise, the use of a fusion protein of melanoma antigens and antibodies directed against the mannose receptor contributed to the generation of multiple HLA-restricted antigen specific T cell responses[58].

As DCs constitute only a small percentage of blood leukocytes (<0.1%), several studies have used the DC growth factors Flt-3 ligand and GM-CSF to expand mouse[59] and human[60] DC subsets *in vivo*. Interestingly, the +/- ten fold increase in the numbers of DCs by administration of these growth factors could induce anti-tumor immunity in mouse tumor models, but to a far greater extend when combined with infusion of DC maturating compounds.

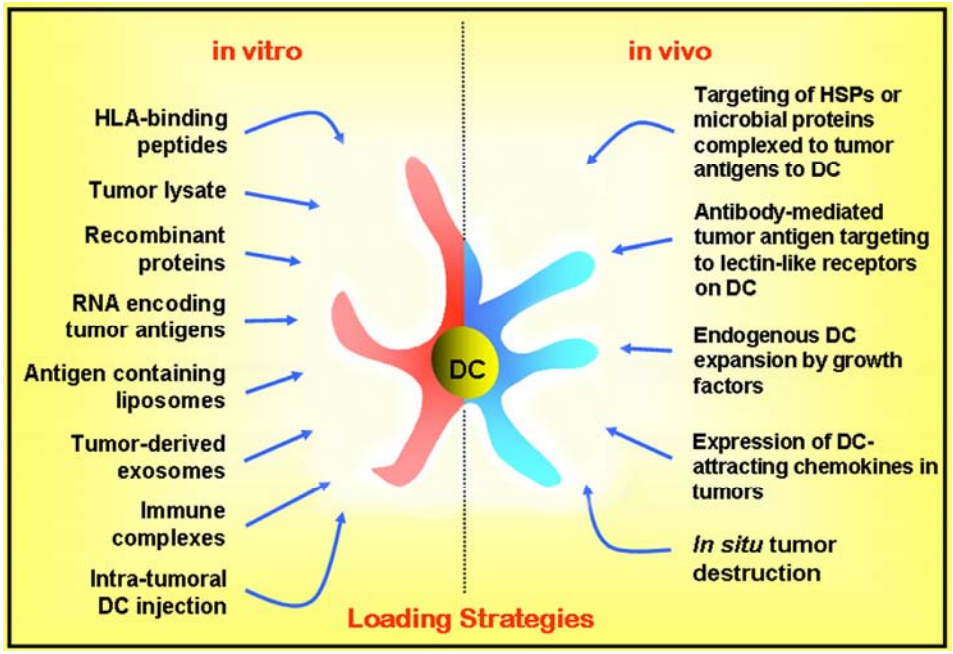

Fig. 1: Approaches for antigen loading on dendritic cells. The potent antigen-processing and -presenting functions of DCs have initiated the search for the optimal strategy to employ DCs as tumor vaccines. The figure shows that DCs can be loaded with tumor antigens *in vitro* as well as *in vivo*. *Ex vivo* generated DCs can also be injected directly into the tumor to accomplish the uptake of tumor-derived antigen *in vivo*. On the other hand, DCs can be loaded *in vivo, e.g.* by increasing DC numbers via growth factors or migration into the tumor via chemokine expression. *In vivo* targeting is done by coupling of antigens to DC specific receptors or HSPs. A relatively new approach involves loading DCs by *in situ* tumor destruction.

These studies suggest that increasing the number of circulating DCs leads to presentation of tumor antigens by DCs, but is in itself not a very efficient way to induce anti-tumor immunity. To increase loading of endogenous DCs with tumor antigens *in vivo*, they also can be attracted to the tumor site by expression of DC attracting chemokines in the tumor tissue (reviewed in [42]). Studies by Furumoto et al. demonstrate that expression of the chemokine CCL20 at the tumor site attracted large numbers of circulating DCs into the tumor mass[61]. Consistent with the requirement of DC maturation for efficient immune response induction, eradication of existing tumors depended on the additional administration of the TLR-ligand CpG-ODN.

The finding that *in vivo* expanded DCs are able to acquire tumor antigens raises the question how efficient this process occurs in the natural situation. DCs and macrophages are both well known for their capacities to phagocytose antigens, but an important distinction between these cells is their differential ability to induce immune responses. The basis for this difference remains to be studied in more detail, but is likely related to the strategic location of certain DC subsets within the LN and their ability to retain antigens within their endocytotic compartments, whereas macrophages rapidly degrade antigens in

their lysosomes[62]. Based on the idea to employ the intrinsic capacity of DCs to scavenge antigens and to retain these antigens for a prolonged time, we and others have investigated *in situ* tumor destruction as a tool to load endogenous DCs. Following cryo ablation or radiofrequency ablation, large amounts of tumor material (e.g. necrotic tumor cells) are released and remain in the tumor bearing individual. Recent data indeed indicate that using radiofrequency ablation or cryo ablation up to 25% of endogenous draining lymph node DCs acquire antigen, whereas other APC were essentially negative (our unpublished results). Tumor eradication by radiotherapy[63] or chemotherapy[64] has also been demonstrated to provide a potential antigen source for the induction of anti-tumor immunity. However, a role for DCs has not yet been addressed in these models. An advantage of *in vivo* loading of DCs by *in situ* tumor destruction over other *in vivo* DC loading approaches is that there is no need for retroviral infection, construction of recombinant proteins or prior knowledge of the right tumor antigens. One of the potential drawbacks of *in situ* tumor destruction approaches could be that *in vivo* loaded DCs suffer from the immune suppressive environment present at the tumor site that might affect DC maturation and/or migration. Furthermore, it remains to be investigated how important exclusive targeting of tumor antigens to DCs is for induction of potent immune responses *in vivo*. Preferential loading of DCs might be optimal in the initiation phase of the immune response, but antigen uptake by other APC, like B cells or macrophages (as occurs following *in situ* tumor destruction), could possibly contribute as well and be important to boost T cell expansion and improve antibody-mediated responses.

In situ release of antigens can be obtained in several ways. Although not studied comprehensively, factors like amount of antigens released, quality of antigens released and time frame of antigen exposure will likely be different in these various strategies, but important for the loading of APC. Table 1 shows some of the approaches to obtain *in situ* tumor cell-death, which are possibly effective in loading DC *in vivo*. The different methods are more extensively discussed in Chapter 7 of this thesis.

Method	Mode of action	
Chemotherapy	Mostly creates apoptosis. Slow release of antigens	Comprises many compounds that are more or less toxic to cells
Tumor ablation	Mostly creates necrosis. More rapid release of antigens	Ablation can be accomplished by cold, radiofrequency, laser, ultra-sound or microwaves
Radiation	Mostly creates apoptosis. Slow release of antigens	

Table 1: Various ways to obtain *in situ* tumor antigen release

Maturation of dendritic cells in vivo

Although solid human tumors are frequently infiltrated by DCs that have captured and presented antigens, the onset of a proper immune response is nevertheless often

hampered. As DC maturation is of crucial importance for immune response induction, the lack of potent DC maturating compounds like microbial PAMPs may well be responsible for this. To efficiently mature DCs *in vivo* following loading with antigens, the same DC maturation stimuli as used for *ex vivo* DC maturation, like pathogen-derived TLR-ligands and CD40 activating mAbs, are commonly applied in mouse models. However, one should be aware that the nature of *in vivo* loading of DCs can also influence maturation. For instance, apoptotic or necrotic cells are both taken up by DCs but have a differential outcome with regard to DC maturation. Moreover, endogenous inflammatory stimuli produced upon inflammation may also have an impact on maturation[27]. This may especially be the case with *in situ* tumor destruction procedures, where endogenous danger signals are readily released from the damaged tissue/tumor, which may contribute to immune activation.

Several studies have suggested that necrotic cell material can efficiently mature DCs and induce systemic immunity, whereas antigens from apoptotic bodies might fail to do so and induce *in vivo* tolerance[65,66]. Opsonisation of apoptotic cells by complement components has demonstrated to revert the observed tolerance induction[67]. Similarly, expression of HSPs in dying tumor cells has proven to efficiently induce immune responses and to be essential in the *in vivo* cross-priming of CD8+ T cells[50]. Interestingly, it has been suggested that HSPs are endogenous ligands for TLR2 and 4[68]. The identification of uric acid as an endogenous immune stimulus[69] further provides evidence that endogenous danger signals may contribute to immune activation and stimulate anti-tumor immunity.

In addition, DCs in the vicinity of the tumor can be loaded with antigen and concomitantly matured by *in situ* administration of exogenous stimuli containing PAMPs or by activating antibodies. For example, systemic or peri-tumoral injections of agonistic anti-CD40 antibody caused expansion of CD8+ T cells that infiltrated and permanently eradicated murine tumors[70]. Also in case of anti-DEC-205 mAb-mediated delivery of antigens to DC, CD40 triggering, inducing DC maturation, was an essential factor for immune response initiation[53].

TLR-triggering has shown to be one of the most potent inducers of DC maturation *in vivo*. Recently, the TLR3 ligand poly I:C was shown to increase the numbers and function of antigen-specific CD8+ T cells in a murine model, which led to protection from a tumor challenge in mice treated with a DC vaccine[71]. The compound imiquimod, an imidazoquinoline that has been recognized as a ligand for TLR7 and TLR8 might also be of interest for combinational therapy. Pre-treatment of the DC injection site with imiquimod induced migration of *ex vivo* generated mouse DCs and obviated the need for *ex vivo* maturation[72]. Although many TLR-ligands are currently under investigation as tools for *in vivo* activation of DCs, there has been a particular focus on the TLR9 ligand CpG-ODN in the past years. Unmethylated CpG-ODN stimulates DCs to express co-stimulatory molecules, increase antigen presentation and secrete particularly Th1-associated cytokines, such as IL-12. Moreover, the addition of CpG-ODN can overcome the inhibitory effects of immune suppressive factors secreted by B16 melanoma cells on bone marrow derived DCs and restore their capacity to activate allogeneic T cells[61]. CpG-ODN also improved the MHC class I peptide-processing pathway, leading to an increased number of cross-presented epitopes on the cell surface of DCs[73]. These effects therefore render CpG-ODN a promising

adjuvant for *in vivo* activation. Indeed, the anti-tumor efficacy of CpG-ODN has been reported in a number of preventive and therapeutic models (reviewed in [74]). In these studies regression of tumors was seen when CpG-ODN was injected intra- or peri-tumorally. The efficacy of CpG-ODN was, however, often negatively correlated with the size of tumors and also seemed to be less when the treated tumor was less immunogenic. Interestingly, CpG-ODN in combination with radiation therapy was shown to delay tumor growth in sarcoma-bearing mice[63] and to induce tumor remission in rats with 9L-glioma[75]. In agreement with this, we have recently found that *in situ* tumor ablation of large established murine tumors by cryo ablation can efficiently be combined with CpG-ODN (Chapter 5).

Future research will have to determine the exact mode of action of TLR-enhanced immune response induction by DCs. It would for instance be of particular interest to gain more insight in how TLR signaling cross-talks with antigen uptake. Another important challenge in future research will be to find inflammatory triggers like for instance activation of members of the TNF receptor superfamily with antibodies that can work synergistically with aforementioned TLR stimuli in creating highly efficient DC vaccines. Finally, one should realize that in contrast to *ex vivo* maturation of purified DC, *in vivo* administration of TLR-ligands or immune activating mAbs will also activate many other cells besides DCs. TLR expression is not limited to DCs and over-stimulation might induce negative side effects.

Migration of in vivo loaded dendritic cells

Studies with *ex vivo* produced DC vaccines have taught us that DC migration is tightly controlled. DC maturation, chemokine receptor and chemokine expression by DCs *in situ* in combination with various tissue-derived factors have all shown to influence DC migration. Although many interesting questions about migration have been answered using *ex vivo* produced and labeled DC vaccines, these studies also indicated that migration of *ex vivo* generated DC is rather inefficient as compared to the *in vivo* situation. For *in vivo* DC vaccines, intra-tumoral expression of the mouse chemokine CCL21 revealed increased DC recruitment to tumor sites with concomitant enhancement of the T cell responses[76]. In addition, CCL20 gene transduction or intra-tumoral injections of CCL20 protein attracted DCs into the tumor core and induced potent anti-tumor responses in CT26 tumor-bearing mice[61]. On the other hand, CCL2/MCP-1 attracted different DC subsets and monocytes, but also promoted tumor angiogenesis through its action on endothelial cells and thus promotes tumor growth[77]. This indicates that careful selection of specific chemokine stimuli is required to attract the right DC subsets. In case antigens are targeted to endogenous DCs, e.g. through surface receptors or by DC specific promoters, natural migration of DCs will not be induced automatically. Although these DCs do not need to travel to the tumor site to take up antigens, they still need to migrate to the lymph nodes to present their antigens. Therefore, it may be beneficial to vigilantly select combinations of chemokines in combination with maturation inducing stimuli to accomplish efficient migration of antigen-loaded DCs to the lymph nodes.

DC-based vaccines: How to overcome immune suppression

Tumors can actively down-regulate anti-tumor immunity and even create a state of immunological unresponsiveness or self-tolerance to tumor antigens (reviewed in [78]). Moreover, in tumor-associated lymph nodes, T cells expressing a CD4+CD25+ regulatory phenotype (Regulatory T cells, Tregs) can be found, that can actively suppress DC function[13]. To exploit the full benefit of DC vaccination, it is therefore important to learn more about the inhibitory pathways that prevent the immune system to eliminate tumors.

Tumor-derived inhibitory factors and dendritic cells

Tumor cells produce immune inhibitory factors, such as IL-10, TGF-β, VEGF and prostaglandins that may lead to a decrease in numbers and function of DCs[78]. For instance, IL-10 and TGF-β have been suggested to affect differentiation, migration and activation of DCs[42]. Moreover, DCs from IL-10 transgenic mice were found to have significantly suppressed IL-12 production and they failed to generate anti-tumor responses[79]. Interestingly, the use of an IL-10 neutralizing antibody increased the capacity of mouse DCs to respond to TLR-ligands[80]. It has also been postulated that tumor-associated factors like IL-10 and TGF-β introduce an imbalance between the expression of co-stimulatory and co-inhibitory molecules, which leads to DCs that support tolerance rather than induce immunity. For example, IL-10 and TGF-β have both been shown to reduce the expression of the co-stimulatory molecules CD80 and CD86 by DC[78], whereas the expression of the inhibitory molecule B7-H1 on DCs is upregulated by factors within the tumor environment. B7-H1 is present on various tumors and on mDC in draining lymph nodes from ovarian carcinomas[81]. Its ligand PD-1, a negative regulator of T cell responses, is also expressed by a significant number of tumor-associated T cells. Antibody-mediated blockade of B7-H1 signaling on human T cells or DC improved DC-mediated T cell activation and inhibited mouse B16 melanoma and CT26 colon cancer growth[82]. These data suggest that forcing expression of B7-H1 on DCs can be regarded as a mechanism of tumor-induced immune evasion.

Also the attraction of tolerance inducing subtypes of DCs may be promoted by the presence of a tumor. In breast carcinoma it was shown that immature DCs appeared to reside within the tumor and mature DCs only in peri-tumoral areas, while total myeloid DC numbers were decreased[83]. Moreover, large numbers of DCs that accumulated in tumor draining lymph nodes of patients were shown to express the immunoregulatory enzyme indoleamine 2,3-diogygenase (IDO)[84], which provides a potential mechanism of DC-mediated suppression of anti-tumor immunity, since IDO inhibits the activation of T cells. The recruitment of a population of IDO-expressing DCs would therefore be advantageous for tumor growth. In agreement, exposing mice to the IDO inhibitor 1-methyl-tryptophan (1MT) indeed delayed Lewis lung carcinoma growth in syngeneic mice *in vivo*[84].

Regulatory T cells and immune suppression

CD4+CD25+ regulatory T cells (Treg) have been shown to be of critical importance in regulation of T cell dependent immune responses and in creating the immunological tolerance to self and non-self (reviewed in [6]). These regulatory T cells are able to suppress

T cell function in a cell contact dependent manner and represent one of the feedback mechanisms used by the immune system to limit excessive immune responses. This suggests that naturally occurring Tregs that are engaged in the maintenance of self-tolerance may at the same time impede immuno surveillance against autologous tumor cells and limit the effect of vaccination. Indeed, Tregs have been found in tumor-associated lymph nodes, where they were shown to suppress conventional T cells, but also DC function[6]. Therefore, the combination of tumor vaccination and manipulation of the suppressing functions of Treg may deliver significant improvement of cancer immunotherapy. In fact, depletion of $CD25^+CD4^+$ T cells before a subsequent tumor challenge provided effective anti-tumor immunity in otherwise non-responding mice[6]. Depletion of Treg can be accomplished by depleting mAbs recognizing molecules present on Treg, but also chemotherapy was shown to decrease circulating regulatory T cell numbers[85]. The latter might be of critical importance when patients are treated with lympho-depleting chemotherapy and subsequent adoptive transfer of tumor directed T cells[86].

Also crosslinking of CTLA-4 on Treg has been suggested to play a role in (Treg-mediated) immune suppression[87]. CTLA-4 blockade is known to lower the threshold for T cell activation, but Tregs also express high levels of CTLA-4. Blockade of CTLA-4 in animal models was shown to improve the effectiveness of cancer immunotherapy, but intriguingly, depletion of CD25+ cells and simultaneous blockade of CTLA-4 even acted synergistically on tumor rejection of B16 melanoma following vaccination[88]. The first human studies applying CTLA-4 blockade have now been reported. The results demonstrated tumor regression in some patients but also various autoimmune manifestations were noted[89,90]. The CTLA-4 injections in these studies were all accompanied by a preceding vaccination. Interestingly, administration of anti-CTLA-4 mAbs in tumor-bearing mice undergoing *in situ* tumor destruction by chemotherapy enhanced tumor regression and survival in the absence of vaccination[91]. Consistently, the release of antigens by radiofrequency ablation of established tumors, followed by anti-CTLA-4 treatment is highly effective in producing *in vivo* anti-tumor immunity, mediated by specific T cells[92].

DC-based vaccines: Where we stand

Dendritic cells are the most professional antigen-presenting cells of our immune system. Mouse studies have clearly demonstrated their unique potency in the induction of anti-tumor immunity. The first studies applying *ex vivo* produced DC-based vaccines in cancer patients have demonstrated that they are safe and have minimal side effects[12]. Although the majority of studies investigated the therapeutic effects of DC vaccines in late-stage cancer patients with metastasis, clinical and immunological responses have been observed. However, the results are variable and long-lasting clinical responses were limited and not observed in all studies. It's fair to state that the use of immature rather than mature DCs in several studies may have affected the immunological and clinical outcome, as immature DC are now well accepted to be less potent for the induction of strong anti-tumor immune responses. The fact that DC maturation is highly complex, combined with the recent

findings that the potency of a DC vaccine is dependent on the type of maturation signals a DC receives, further imply that even the mature DC used could be considered as sub-optimal. In our opinion, small two-armed studies in combination with appropriate characterization of both the DC vaccine and immunomonitoring of the response induced is crucial for further development of DC-based vaccines. We believe that the true potential of these immunostimulatory cells has not yet been fully exploited.

For a broad applicability of DC-based vaccines in the future it is important to be able to load and mature DC directly *in vivo* by for example *in situ* tumor destruction in combination with DC maturation stimuli. This also will allow optimal cross-talk of the DC with all the other components of the immune system especially when combined with other approaches that counteract the immune-suppressive conditions frequently observed in cancer patients (See also Figure 2).

DC-based vaccines: Where we go

Immunotherapy applying tumor-antigen-loaded DCs has proven to be feasible and effective in some patients, but a better understanding of how DCs succeed to induce and modulate immunity is necessary to optimally exploit the DC's full potential in vaccines. Variables that have to be optimized in the next years include the mode of DC preparation, the DC subtype, the dose and timing interval of vaccination, the route of administration, the mode of antigen loading and especially DC maturation. Novel approaches aiming at the *in vivo* loading of DC vaccines have shown promising results, but will nevertheless only be effective when we learn to define the most optimal maturation stimuli *in vivo*. Understanding the complex interactions between the processing of the *in vivo* provided antigens and maturation stimuli, including their time constraints, will be of critical importance to induce optimal cross-presentation and subsequent CD8 T cell responses. Finally, it will be of particular interest to design DC vaccines that can be combined with other anti-tumor therapies. One could think of therapies that help the DC vaccine in performing its task, like switching off regulatory T cells, or therapies that affect other aspects of tumor growth, like anti-angiogenesis treatments.

The challenge for scientists in the next five years will be to further extend our fundamental knowledge of DC immuno-biology and to implement these findings in the rational design of DC immunotherapy for the treatment of cancer patients. Such DC-based vaccination studies, either alone or in combination with other therapies, in patients with low tumor burden will uncover the full potential of DC vaccines and determine its position alongside the conventional cancer treatment modalities.

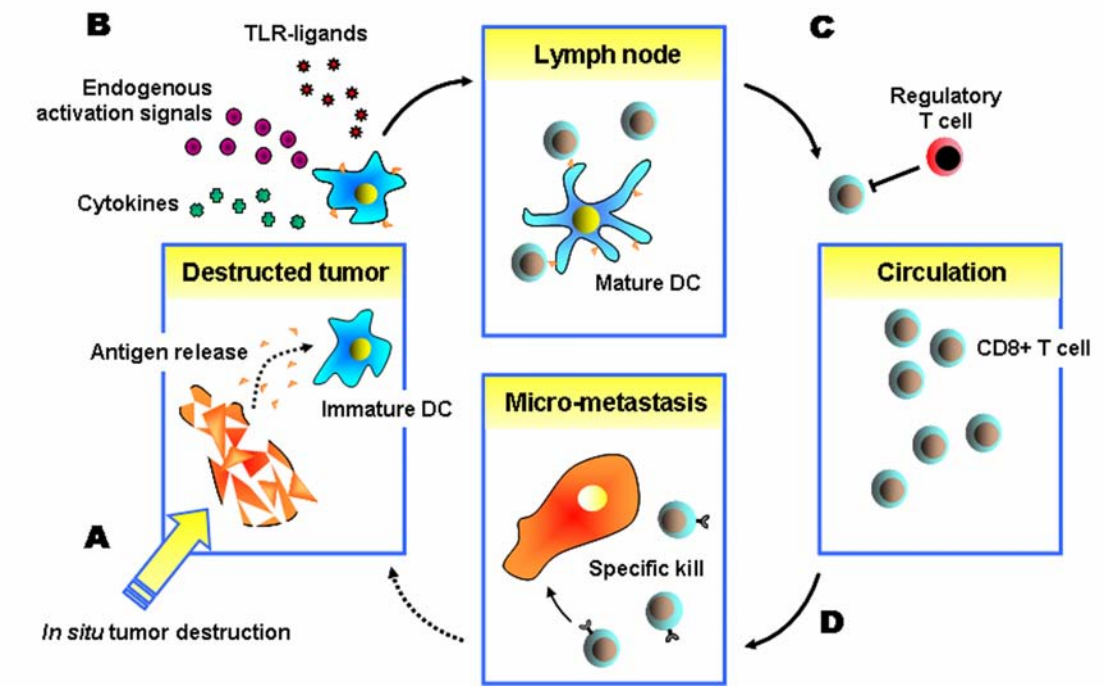

Fig. 2: *In vivo* interventions to obtain an optimal CD8+ T cell anti-tumor response using *in situ* tumor destruction. A) *In situ* tumor destruction provides large amounts of antigen available for uptake and processing by DCs. Tumor antigens are loaded on DCs that migrate towards the draining lymph nodes or are already present in these lymph nodes. **B)** Exogenously administered or endogenously produced stimuli, such as inflammatory cytokines, HSPs or TLR-ligands can work synergistically in creating effective, mature DCs. **C)** The antigens are processed and are presented on MHC molecules to T cells in the lymph node. Interventions like the blockade of CTLA-4 or prevention of immune regulatory processes by for instance regulatory T cells may further stimulate T cell immunity. **D)** Activated T cells migrate to the destructed tumor or to micrometastases, where they kill remaining live tumor cells. The killed tumor cells, might subsequently provide new antigens for DCs.

References

1. Janeway, C.A., Jr. How the immune system works to protect the host from infection: a personal view. *Proc Natl Acad Sci U S A* **98**, 7461-8 (2001).
2. Bevan, M.J. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**, 1283-8 (1976).
3. O'Garra, A. & Murphy, K. Role of cytokines in determining T-lymphocyte function. *Curr Opin Immunol* **6**, 458-66 (1994).
4. Hoffmann, M.W., Heath, W.R., Ruschmeyer, D. & Miller, J.F. Deletion of high-avidity T cells by thymic epithelium. *Proc Natl Acad Sci U S A* **92**, 9851-5 (1995).
5. Sutmuller, R.P., Offringa, R. & Melief, C.J. Revival of the regulatory T cell: new targets for drug development. *Drug Discov Today* **9**, 310-6 (2004).
6. Sakaguchi, S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* **6**, 345-52 (2005).
7. Adams, S., O'Neill, D.W. & Bhardwaj, N. Recent advances in dendritic cell biology. *J Clin Immunol* **25**, 87-98 (2005).
8. Banchereau, J. & Palucka, A.K. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* **5**, 296-306 (2005).
9. McKenna, K., Beignon, A.S. & Bhardwaj, N. Plasmacytoid dendritic cells: linking innate and adaptive immunity. *J Virol* **79**, 17-27 (2005).
10. Schreurs, M.W., Eggert, A.A., Punt, C.J., Figdor, C.G. & Adema, G.J. Dendritic cell-based vaccines: from mouse models to clinical cancer immunotherapy. *Crit Rev Oncog* **11**, 1-17 (2000).
11. Gilboa, E., Nair, S.K. & Lyerly, H.K. Immunotherapy of cancer with dendritic-cell-based vaccines. *Cancer Immunol Immunother* **46**, 82-7 (1998).
12. Figdor, C.G., de Vries, I.J., Lesterhuis, W.J. & Melief, C.J. Dendritic cell immunotherapy: mapping the way. *Nat Med* **10**, 475-80 (2004).
13. Rosenberg, S.A., Yang, J.C. & Restifo, N.P. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* **10**, 909-15 (2004).
14. De Vries, I.J. et al. Maturation of Dendritic Cells Is a Prerequisite for Inducing Immune Responses in Advanced Melanoma Patients. *Clin Cancer Res* **9**, 5091-5100 (2003).
15. Gilboa, E. & Vieweg, J. Cancer immunotherapy with mRNA-transfected dendritic cells. *Immunol Rev* **199**, 251-63 (2004).
16. Nestle, F.O. et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* **4**, 328-32 (1998).
17. Brossart, P. & Bevan, M.J. Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* **90**, 1594-9 (1997).
18. van Broekhoven, C.L., Parish, C.R., Demangel, C., Britton, W.J. & Altin, J.G. Targeting dendritic cells with antigen-containing liposomes: a highly effective procedure for induction of antitumor immunity and for tumor immunotherapy. *Cancer Res* **64**, 4357-65 (2004).

19. Zitvogel, L. et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* **4**, 594-600 (1998).
20. Schuurhuis, D.H. et al. Antigen-antibody immune complexes empower dendritic cells to efficiently prime specific CD8+ CTL responses *in vivo*. *J Immunol* **168**, 2240-6 (2002).
21. Mazzolini, G. et al. Intratumoral injection of dendritic cells engineered to secrete interleukin-12 by recombinant adenovirus in patients with metastatic gastrointestinal carcinomas. *J Clin Oncol* **23**, 999-1010 (2005).
22. Wolkers, M.C., Brouwenstijn, N., Bakker, A.H., Toebes, M. & Schumacher, T.N. Antigen bias in T cell cross-priming. *Science* **304**, 1314-7 (2004).
23. Delamarre, L., Holcombe, H. & Mellman, I. Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *J Exp Med* **198**, 111-22 (2003).
24. Datta, S.K. et al. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J Immunol* **170**, 4102-10 (2003).
25. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-46 (2003).
26. Matzinger, P. The danger model: a renewed sense of self. *Science* **296**, 301-5 (2002).
27. Skoberne, M., Beignon, A.S. & Bhardwaj, N. Danger signals: a time and space continuum. *Trends Mol Med* **10**, 251-7 (2004).
28. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* **5**, 987-95 (2004).
29. Bullock, T.N. & Yagita, H. Induction of CD70 on dendritic cells through CD40 or TLR-stimulation contributes to the development of CD8+ T cell responses in the absence of CD4+ T cells. *J Immunol* **174**, 710-7 (2005).
30. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat Rev Immunol* **4**, 499-511 (2004).
31. Yarovinsky, F. et al. TLR11 Activation of Dendritic Cells by a Protozoan Profilin-Like Protein. *Science* (2005).
32. Hasan, U. et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol* **174**, 2942-50 (2005).
33. Kadowaki, N. et al. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* **194**, 863-9 (2001).
34. Jarrossay, D., Napolitani, G., Colonna, M., Sallusto, F. & Lanzavecchia, A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur J Immunol* **31**, 3388-93 (2001).
35. Steinman, R.M., Hawiger, D. & Nussenzweig, M.C. Tolerogenic dendritic cells. *Annu Rev Immunol* **21**, 685-711 (2003).

36. Jonuleit, H. et al. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T cell responses in humans following intranodal injection. *Int J Cancer* **93**, 243-51 (2001).
37. Mailliard, R.B. et al. alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res* **64**, 5934-7 (2004).
38. Sporri, R. & Reis e Sousa, C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* **6**, 163-70 (2005).
39. Pulendran, B. Variegation of the immune response with dendritic cells and pathogen recognition receptors. *J Immunol* **174**, 2457-65 (2005).
40. De Vries, I.J. et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res* **63**, 12-7 (2003).
41. Adema, G.J., de Vries, I.J., Punt, C.J. & Figdor, C.G. Migration of dendritic cell based cancer vaccines: *in vivo* veritas? *Curr Opin Immunol* **17**, 170-4 (2005).
42. Vicari, A.P. et al. *In vivo* manipulation of dendritic cell migration and activation to elicit antitumour immunity. *Novartis Found Symp* **256**, 241-54; discussion 254-69 (2004).
43. Forster, R. et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* **99**, 23-33 (1999).
44. Ohl, L. et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* **21**, 279-88 (2004).
45. Angeli, V. et al. Dyslipidemia associated with atherosclerotic disease systemically alters dendritic cell mobilization. *Immunity* **21**, 561-74 (2004).
46. Katagiri, K. et al. Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. *Nat Immunol* **5**, 1045-51 (2004).
47. Eggert, A.A. et al. Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res* **59**, 3340-5 (1999).
48. Josien, R. et al. TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells *in vivo*. *J Exp Med* **191**, 495-502 (2000).
49. Mullins, D.W. et al. Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. *J Exp Med* **198**, 1023-34 (2003).
50. Binder, R.J. & Srivastava, P.K. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8(+) T cells. *Nat Immunol* **6**, 593-9 (2005).
51. Eriksson, K. et al. Coupling of antigen to cholera toxin for dendritic cell vaccination promotes the induction of MHC class I-restricted cytotoxic T cells and the rejection of a cognate antigen-expressing model tumor. *Eur J Immunol* **34**, 1272-81 (2004).
52. Moron, V.G., Rueda, P., Sedlik, C. & Leclerc, C. *In vivo*, dendritic cells can cross-present virus-like particles using an endosome-to-cytosol pathway. *J Immunol* **171**, 2242-50 (2003).

53. Bonifaz, L.C. et al. *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* **199**, 815-24 (2004).
54. Hawiger, D. et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. *J Exp Med* **194**, 769-79 (2001).
55. Mahnke, K., Qian, Y., Knop, J. & Enk, A.H. Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. *Blood* **101**, 4862-9 (2003).
56. Tacken, P.J. et al. Effective induction of naive and recall T cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. *Blood* (2005).
57. Engering, A. et al. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J Immunol* **168**, 2118-26 (2002).
58. Ramakrishna, V. et al. Mannose receptor targeting of tumor antigen pmel17 to human dendritic cells directs anti-melanoma T cell responses via multiple HLA molecules. *J Immunol* **172**, 2845-52 (2004).
59. Merad, M., Sugie, T., Engleman, E.G. & Fong, L. *In vivo* manipulation of dendritic cells to induce therapeutic immunity. *Blood* **99**, 1676-82 (2002).
60. Pulendran, B. et al. Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets *in vivo*. *J Immunol* **165**, 566-72 (2000).
61. Furumoto, K., Soares, L., Engleman, E.G. & Merad, M. Induction of potent antitumor immunity by *in situ* targeting of intratumoral DCs. *J Clin Invest* **113**, 774-83 (2004).
62. Delamarre, L., Pack, M., Chang, H., Mellman, I. & Trombetta, E.S. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* **307**, 1630-4 (2005).
63. Milas, L. et al. CpG oligodeoxynucleotide enhances tumor response to radiation. *Cancer Res* **64**, 5074-7 (2004).
64. Weigel, B.J., Rodeberg, D.A., Krieg, A.M. & Blazar, B.R. CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma. *Clin Cancer Res* **9**, 3105-14 (2003).
65. Voll, R.E. et al. Immunosuppressive effects of apoptotic cells. *Nature* **390**, 350-1 (1997).
66. Steinman, R.M., Turley, S., Mellman, I. & Inaba, K. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med* **191**, 411-6 (2000).
67. Taylor, P.R. et al. A hierarchical role for classical pathway complement proteins in the clearance of apoptotic cells *in vivo*. *J Exp Med* **192**, 359-66 (2000).
68. Vabulas, R.M. et al. The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. *J Biol Chem* **277**, 20847-53 (2002).
69. Shi, Y., Evans, J.E. & Rock, K.L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-21 (2003).

70. van Mierlo, G.J. et al. CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. *Proc Natl Acad Sci U S A* **99**, 5561-6 (2002).
71. Salem, M.L., Kadima, A.N., Cole, D.J. & Gillanders, W.E. Defining the antigen-specific T cell response to vaccination and poly(I:C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T cell responses and antitumor immunity. *J Immunother* **28**, 220-8 (2005).
72. Nair, S. et al. Injection of immature dendritic cells into adjuvant-treated skin obviates the need for *ex vivo* maturation. *J Immunol* **171**, 6275-82 (2003).
73. van Mierlo, G.J. et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. *J Immunol* **173**, 6753-9 (2004).
74. Krieg, A.M. Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. *Curr Oncol Rep* **6**, 88-95 (2004).
75. Meng, Y. et al. Successful combination of local CpG-ODN and radiotherapy in malignant glioma. *Int J Cancer* (2005).
76. Vicari, A.P. et al. Antitumor effects of the mouse chemokine 6Ckine/SLC through angiostatic and immunological mechanisms. *J Immunol* **165**, 1992-2000 (2000).
77. Salcedo, R. et al. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* **96**, 34-40 (2000).
78. Zou, W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* **5**, 263-74 (2005).
79. Sharma, S. et al. T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function. *J Immunol* **163**, 5020-8 (1999).
80. Vicari, A.P. et al. Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. *J Exp Med* **196**, 541-9 (2002).
81. Curiel, T.J. et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* **9**, 562-7 (2003).
82. Iwai, Y., Terawaki, S. & Honjo, T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol* **17**, 133-44 (2005).
83. Bell, D. et al. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J Exp Med* **190**, 1417-26 (1999).
84. Mellor, A.L. & Munn, D.H. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* **4**, 762-74 (2004).
85. Ercolini, A.M. et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J Exp Med* **201**, 1591-602 (2005).
86. Dudley, M.E. et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* **23**, 2346-57 (2005).

87. Egen, J.G., Kuhns, M.S. & Allison, J.P. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* **3**, 611-8 (2002).
88. Sutmuller, R.P. et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* **194**, 823-32 (2001).
89. Phan, G.Q. et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* **100**, 8372-7 (2003).
90. Hodi, F.S. et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. *Proc Natl Acad Sci U S A* **100**, 4712-7 (2003).
91. Mokyr, M.B., Kalinichenko, T., Gorelik, L. & Bluestone, J.A. Realization of the therapeutic potential of CTLA-4 blockade in low-dose chemotherapy-treated tumor-bearing mice. *Cancer Res* **58**, 5301-4 (1998).
92. den Brok, M.H. et al. *In situ* tumor ablation creates an antigen source for the generation of antitumor immunity. *Cancer Res* **64**, 4024-9 (2004).

Chapter 3

In situ tumor ablation creates an antigen source for the generation of anti-tumor immunity

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Abstract

Tumor-destructing techniques, like radiofrequency ablation (RFA), allow eradication of large tumors. Potentially, *in situ* tumor destruction can also provide the immune system with an antigen source for the induction of anti-tumor immunity. Antigen presenting cells could take up antigens in the periphery after which they induce specific immune responses. Recent data show that especially antigen presenting dendritic cells (DC) are crucial for the induction of potent immune responses. However, virtually nothing is known regarding the induction of immune responses after *in situ* tumor destruction in neither mice nor men. Herein, we used the well-defined murine B16-OVA melanoma cell line to develop a novel tumor model to explore: 1) the immunological consequences of *in situ* tumor destruction and 2) the efficacy of a combination approach of tumor destruction and immunostimulation. Applying this model system we demonstrate that following RFA, a weak but detectable immune response develops, directed against OVA, but also against a broader range of B16 antigens. Adoptive transfer experiments further indicate that anti-tumor reactivity can be transferred to naïve mice by splenocytes. To augment the response observed, we administered a blocking mAb against cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) at the time of tumor destruction. Interestingly, this strongly enhanced anti-tumor immunity resulting in long-lasting tumor protection. These results illustrate that *in situ* tumor destruction can provide a useful antigen source for the induction of anti-tumor immunity, provided that additional immunostimulatory signals are co-administered.

Introduction

In situ tumor ablation with a thermal energy source such as Radiofrequency (RFA), Laser, Microwave, or Cryo ablation has received increasing attention over the past decades as a minimally invasive technique for treatment of focal cancer, and encouraging results have been obtained. Especially, RFA treatment is widely used for local ablation of liver tumors with a size of up to 4 cm[1,2]. RFA has been proven to be a safe procedure with a complication rate below 10%. The technique can be performed during open surgery or as a minimal invasive procedure when applied percutaneously or via laparoscopy. RFA has been used successfully in the treatment of bone tumors[3], lung tumors[4], renal cancer[5] and primary or metastatic liver tumors[1,2,6,7]. Although RFA treatment is not applicable to all patients, RFA and comparable techniques require fewer resources, result in faster recovery, and in most cases, offer reduced morbidity and mortality compared to surgical resection. Unfortunately, many of these patients will die from multiple metastases that remain untreated. Therefore, the addition of a relevant systemic therapy would be highly valuable.

Upon tumor ablation *in situ,* large amounts of tumor debris are released that could potentially be taken up by the immune system. For long it has been discussed whether or not ablated tumor debris is able to induce a systemic immune response, but a systematic analysis has not been reported yet and convincing evidence is therefore lacking. Few studies report on an occasional patient with spontaneously regressing metastases and reduced numbers of developing secondary foci post-ablation[8,9]. However, based on the results of vaccination studies with large amounts of irradiated autologous tumor cells or tumor lysates[10,11], it is not very likely that a large amount of tumor debris by itself is sufficient to induce a potent anti-tumor response. Moreover, recent insights in the requirements for the induction of an effective immune response demonstrate that maturation of antigen presenting cells, especially dendritic cells, is a prerequisite for the induction of adaptive immunity[12,13]. The importance of immune activation for the induction of anti-tumor immunity has been well established. Expression of, for instance, the B7 molecules appeared to be sufficient to induce T cell mediated rejection of a variety of tumors[14,15]. Likewise, local injections of stimulating antibodies against the co-stimulatory molecule CD40 lead to enhanced systemic anti-tumor responses in mice[16]. Similar results have been obtained by antibody triggering of other stimulatory members of the tumor-necrosis factor receptor (TNFR) family: OX40, 4-1BB, CD27, CD30 (reviewed in [17]). In addition to these stimulatory pathways, also blockade of inhibitory receptors, e.g. CTLA-4, has been applied successfully to induce tumor rejection[18]. In recent years, *ex vivo* generated dendritic cells (DC) loaded with tumor antigens have been studied and shown to evoke tumor-specific responses in cancer patients[19,20]. Potentially, the tumor debris generated after *in situ* tumor destruction can be used as an antigen source for the immune system. Combining *in situ* tumor destruction with immune-potentiating strategies might represent a relatively simple way of *in situ* immune response induction.

Here we report the development of a mouse tumor model that allowed us to explore the potential of *in situ* tumor destruction alone or in combination with immunomodulatory approaches. The results demonstrate that following RFA a weak but detectable immune response is induced. Furthermore, administration of CTLA-4 blocking

antibodies that lower the threshold for T cell activation potentiates the immune response leading to increased tumor protection.

Results

Development of an in situ tumor ablation model

To investigate the induction of anti-tumor immune responses following *in situ* tumor destruction, we developed a mouse B16-OVA model in which Radiofrequency ablation (RFA) was used to destruct established tumors. Initial experiments with the RFA equipment indicated a tight balance between incomplete ablation of B16-OVA tumors resulting in recurrences and too severe ablation of the tumor-surrounding tissue. Therefore, we optimized the size of the tumor at the moment of destruction, duration of the ablative cycles, and the impedance. Two consecutive treatment cycles of 80 sec. with an impedance of 400 ohms, together covering the whole tumor area (7 mm in diameter), yielded the best results (not shown and Fig. 1*b*). Using this treatment regimen, post operation survival rates of 100% were obtained with a recurrence rate of 20% (Fig. 1*b*). We observed that whenever recurrences occurred a few days after treatment, they appeared at the edge of the treated area, and progressed rapidly. Wounds healed completely in 20 to 30 days.

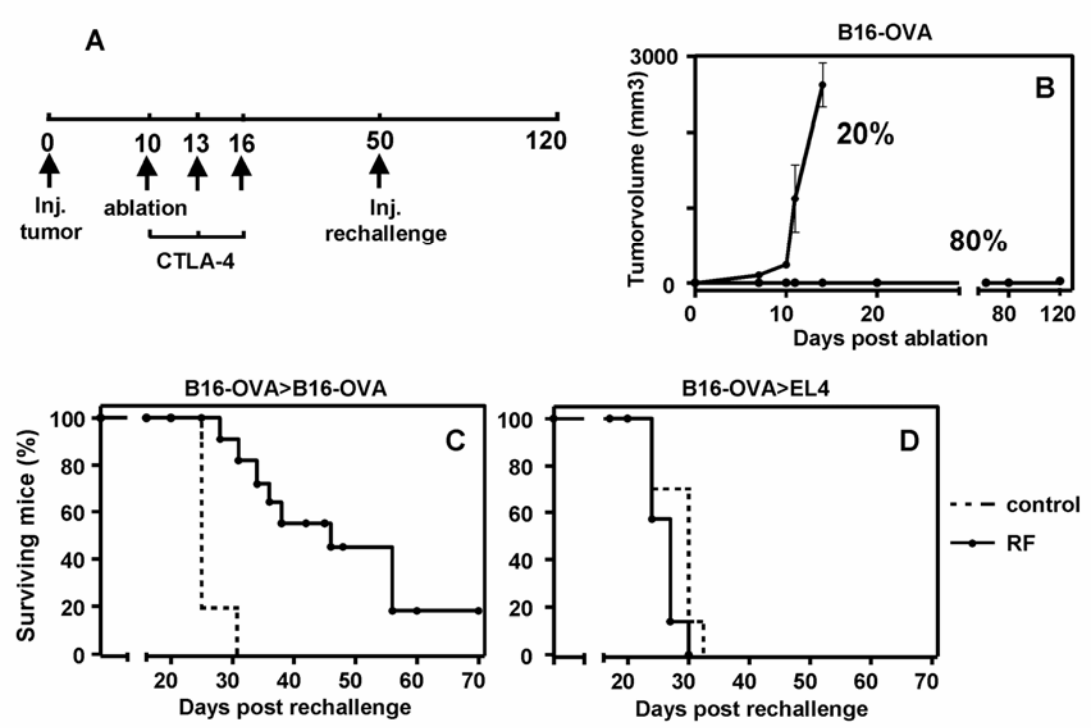

Fig. 1: Induction of a tumor specific immune response following RFA. (A) Time schedule outlining the different treatments as used in the experiments. Ten days after tumor inoculation the B16-OVA melanoma tumors (7mm) were ablated by RFA. Forty days after RFA mice were re-challenged. (B) The overall survival of animals after ablation. T=0 corresponds to the time of ablation. (C+D) Forty days after ablation $15x10^3$ B16-OVA cells (C) or $15x10^3$ EL4 mouse thymoma cells (D) were injected s.c. in the contralateral leg (●). Normal growth was monitored by injection of $15x10^3$ B16-OVA cells into naïve mice (dotted lines). T=0 corresponds to the time of injection of the tumor re-challenge. $P<0,005$ for panel c (n=7-11, one representative experiment out of three independent experiments is shown).

Immune responses after RFA

Using the aforementioned model, we determined whether specific anti-tumor reactivity could be detected after RFA. Hereto, B16-OVA tumor-bearing mice were RFA treated and then re-challenged with either B16-OVA cells or non-related EL4 thymoma cells. A detailed time schedule of the different treatments is given in figure 1*a*. Re-challenges were given 40 days after ablation to exclude direct effects of the RFA treatment on the tumor re-challenge. As surgical excision of the established tumor was not possible due to the occurrence of local recurrences in the majority of these mice, age-matched, untreated naïve mice were used as controls. As shown in figure 1*c*, RFA of B16-OVA resulted in a clear delay in the outgrowth of B16-OVA tumor cells and a low level of protection (20% of the mice). In contrast, no delay in outgrowth of the non-related EL4 mouse thymoma was observed (Fig. 1*d*). These data imply that a weak but tumor-specific immune response had developed after *in situ* tumor destruction by RFA.

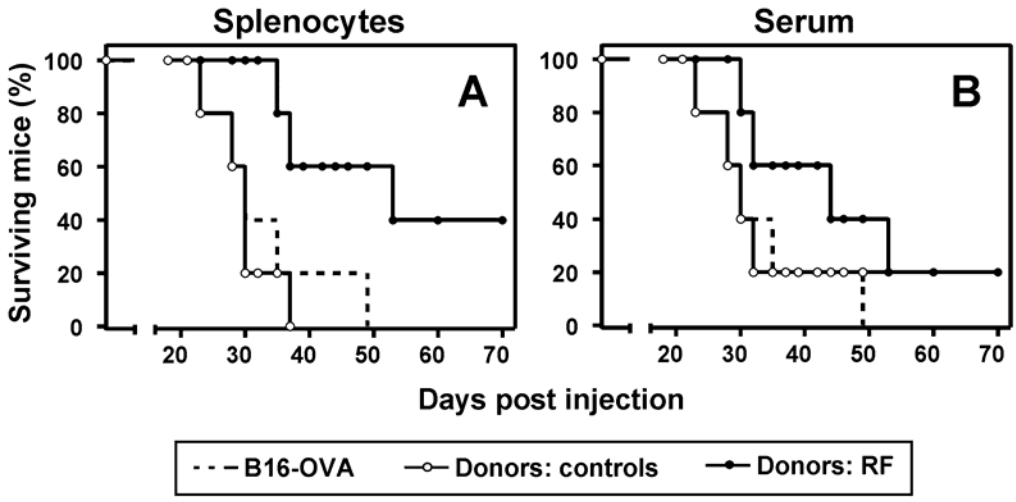

Fig. 2: Adoptive transfer of immune reactivity. Forty days after RFA, mice were injected with $25x10^3$ B16-OVA cells to boost the response. Ten days later, splenocytes (A) or serum (B) of these mice were harvested and transferred to naïve mice. Three days later, recipient mice received a challenge with $15x10^3$ B16-OVA cells (●). Control transfers were performed using spleen cells and serum from mice that received $25x10^3$ B16-OVA 10 days prior to the isolation of spleen and serum (○). Normal growth was monitored by injection of $15x10^3$ B16-OVA cells into naïve mice (dotted lines). T=0 corresponds to the time of injection of the tumor challenge. $P<0,02$ for RF vs. control donor in panel A (n=5 per group, one out of two independent experiments is shown).

Adoptive transfer of specific immunity

To further demonstrate the involvement of the immune system, we investigated whether the observed effects on tumor growth and protection could be transferred from RFA-treated mice to naïve mice via serum or splenocytes. Interestingly, transfer of $35x10^6$ splenocytes of RFA treated mice resulted in delayed tumor outgrowth and partial protection against a lethal B16-OVA challenge (Fig. 2*a*). Transfer of serum of the same mice only

resulted in a minor delay in tumor outgrowth (Fig. 2*b*). No delay in tumor growth was observed after transfer of control splenocytes or serum from mice that received B16-OVA cells 10 days prior to the isolation of splenocytes and serum. (Fig. 2*a*+*b*). These results indicate that the observed immune response is mainly cell-mediated. The finding that splenocytes derived from mice that carry non-ablated B16-OVA tumors are unable to transfer anti-tumor reactivity, demonstrates that specific immunity is not simply induced by a growing B16-OVA tumor, but is in addition dependent on ablation of the tumor.

Long lasting immunity against B16-OVA

Next, we investigated whether the RFA-treated mice that had rejected the initial re-challenge, were protected against a second re-challenge with B16-OVA and wildtype B16-F10, its less immunogenic counterpart. As only a limited number of surviving mice were available, groups of 3 to 4 mice were re-challenged with a lethal dose of B16-OVA in one flank, while B16-F10 was given contra laterally. Interestingly, mice that had rejected the first B16-OVA challenge were completely protected against a second B16-OVA re-challenge 70 days later (Fig. 3). This observation demonstrates that immunological memory was present in these mice. Furthermore, also a delayed outgrowth of the wild type B16-F10 tumor was observed as compared to controls (Fig. 3). These data indicate that the immune response is not only directed against the immuno-dominant OVA epitope but also towards a broader range of B16 antigens.

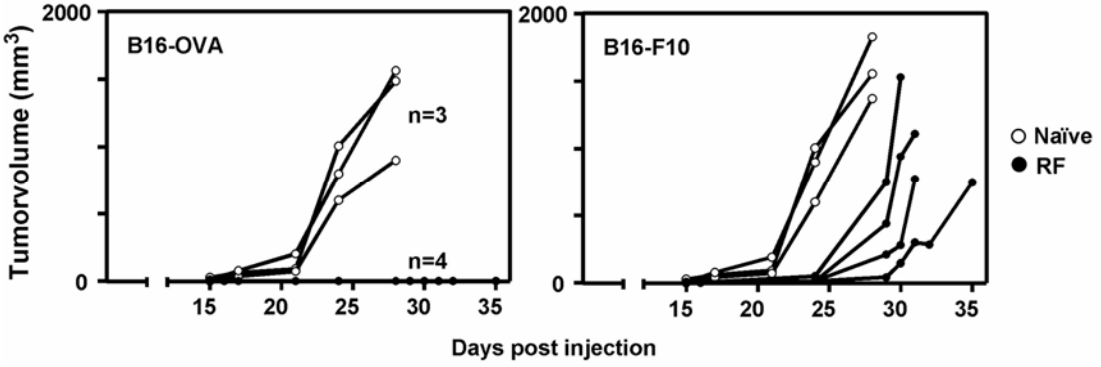

Fig. 3: Memory- and anti-B16 immune response. Mice that had rejected a B16-OVA re-challenge after RFA treatment of a B16-OVA tumor (see Fig. 1) received a second set of re-challenges (●). On the left flank $15x10^3$ B16-OVA cells (left panel) and on the right flank $10x10^3$ B16-F10 wildtype cells (right panel) were injected. The same tumor cell injections were given to naïve mice (○). T=0 corresponds to the time of injection of the second tumor re-challenge. (n=3-4 per group, one out of two independent experiments is shown).

a-CTLA-4 enhances immunity against B16-OVA and induces specific CTL

Administration of blocking mAbs against CTLA-4 has previously been used to facilitate the induction of T cell responses by disrupting the negative regulatory function of CTLA-4[23]. Therefore, we explored whether *in situ* tumor destruction in combination with CTLA-4 blockade could enhance the anti-tumor immunity in our model. Hereto, blocking anti-CTLA-

4 antibody 9H10 or a control antibody was administered on days 0, 3 and 6 after RFA and mice were re-challenged 40 days later. As shown in Fig. 4, the combination of RFA and CTLA-4 treatment resulted in an increase in protection against a lethal B16-OVA injection from 25 to 75% of the mice. No increase in protection was observed when control IgG was administered after RFA (Fig. 4). Furthermore, anti-CTLA-4 treatment without RFA was not sufficient to eradicate either the primary tumor or a tumor challenge given 40 days post antibody injection (not shown).

To determine whether antigen specific T cells are induced after RFA with or without CTLA-4 blockade, we analysed the presence of OVA-specific CD8+ T cells by MHC-tetramer staining after a single re-stimulation of spleen and lymph node cells. As shown in figure 5*a*, a low number of OVA specific cytotoxic T cells (CTL) was detected 10 days after RFA treatment plus control IgG. However, in RFA mice that in addition received CTLA-4 treatment, a 20-fold increase in OVA-specific CTL was observed (Fig. 5*a*). No OVA tetramer-positive cells were discerned in naïve mice.

Analysis of IFN-γ production in response to B16-OVA tumor cells confirmed the increased anti-tumor reactivity in RFA plus CTLA-4-treated mice and further demonstrated that these specific CTL are functional (Fig. 5*b*).

Collectively, these findings demonstrate that *in situ* tumor destruction can provide a useful antigen source for the induction of anti-tumor immunity. Weak anti-tumor T cell responses are generated after RFA-mediated tumor ablation that can be significantly enhanced by co-administration of blocking CTLA-4 antibodies.

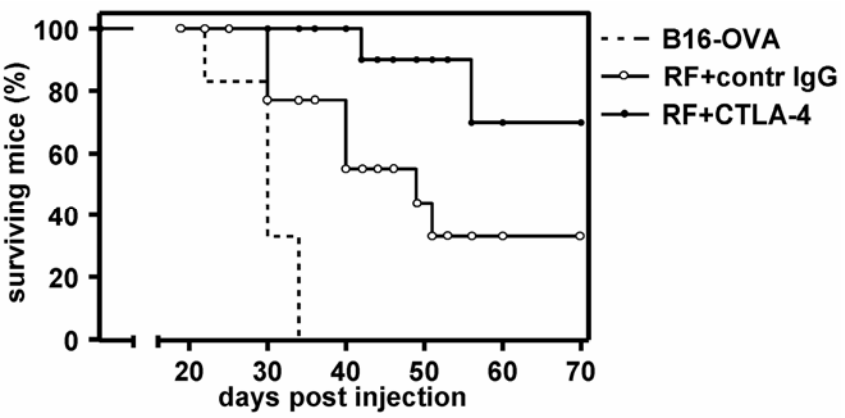

Fig. 4: Co-administration of blocking CTLA-4 mAbs enhances the anti-tumor effect. At day 0, 3 and 6 after RF ablation of B16-OVA, mice received 200μg of anti-CTLA-4 antibody (●) or control IgG (○). Forty days after ablation a re-challenge with $15x10^3$ B16-OVA cells was given in the contralateral leg. Normal tumor growth was monitored by injection of the same amount of cells into naïve mice (dotted line). T=0 corresponds to the time of injection of the tumor re-challenge. *P*<0,05 for both lines. (n=7-11 per group, one out of two independent experiments is shown).

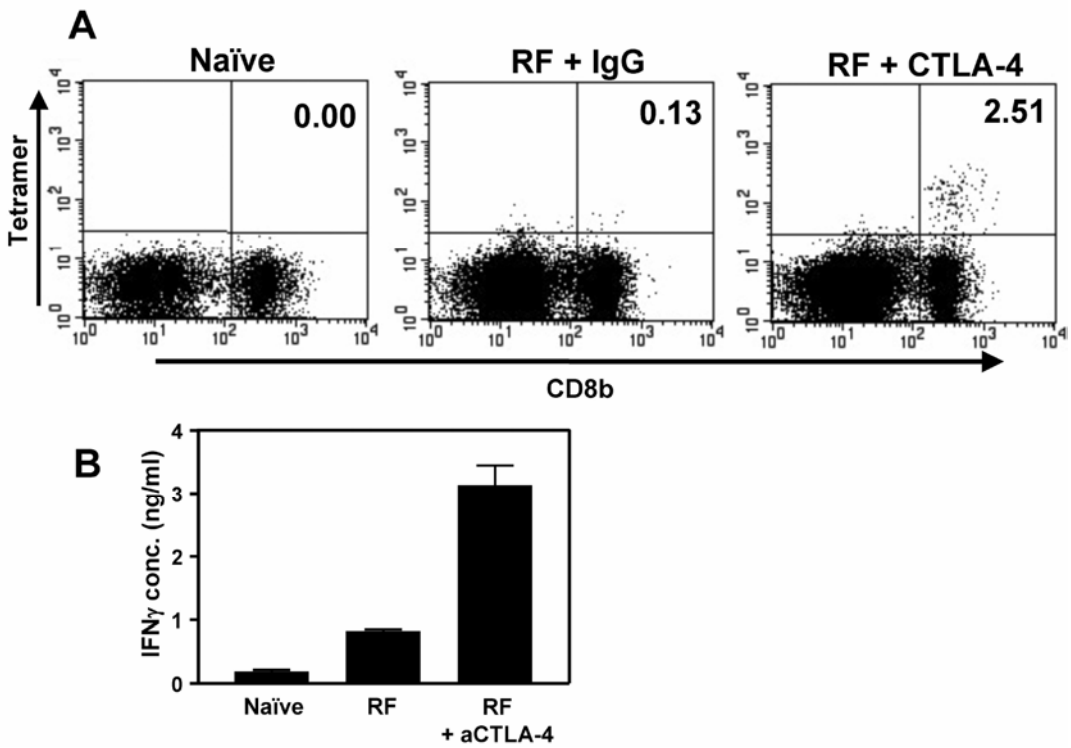

Fig. 5: Increased OVA-specific CTL numbers and IFN-γ production following CTLA-4 blockade. (A) At day 10 after ablation, a mix of lymph node and spleen cells was obtained from mice treated with RFA and control IgG, mice treated with RFA and anti-CTLA-4 antibodies, or naïve mice. T cells were harvested and cultured in the presence of irradiated B16-OVA cells and IL-2 for 10 days, followed by staining with OVA-Kb tetramers and anti-CD8b. Depicted numbers are percentages tetramer-positive cells within the CD8b+ population. (B) IFN-γ production by the stimulated T cell cultures indicated above was analyzed by ELISA. * = p<0,01 when compared to naïve, as determined by St. t-test. Mean values with SD from triplicates are shown, from one out of two independent experiments.

Discussion

Radiofrequency ablation (RFA) is a minimally invasive treatment for local tumor destruction that generates large amounts of tumor debris[2]. Using a newly developed *in situ* tumor destruction model, we now demonstrate that following RFA a weak, but tumor-specific immune response is induced, resulting in protection against a lethal tumor re-challenge in 20% of the mice. The anti-tumor reactivity can be transferred to naïve mice by splenocytes and is directed against multiple tumor antigens. Moreover, the response induced by RFA can be potentiated by co-administration of blocking CTLA-4 antibodies.

For long it has been recognized that heating or freezing of tumors is an effective way of tumor destruction *in situ*. In recent years, technical developments in equipment and monitoring devices have strongly increased the applicability of RFA[1,2,5,6,25,26]. Currently, RFA is widely used for destruction of tumors, particularly in the treatment of inoperable liver metastases. Tumor debris remaining after *in situ* tumor destruction is a potential antigen source for the induction of anti-tumor immunity. However, little is known regarding the induction of anti-tumor immune responses following RFA. Preliminary studies in pig and rabbit revealed an influx of immune cells in the periphery of the coagulation area directly after RFA, together with an increased T cell proliferation[27,28].

We have now demonstrated in a mouse B16-OVA tumor model that *in situ* tumor ablation results in the induction of immunity against a lethal tumor re-challenge given 40 days after tumor ablation. The time span between tumor ablation and tumor re-challenge excludes any direct or non-specific immune effect of the RFA treatment on the growth of the tumor re-challenge. Our finding that ablation of 5-7 mm large B16-OVA tumors resulted in a delay in tumor growth and partial protection against a subsequent B16-OVA but not EL4 tumor re-challenge is indicative for the involvement of a tumor-specific immune response. Adoptive transfer experiments of splenocytes of ablated mice further demonstrated for the first time, that the protective anti-tumor response observed after RFA is predominantly mediated via the cellular arm of the immune system. The finding that no delay in tumor growth or protection was observed after transfer of splenocytes from mice that carry B16-OVA tumors for 10 days without RFA, demonstrates that the effect is at least in part dependent on RFA treatment of the primary tumor.

The mechanism by which RFA induces or enhances immune responses is still poorly understood. Ablative treatments are known to release tumor debris, and a rise in the tumor-associated 'carcinoembryonic antigen' (CEA) has indeed been detected following ablation of colorectal liver metastasis (own observations). A limited number of studies also reported a rise in C-reactive protein, IL-6, soluble TNF-RI and IL-2 after cryoablation of tumor tissue[29,30], but little is known about the cytokine release after RFA. Potentially, scavenging of the tumor debris by immune phagocytes in combination with the release of inflammatory cytokines could be responsible for the observed weak immune responses after RFA. The experiments now indicate that low, but detectable amounts of specific

CD8+ T cells are induced following RFA, which likely also requires CD4+ T cells. Upon combination with CTLA-4 blockade (see below) these CD8+ T cells further expand. The involvement of NK cells and elevated cytokine levels in the delayed outgrowth of tumors can formally not be excluded. However, we consider this unlikely because of the time-span of 40 days between ablation and tumor re-challenge. Moreover, the absence of anti-tumor effects upon a re-challenge with EL-4 cells following ablation of a B16-OVA tumor and vice versa is indicative for the induction of a tumor-specific immune response (Fig. 1 and not shown). Whether the effect of RFA is solely based on the induction of a 'de novo' immune response, occurs as a consequence of the elimination of the suppressive effect of the B16OVA tumor on T cell function, or both, remains to be determined.

Re-challenge experiments in surviving mice, revealed that once mice had rejected B16-OVA, they were completely protected against a new tumor challenge. These data indicate that in the few mice surviving the first challenge, a bonafide systemic memory response was generated. A clear delay in the outgrowth of the wildtype B16-F10 tumor was also observed in the surviving mice. We note that a direct re-challenge with wild-type B16-F10 after ablation of B16-OVA only resulted in a small delay in outgrowth (not shown). These data indicate that the immune response induced by RFA is directed against multiple B16 antigens. However, they also suggest that upon rejection of B16-OVA tumors the pool of B16-F10-reactive T cells is expanded or further broadened by epitope-spreading[31] to obtain the delay in B16-F10 outgrowth in the surviving mice.

The aforementioned data indicate that in our B16-OVA model a weak, but specific anti-tumor response can be achieved following RFA in a limited number of mice. Data on anti-tumor responses in patients after local ablative treatment have only incidentally been reported[8,9]. Based on our current knowledge on immune response induction in general, and the unique requirement for mature professional antigen presenting cells in particular, the aforementioned results imply that immune activation following RFA is sub-optimal. Similarly, vaccination with irradiated tumor cells or tumor cell lysates alone does not result in the induction of a potent anti-tumor immune response in mice or man[10,11].

Several strategies have been reported to augment an immune response, including the administration of stimulatory antibodies to CD40, 4-1BB or blocking mAbs against CTLA-4. Repetitive administration of blocking CTLA-4 mAb has been shown to cure mice from established tumors [32,33] and in combination with anti-tumor vaccination CTLA-4 blockade significantly enhanced the potency of the vaccine[34,35]. In our *in situ* tumor destruction model, we demonstrate that the anti-tumor effect is markedly improved if ablation was accompanied by CTLA-4 blockade. Since CTLA-4 blockade by itself could not abrogate the outgrowth of the tumor (not shown), these data indicate that the ablation of the tumor and the anti-CTLA-4 treatment act synergistically. CTLA-4 has previously been shown to maintain the threshold for a T cell to proceed to full activation and to limit the proliferative ability of activated antigen-specific T cells[18]. Indeed, upon combining CTLA-4 blockade with RFA increased numbers of interferon-γ producing, and OVA-specific CTL were detected. Recent reports also suggest an important role of CTLA-4 in the function of

CD4+CD25+ regulatory T cells[36,37]. Involvement of these regulatory T cells in the tumor destruction model can therefore not be excluded, and is currently under investigation.

In the clinical setting, RFA has been successfully applied for treatment of colorectal liver metastases[7]. More recently, encouraging results have also been obtained in the treatment of breast cancer patients[25]. However, these studies are mainly focused on the successful eradication of the treated tumor foci, whereas little or no clinical benefits have been reported for untreated lesions. Furthermore, when RFA is applied for the destruction of large-sized tumors (>4cm), local recurrence rates increase strongly. Therefore, an efficient systemic anti-tumor therapy in addition to ablation could be highly beneficial. The results described here, indicate that RFA, in combination with immunomodulation, can induce a systemic immune response and might therefore enhance the efficacy of RFA treatment and protection against local recurrences as well as the development of metastasis. Therefore, RFA treatment might offer interesting novel possibilities in combination with existing immunotherapy strategies. The data in this paper suggest that tumor debris is a useful antigen source for the immune system, provided that additional immunostimulatory signals are co-administered.

Materials and Methods

Animals

Male and female C57BL/6n mice were purchased from Charles River Wiga (Sulzfeld, Germany) and kept under specified pathogen-free conditions in the Central Animal Laboratory, Nijmegen University (Nijmegen, the Netherlands). All experiments were performed according to the guidelines for animal care of the Nijmegen Animal Experiments Committee. For ablations and tumor experiments, 9-11 week-old mice were used.

Tumors

Mice were injected subcutaneously in the middle of the right femur with $5x10^5$ cells of the OVA-transfected murine melanoma cell line B16-F10 (B16-OVA, clone MO5), which was kindly provided by dr. Kenneth Rock (Dana Farber Cancer Institute, Boston, MA, USA)[21] or with the murine thymoma cell line EL4 (ATCC). Cells were cultured as described, harvested and injected in a 1:3 mixture of Matrigel (BD Biosciences, Alphen a/d Rijn, the Netherlands) and PBS in a total volume of 50 μl as used before[22]. Evaluation of tumor size was performed every three days using calipers. Tumor volumes were scored with the formula $(A \times B^2) \times 0,4$, in which A is the largest and B is the shortest dimension. Tumors were selected for ablation when their diameter measured between 5 to 7 mm. Mice carrying macroscopic satellite lesions (2 out of 50 mice) were excluded from the experiments, as complete ablation of such lesions appeared difficult. In tumor re-challenge experiments, mice were sacrificed when tumors reached a volume of $\pm$ 850 mm^3.

Radiofrequency ablation

Animals were anaesthetized by isoflurane inhalation and properly shaved at the tumor area and on the contra lateral flank. After placement and proper attachment of the contra lateral side onto an electricity-conducting pad (grounding pad), the tumor area was disinfected with alcohol. A RFA needle with active tip of 8 mm (SMK-15, Cotop, Amsterdam, the Netherlands) was inserted s.c. and placed in the middle of the tumor. After placement of the RFA needle, impedance could be evaluated on the RF lesion generator system (Model RFG-3B, Radionics, Burlington, MA, USA). Next, treatment was started by delivering RFA energy. During two treatment cycles of 80 sec., temperature could be monitored by means of a thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of 75-80°C could be reached.

Re-challenges

Forty days after ablation of tumors, mice were challenged by subcutaneous injection on the contra lateral femur with either $15x10^3$ B16-OVA cells, $10x10^3$ B16-F10 cells (kindly provided by I.J. Fidler) or $15x10^3$ EL4 cells. The amount of tumor cells was based on titration experiments demonstrating that this number of cells yielded a solid 100% tumor

take, while leaving an appropriate experimental window to study the effects of RF ablation. Some mice that rejected the first re-challenge received a second re-challenge. These were inoculated on the left and the right flank. Injections were performed in PBS in a total volume of 100 μl. Statistical analysis for all re-challenges was performed using the log rank Kaplan Meier estimation.

Adoptive transfers

At day 35 after ablation, mice were boosted with a subcutaneous challenge of $25x10^3$ B16-OVA cells to enhance an active immune response. Control mice also received $25x10^3$ B16-OVA cells. Ten days afterwards, serum and spleens were isolated and single cell suspensions were obtained by crushing and a passage through nylon mesh. Lymphocytes were concentrated by density gradient centrifugation (Lympholyte-M, Cedarlane Laboratories, Sanbio, Uden, the Netherlands). Then, recipient naïve mice received $35x10^6$ lymphocytes or 200 μl serum intravenously in the tail vein from RFA-treated or control donor mice. These mice were challenged with $15x10^3$ B16-OVA cells three days later.

CTLA-4 treatment

The hamster hybridoma 9H10[23] was cultured and, using standard isolation procedures, IgG was collected. Total hamster IgG was used as control antibody (Jackson Immunoreagents, West Baltimore Pike, PE, USA). Directly after RFA, at day 3 and at day 6 mice received an intraperitoneal injection of 200 μg anti-CTLA-4 or hamster IgG in a total volume of 200 μl.

Tetramer stainings

A T cell culture was obtained from splenocytes and draining lymph nodes of mice 10 days after ablation of a B16-OVA tumor. Cells obtained from naïve age-matched mice were used as controls. Stimulation of these cells ($1x10^5$) was performed by addition of irradiated, IFN-γ-treated, B16-OVA cells ($5x10^4$) in IL-2 (10 Cetus U/ml) supplemented culture medium. At day 5 and day 10 (prior to staining) cells were collected, after which dead cells were removed by a Ficol-Hypaque gradient. The OVA specific CTL clone OVA-2 was cultured as described [24] and used as positive readout (data not shown). At day 10 of culture, cells were stained for 15 minutes at RT by OVA-tetramers (H2Kb) conjugated to APC, which were a kind gift of S.H. van der Burg (LUMC, Leiden, The Netherlands). Subsequently, cells were counterstained for CD8b-FITC (BD Pharmingen) and propidium iodide (Sigma) and analyzed on a FACS-Calibur™ system (BD) with the CELLQuest software. Values are presented as percentages of tetramer positive cells within the total CD8b+ population.

IFN-γ ELISA

The same bulk cultures as described for the tetramer stainings were used to collect supernatant 24 hours after stimulation. Capture and biotinylated detection antibodies directed to mouse IFN-γ were purchased from BD Pharmingen and, using standard ELISA

procedures, IFN-γ concentration was measured in 50 μl of supernatant. Data were analyzed for statistical significance by Student's T-test.

References

1. Curley, S.A. Radiofrequency ablation of malignant liver tumors. *Ann Surg Oncol* **10**, 338-47 (2003).
2. Garcea, G., Lloyd, T.D., Aylott, C., Maddern, G. & Berry, D.P. The emergent role of focal liver ablation techniques in the treatment of primary and secondary liver tumours. *Eur J Cancer* **39**, 2150-64 (2003).
3. Rosenthal, D.I., Hornicek, F.J., Torriani, M., Gebhardt, M.C. & Mankin, H.J. Osteoid osteoma: percutaneous treatment with radiofrequency energy. *Radiology* **229**, 171-5 (2003).
4. Zagoria, R.J., Chen, M.Y., Kavanagh, P.V. & Torti, F.M. Radiofrequency ablation of lung metastases from renal cell carcinoma. *J Urol* **166**, 1827-8 (2001).
5. Raj, G.V., Reddan, D.J., Hoey, M.B. & Polascik, T.J. Management of small renal tumors with radiofrequency ablation. *Urology* **61**, 23-9 (2003).
6. Curley, S.A. & Izzo, F. Radiofrequency ablation of primary and metastatic hepatic malignancies. *Int J Clin Oncol* **7**, 72-81 (2002).
7. Ruers, T.J., Joosten, J., Jager, G.J. & Wobbes, T. Long-term results of treating hepatic colorectal metastases with cryosurgery. *Br J Surg* **88**, 844-9 (2001).
8. Soanes, W.A., Ablin, R.J. & Gonder, M.J. Remission of metastatic lesions following cryosurgery in prostatic cancer: immunologic considerations. *J Urol* **104**, 154-9 (1970).
9. Sanchez-Ortiz, R.F., Tannir, N., Ahrar, K. & Wood, C.G. Spontaneous regression of pulmonary metastases from renal cell carcinoma after radiofrequency ablation of primary tumor: an *in situ* tumor vaccine? *J Urol* **170**, 178-9 (2003).
10. Jager, E., Jager, D. & Knuth, A. Antigen-specific immunotherapy and cancer vaccines. *Int J Cancer* **106**, 817-20 (2003).
11. Sinkovics, J.G. & Horvath, J.C. Vaccination against human cancers (review). *Int J Oncol* **16**, 81-96 (2000).
12. De Vries, I.J. et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res* **63**, 12-7 (2003).
13. De Vries, I.J. et al. Maturation of Dendritic Cells Is a Prerequisite for Inducing Immune Responses in Advanced Melanoma Patients. *Clin Cancer Res* **9**, 5091-5100 (2003).
14. Chen, L. et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* **71**, 1093-102 (1992).
15. Townsend, S.E. & Allison, J.P. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science* **259**, 368-70 (1993).
16. van Mierlo, G.J. et al. CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. *Proc Natl Acad Sci U S A* **99**, 5561-6 (2002).
17. Croft, M. Co-stimulatory members of the TNFR family: keys to effective T cell immunity? *Nat Rev Immunol* **3**, 609-20 (2003).

18. Egen, J.G., Kuhns, M.S. & Allison, J.P. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* **3**, 611-8 (2002).
19. Schreurs, M.W., Eggert, A.A., Punt, C.J., Figdor, C.G. & Adema, G.J. Dendritic cell-based vaccines: from mouse models to clinical cancer immunotherapy. *Crit Rev Oncog* **11**, 1-17 (2000).
20. Banchereau, J., Schuler-Thurner, B., Palucka, A.K. & Schuler, G. Dendritic cells as vectors for therapy. *Cell* **106**, 271-4 (2001).
21. Falo, L.D., Jr., Kovacsovics-Bankowski, M., Thompson, K. & Rock, K.L. Targeting antigen into the phagocytic pathway *in vivo* induces protective tumour immunity. *Nat Med* **1**, 649-53 (1995).
22. Luo, Y. et al. Transcription factor Fos-related antigen 1 is an effective target for a breast cancer vaccine. *Proc Natl Acad Sci U S A* **100**, 8850-5 (2003).
23. Krummel, M.F. & Allison, J.P. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182**, 459-65. (1995).
24. Schreurs, M.W., Eggert, A.A., de Boer, A.J., Figdor, C.G. & Adema, G.J. Generation and functional characterization of mouse monocyte-derived dendritic cells. *Eur J Immunol* **29**, 2835-41 (1999).
25. Noguchi, M. Minimally invasive surgery for small breast cancer. *J Surg Oncol* **84**, 94-101; discussion 102 (2003).
26. Greve, J.W. Alternative techniques for the treatment of colon carcinoma metastases in the liver: current status in The Netherlands. *Scand J Gastroenterol Suppl*, 77-81 (2001).
27. Hansler, J. et al. Cellular and vascular reactions in the liver to radiofrequency thermo-ablation with wet needle applicators. Study on juvenile domestic pigs. *Eur Surg Res* **34**, 357-63 (2002).
28. Wissniowski, T.T. et al. Activation of tumor-specific T lymphocytes by radiofrequency ablation of the VX2 hepatoma in rabbits. *Cancer Res* **63**, 6496-500 (2003).
29. de Jong, K.P. et al. Serum response of hepatocyte growth factor, insulin-like growth factor-I, interleukin-6, and acute phase proteins in patients with colorectal liver metastases treated with partial hepatectomy or cryosurgery. *J Hepatol* **34**, 422-7 (2001).
30. Huang, A. et al. Phase I study of percutaneous cryotherapy for colorectal liver metastasis. *Br J Surg* **89**, 303-10 (2002).
31. Ribas, A., Timmerman, J.M., Butterfield, L.H. & Economou, J.S. Determinant spreading and tumor responses after peptide-based cancer immunotherapy. *Trends Immunol* **24**, 58-61 (2003).
32. Leach, D.R., Krummel, M.F. & Allison, J.P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* **271**, 1734-6. (1996).
33. Shrikant, P., Khoruts, A. & Mescher, M.F. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity* **11**, 483-93 (1999).
34. Sutmuller, R.P. et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals

alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* **194**, 823-32. (2001).

35. van Elsas, A., Hurwitz, A.A. & Allison, J.P. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* **190**, 355-66 (1999).
36. Takahashi, T. et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* **192**, 303-10. (2000).
37. Read, S., Malmstrom, V. & Powrie, F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* **192**, 295-302. (2000).

Chapter 4

Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumor immunity

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Submitted

Abstract

Dendritic cells (DC) are professional antigen presenting cells that play a pivotal role in the induction of immunity. *Ex vivo*-generated, tumor antigen-loaded mature DC are currently exploited as cancer vaccines in clinical studies. However, antigen loading and maturation of DC directly *in vivo* would greatly facilitate the application of DC-based vaccines. We formerly showed in murine models that radiofrequency-mediated tumor destruction can provide an antigen source for the *in vivo* induction of anti-tumor immunity, but the role of DC herein has not been elucidated. In this paper we evaluate radiofrequency and cryo ablation for their ability to provide an antigen source for DC and compare this with an *ex vivo*-loaded DC vaccine. The data demonstrate that upon tumor destruction by radiofrequency ablation, up to 7% of the total draining lymph node DC contained antigen, whereas only few DC from the conventional vaccine reached the lymph node. Interestingly, following cryo ablation the amount of antigen-loaded DC is almost doubled. Analysis of surface markers revealed that both destruction methods were able to induce DC maturation. Finally, we show that *in situ* tumor ablation can be efficiently combined with immune modulation by anti-CTLA-4 antibodies or regulatory T cell depletion. These combination treatments protected mice from the outgrowth of tumor challenges, and led to *in vivo* enhancement of tumor-specific T cell numbers, which produced more IFN-γ upon activation. Therefore, *in situ* tumor destruction in combination with immune modulation creates a unique, '*in situ* DC-vaccine' that is readily applicable in the clinic without prior knowledge of tumor antigens.

Introduction

Dendritic cells (DC) are crucial antigen presenting cells for the initiation of primary T cell responses[1,2]. Immature DC are well equipped to take up and process antigen from their surroundings, but they lack sufficient co-stimulatory signals required for productive T cell activation. In a stimulatory environment, like in an infection, immature DC undergo activation, maturation and acquire the capacity to cross-present exogenous antigens in MHC class I. Particularly, the Toll-Like Receptor (TLR) family of proteins initiates the DC maturation process upon recognition of conserved pathogen-associated molecular patterns (PAMPs), like LPS or unmethylated CpG oligodeoxynucleotides (CpG-ODN). Upon maturation, co-stimulatory molecule and MHC-peptide complex expression increases and cytokines like IL-12 skew the functional outcome of the response[3]. DC that did not perceive an activating environment while taking up antigen do not mature and induce tolerance rather than immunity. The importance of immune activation for the induction of anti-tumor immunity has been well established. Next to direct activation of DC by PAMPs, a broad range of indirect strategies has been explored to accomplish activation of the immune system. Expression of, for instance, co-stimulatory molecules on tumors can induce T cell-mediated rejection of a variety of tumors[4]. In addition to these stimulatory pathways, also blockade of inhibitory receptors, e.g. CTLA-4, has been applied successfully to induce tumor rejection[5,6]. In this respect, also *in vivo* depletion of regulatory T cells, that are able to suppress conventional T cell expansion, has been shown to evoke anti-tumor immunity[7,8].

Since murine models demonstrate that especially DC are effective in inducing effective immune responses, *ex vivo* generated DC are currently applied to stimulate anti-tumor immunity in clinical trials[9-13]. Although tumor-specific responses have been obtained with tumor antigen-loaded DC-based vaccines, many questions remain unanswered[10,14]. Especially the migration of *ex vivo* generated DC-based vaccines from i.d./s.c. injected depots to the draining lymph nodes has been shown to be inefficient in both mouse models and patients[11,15,16]. Moreover, *ex vivo* generation and loading of DC is time consuming and costly. *In vivo* loading and maturation of DC would therefore improve the applicability of DC vaccination to a great extend.

Recent studies using antigens coupled to antibodies directed against the mouse DC antigen DEC-205[17] or attraction of DC to the tumor via retrovirus-mediated expression of the DC-attracting chemokine CCL20[18] illustrate the possibility to directly load tumor antigens onto DC *in vivo*. We previously showed in a murine model that the tumor debris left in the body after *in situ* tumor destruction by radiofrequency ablation is an *in vivo* tumor antigen source for the immune system. Adoptive transfer experiments demonstrated that the immunity induced is T cell-dependent, but the role of DC herein has not been described. Tumor ablative treatments, like cryo or radiofrequency ablation, are successfully used in clinical settings to destruct different types of tumors[19-23]. Nevertheless, cancer patients treated with ablative regimens mostly develop systemic recurrences as a consequence of the outgrowth of distant micro-metastases, implying that in general no protective immune response is induced. This observation is consistent with our findings

that efficient induction of immunity following ablation requires that additional immune activation stimuli are given simultaneously[24].

We now show that *in situ* tumor destruction by either cryo or radiofrequency ablation can be employed to efficiently provide antigens to DC *in vivo*. A side by side comparison of the two ablative techniques demonstrates that following cryo ablation of established B16 tumors (5-7mm) up to 13% of DC in the draining LN acquire tumor-derived antigen. Radiofrequency ablation results in 7% of LN DC being loaded with tumor antigen, which is still a far better yield compared to a conventional *in vitro* generated DC vaccine. We further show that both destruction methods in itself were able to enhance DC maturation *in vivo* to an equal extend. Finally, we report that both tumor ablation techniques can be efficiently combined with immuno-modulatory approaches, like blockade of CTLA-4 signaling or regulatory T cell depletion, to induce functional CD8+ T cells creating systemic anti-tumor-immunity.

Therefore, *in situ* tumor destruction combined with immuno-modulatory approaches constitutes a powerful '*in situ* DC-vaccine' for which no prior knowledge of tumor antigens is needed.

Results

Immune responses following radiofrequency or cryo ablation

We previously demonstrated that radiofrequency ablation of established (5-7mm) murine tumors resulted in weak, but tumor specific anti-tumor reactivity. However, the mechanism by which immunity is induced and the role of DC herein remain largely unknown. Therefore, we explored the fate of tumor debris generated by two distinct tumor ablation approaches and the putative role of DC in the subsequent induction of immune responses. To first compare the induction of immunity after both techniques, B16-OVA tumor-bearing mice were treated with either radiofrequency or cryo ablation and then re-challenged with either B16-OVA cells or non-related EL4 thymoma cells. A detailed time schedule is given below figure 1. Re-challenges were given 40 days after ablation to exclude direct effects of the ablations on the tumor re-challenge. As shown in figure 1, radiofrequency ablation of B16-OVA resulted in a clear delay in the outgrowth of B16-OVA tumor cells as compared to naïve controls and a low level of protection (20% of the mice, lower right panel). Interestingly, when mice received cryo ablation, slightly more mice were protected (50% of the mice, upper right panel). In contrast, no delay in outgrowth of the non-related EL4 mouse thymoma was observed (left panels). These data imply that a weak, but tumor-specific immune response had developed after both *in situ* tumor destruction techniques. In all experiments, cryo ablation was slightly more effective than radiofrequency ablation.

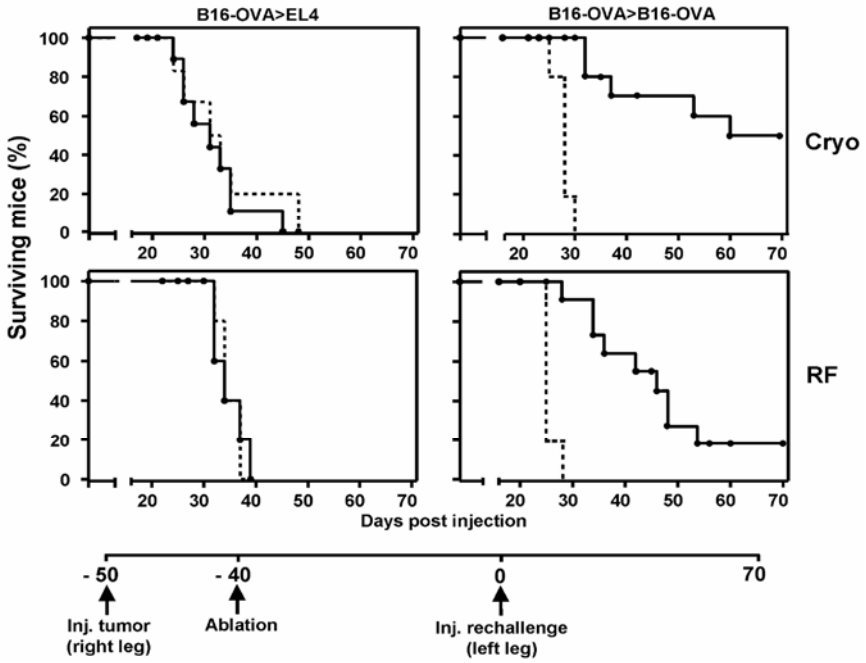

Fig. 1: Anti-tumor immunity following radiofrequency or cryo ablation. Mice with established B16-OVA melanomas (5-7mm) were ablated by cryo (cryo, upper panels) or radiofrequency ablation (RF, lower panels). Forty days later, a re-challenge with $15x10^3$ EL4 (left panels) or $15x10^3$ B16-OVA cells (right panels) was given s.c. in the contra-lateral leg. Figures depict survival curves demonstrating specificity of the response and growth reduction/protection after ablation. As a control, tumor growth was monitored by injection of the same tumor dose into naïve mice (dotted lines). T=0 corresponds to the time of injection of the tumor re-challenge. $P<0,005$ for both B16 lines vs. control. One out of four representative experiments is shown (n=5-11).

Efficient in vivo antigen acquisition by LN CD11c(+) DC following ablation

To determine the fate of tumor antigens and the involvement of DC in the observed anti-tumor immune responses, we studied *in vivo* antigen acquisition by DC following radiofrequency and cryo ablation. Mice carrying established B16-OVA tumors (5-7mm) received an intra-tumoral injection of ^{111}Indium-labeled KLH or ^{111}In-OVA tracer proteins prior to ablation to monitor the fate of tumor-debris. γ-Camera imaging demonstrated that ^{111}In proteins remained in the tumor for at least 72 hrs, whereas unbound $^{111}InCl_3$ rapidly distributes throughout the mice (Fig. 2*a*). Even following both ablation procedures ^{111}In-KLH and OVA largely remained at the treated site whereas only minor local spreading was observed. In case ^{111}In-OVA was used slightly more spreading to the liver was seen compared to KLH, which is likely dependent on the molecular characteristics of OVA. Biodistribution analysis revealed an ablation dependent accumulation of radioactivity in the draining LN's when ^{111}In-KLH (Fig. 2*b-d*) or ^{111}In-OVA (not shown) was used. Although significantly different from untreated tumor-bearing mice, radiofrequency ablated mice showed significantly less accumulation of radioactivity compared to cryo ablation (Fig. 2*b*). Little or no activity was found in the non-draining LN's, liver, kidneys or spleen with or without ablation (Fig. 2*c*), whereas the organs contained high concentrations of radioactivity following injection of unbound $^{111}InCl_3$ (not shown). Applying magnetic bead sorting on the pan-DC marker CD11c we analyzed the cells in the draining LN's containing ^{111}In-KLH. As shown in Fig. 2*d*, following ablation the cell-associated radioactivity was largely present in the CD11c(+) fraction at one and even three days after ablation. Despite the fact that the CD11c(+) DC only comprised a minor fraction of total LN cells, they accounted for up to 25% of total cell-associated activity present in the LN's (cryo ablation, not shown). Consistent with Fig. 2*b*, uptake of ^{111}In-KLH following radiofrequency ablation was significantly lower compared to cryo ablation.

These data thus demonstrate that the tumor-debris created by ablation acts as an antigen depot and that released antigens preferentially accumulate in CD11c(+) DC in the draining LN. They also show that the method of destructing a tumor is highly relevant for the subsequent uptake dynamics of tumor antigens.

Accumulation of antigen positive LN DC following ablation or conventional DC vaccination.

To compare the *in vivo* loading of DC by ablation with an externally loaded conventional DC vaccine, we analyzed the numbers of antigen positive LN DC after performing both techniques. In order to trace the antigen-experienced DC by flowcytometry, ovalbumin conjugated to the fluorophore Alexa-488 (OVA-Alexa) was injected i.t. prior to ablation and DC derived from GFP-transgenic mice were used as an *ex vivo* DC vaccine. These GFP DC were matured with LPS, loaded with the relevant OVA-K^b peptide (SIINFEKL) and $1x10^6$ DC were next injected subcutaneously (s.c.) or around an established (5-7mm) B16-OVA tumor (p.t.). As shown in Fig. 3*a*, over 12% of all draining LN CD11c(+) DC became OVA-Alexa(+) after cryo ablation, whereas after radiofrequency ablation 7% of DC acquired the antigen. These data are in line with the accumulation of antigens in DC observed with ^{111}In-proteins (see also Fig. 2). Consistent with other studies[11,16,25], only small numbers (<1%) of the *ex vivo* generated GFP DC reached the draining lymph node (Fig. 3*b*).

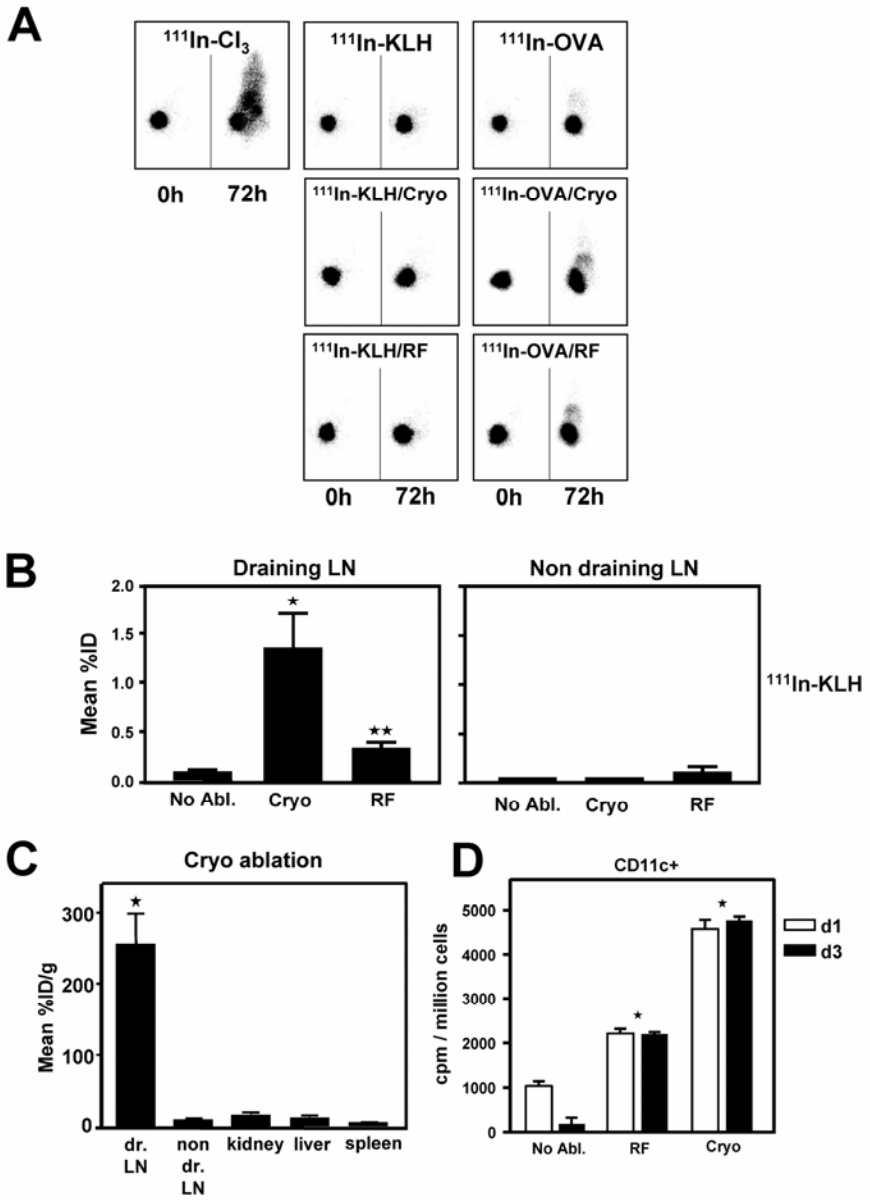

Fig. 2: Preferential uptake of tumor-derived antigens by LN dendritic cells. (A) To study the fate of tumor antigens after ablation, 20 μCi of ^{111}In-Cl_3 (left panel), ^{111}In-KLH (middle panels) or ^{111}In-OVA (right panels) was injected into established B16-OVA tumors (5-7mm). Tumors were left untreated or ablated by cryo ablation (cryo) or radiofrequency ablation (RF) directly after these injections. γ-camera imaging was performed at the indicated time points. For ^{111}In-Cl_3-injected mice the contours, tumor and liver are visible. One representative mouse out of three is shown. (B-C) Biodistribution of ^{111}In-KLH was determined in dissected LNs and organs of mice injected i.t. one day before. Tumors were either left untreated or ablated directly after KLH injection. Radioactivity values from LN's of 4 mice per group are presented as mean percentages of injected dose with s.d., whereas the values for the organs are also corrected for weight. Mice in panel C received cryo ablation, but comparable results were obtained with RF ablation. (D) LN suspensions from non-ablated and ablated mice (5 mice pooled per group) that received i.t. ^{111}In-KLH were subjected to magnetic bead sorting of CD11c(+) cells. After sorting at day 1 and 3 after ablation, the cell-associated radioactivity was measured in the CD11c(+) and CD11c(-) (not shown) cell fraction. Values are presented as counts per minute, corrected for $1x10^6$ cells and natural decay, with sd from triplicates. *= P<0,005 compared to no ablation.

Moreover, plotting the *absolute* numbers of DC present in the draining lymph node further revealed a significant increase in total DC numbers after ablation, accompanied by an increased lymph node volume (Fig. 4 and not shown). This implies that the absolute number of DC actually loaded with tumor antigen is even higher than can be concluded from the relative percentages of antigen-loaded DC shown in Fig. 3*a*.

These data thus indicate that ablation is far more effective in obtaining tumor antigen-loaded DC in the draining LN as compared to conventional DC vaccination.

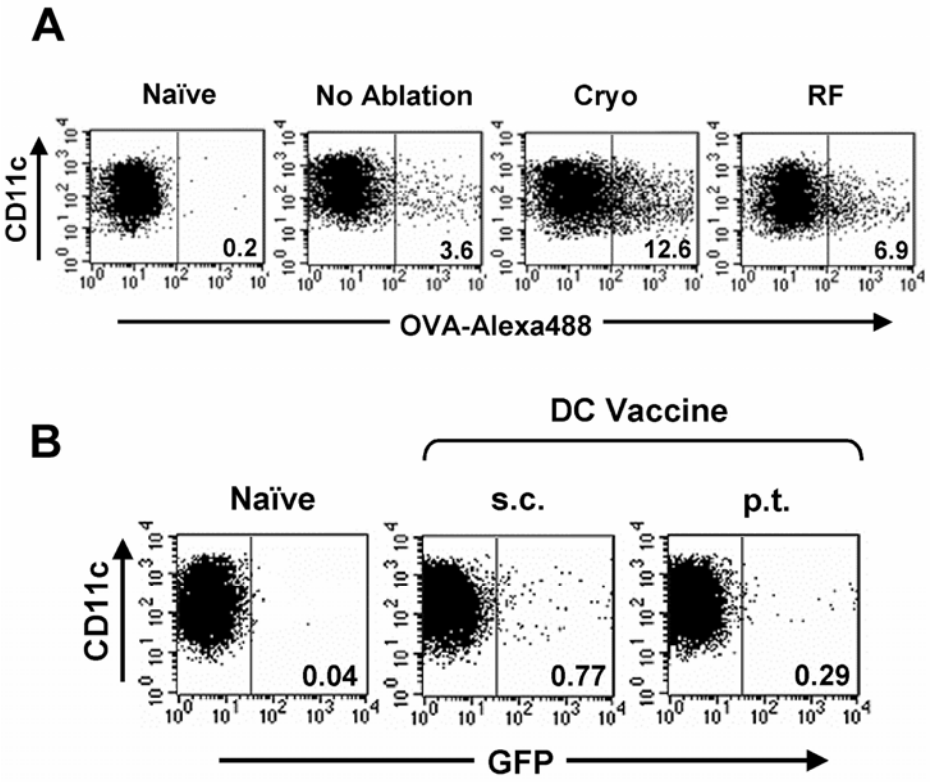

Fig. 3: Increased numbers of antigen positive DC in draining LN following ablation. (A) FACS analysis of CD11c(+) DC isolated from pooled LN suspensions of naïve, tumor-bearing or tumor-ablated mice (n=6 per group). Mice received 20 μg ovalbumin conjugated to Alexa-488 (OVA-Alexa488) i.t. just prior to the time point of ablation. Two days after the indicated treatments, CD11c(+) DC were isolated from draining lymph nodes, stained for CD11c (clone HL3), gated, and plotted for OVA-Alexa488 content. Values shown are percentages of OVA-Alexa488(+) cells within the CD11c(+) fraction. (B) BMDC were cultured from GFP-transgenic mice, loaded with peptides and matured *ex vivo* with LPS. $1x10^6$ DC were injected peri-tumoral (p.t.) in tumor-bearing mice or subcutaneously into naïve mice (s.c.). Two days later CD11c(+) DC were isolated from pooled LN suspensions (n=6 per group), stained, gated, and plotted for GFP. As controls for all experiments, naïve mice were used that did not receive any injection (naïve).

Ablation-dependent maturation of antigen positive LN DC.

Next, we studied the maturation of DC in relation to antigen uptake. Therefore, OVA-Alexa(+) or OVA-Alexa(-) (in vaccination: GFP(+) or (-)) DC were analyzed for expression of the DC maturation marker CD80. OVA-Alexa(+) DC showed a 3-fold increase in CD80 expression relative to OVA-Alexa(-) DC in tumor-bearing and naïve mice (MFI's 987, 318 and 310 resp.) (Fig 5*a*). Moreover, CD80 expression further increased following cryo or

radiofrequency ablation on OVA-Alexa(+) DC but had no effect on OVA-Alexa(-) DC (MFI 1690 vs. 362 and 1567 vs. 361 resp.).

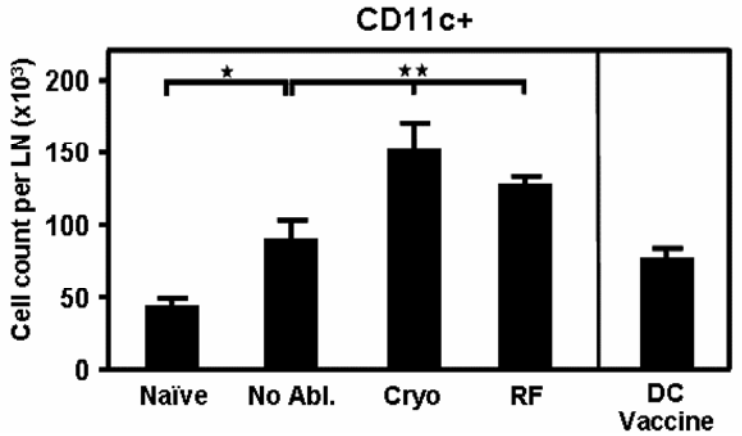

Fig. 4: Absolute CD11c(+) cell count per LN. Data are obtained from experiments described in Fig. 3 and presented as means with sd from three independent experiments. *=*P*<0,005 vs. naïve, **=*P*<0,01 both vs. no ablation.

This indicates that DC that acquired OVA-Alexa, as an indicator of tumor-debris, preferentially up-regulate CD80, while this effect is further enhanced by ablation. Importantly, the induction of maturation by radiofrequency ablation is equal to the induction by cryo ablation, even though less antigen uptake was observed. Analysis of the GFP-DC in the LN for CD80 expression, demonstrated that the exogenously loaded GFP(+) DC were significantly more mature than the resident endogenous GFP(-) DC (Fig. 5*b*) The observed MFI's were comparable to those seen on antigen positive DC after ablation and were similar after peri-tumoral or subcutaneous injection (Fig. 5*b*).

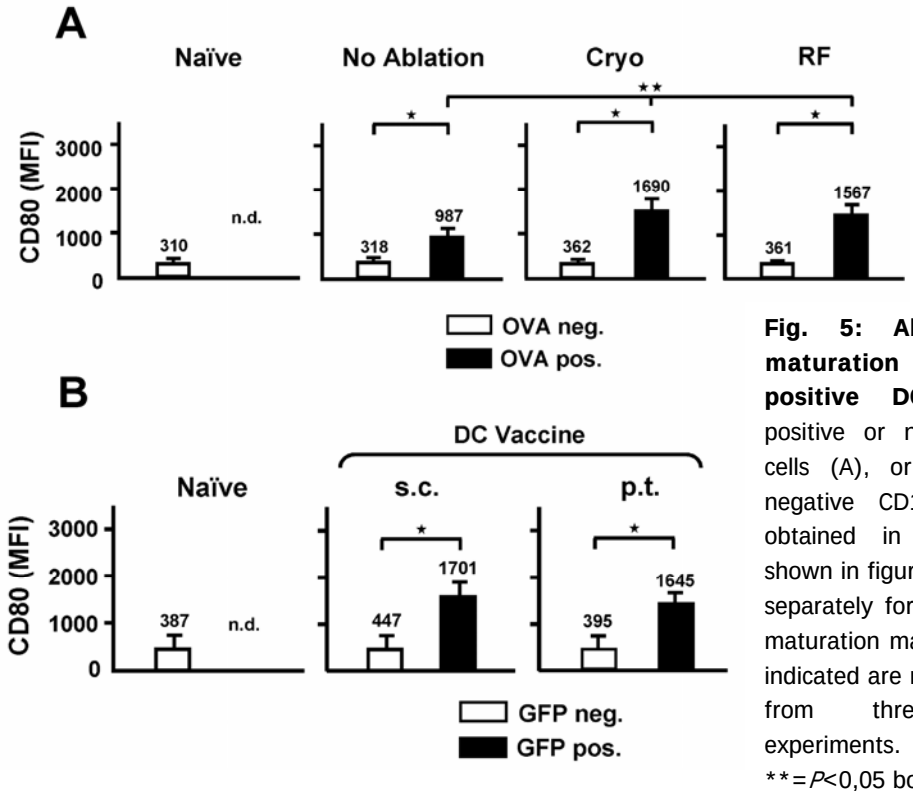

Fig. 5: Ablation induces maturation of antigen positive DC. OVA-Alexa488 positive or negative CD11c(+) cells (A), or GFP positive or negative CD11c(+) cells (B), obtained in the experiments shown in figure 3, were analyzed separately for expression of the maturation marker CD80. Values indicated are mean MFI's with sd from three independent experiments. *=*P*<0,005, **=*P*<0,05 both vs. no ablation.

Similar, but somewhat less profound results were obtained with CD86 expression (not shown).

The combined data thus not only indicate that ablation results in more efficient DC loading but also that ablation-induced CD80 expression of the OVA-Alexa(+) DC in the LN equals the CD80 expression on the DC from the LPS-matured *ex vivo* DC vaccine.

CTLA-4 blockade following ablation enhances systemic anti-tumor immunity.

We previously showed that immune modulation by blockade of CTLA-4 signaling enhances anti-tumor immunity after radiofrequency ablation. We now studied whether CTLA-4 blockade could be also used in the cryo ablation model. Figure 6 shows that the weak anti-tumor response observed after cryo ablation alone indeed could be enhanced by combination with CTLA-4 blockade. Comparable results were obtained when radiofrequency ablation was applied (Fig. 6*a*). Control experiments showed that CTLA-4 injection alone had no significant effect on primary tumors or rechallenges (Fig. 6*b*, left and right panel resp.). Analysis of the mice for OVA-specific T cells revealed that far more specific T cells were present ten days after the combination treatment as compared to ablation alone (Fig. 6*c*). To determine the activation status of these T cells, IFN-γ production was measured after activation with B16-OVA cells. Interestingly, T cells derived from mice treated with anti-CTLA-4 and ablation showed increased IFN-γ production upon recognition of antigen compared to the IgG treatment (Fig. 6*d*). In correspondence with the rechallenge model, somewhat less IFN-γ producing, specific T cells were detected after radiofrequency ablation than after cryo ablation.

Depletion of regulatory T cells prior to ablation enhances systemic anti-tumor immunity.

Next, we investigated whether depletion of regulatory T cells prior to ablation was also able to enhance tumor immunity. Hereto, mice received anti-CD25 antibodies 4 days before ablation of their B16-OVA tumors. The anti-CD25 antibodies did not have any effect on the outgrowth of the B16-OVA tumors, yielding similar tumor-sizes at the day of ablation (see also Fig. 7*b*). Figure 7*a* shows that Treg depletion enhances the initially weak anti-tumor responses after both ablative techniques. This effect is comparable to the effects seen with anti-CTLA-4 treatment (see also figure 6). Treg depletion alone had no significant effect on primary tumors or rechallenges (Fig. 7*b*). Tetramer analysis confirmed the presence of IFN-γ producing, tumor-specific T cells after the combination treatment, whereas after each individual treatment these cells were absent (Fig. 7*c* and 7*d*). In correspondence with the rechallenge model, somewhat less specific T cells were detected after radiofrequency ablation then after cryo ablation.

Collectively, these data thus suggest that antigen uptake following ablation does only lead to enhanced numbers of activated tumor-specific T cells, when suppressive regulation is switched off. *In situ* tumor destruction plus immune activation leads to a more potent systemic anti-tumor response than either treatment modality alone. This treatment regimen allows for direct antigen-loading of DC *in vivo* without delivery of defined tumor antigens as in conventional DC vaccination.

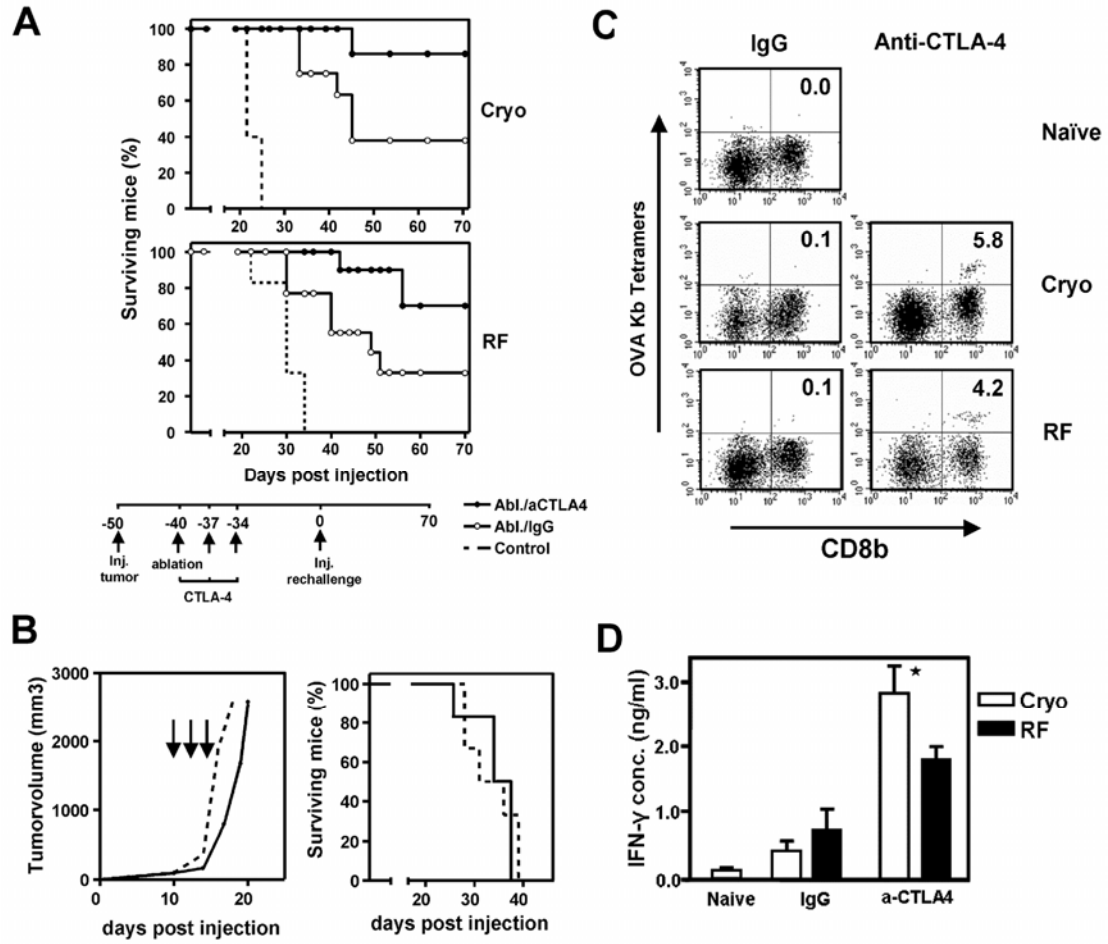

Fig. 6: Anti-CTLA-4 improves therapeutic outcome of ablation. 0, 3 and 6 days after ablation of B16-OVA tumors, mice were injected i.p. with 200 μg anti-CTLA-4 antibodies or control IgG. (A) 40 days following ablation of tumor-bearing mice receiving antibodies, a tumor rechallenge was performed as described before. Figures depict survival curves demonstrating growth reduction/protection after ablation plus CTLA-4 treatment. As a control, tumor growth was monitored by injection of the same tumor dose into naïve mice (dotted lines). T=0 corresponds to the time of injection of the tumor re-challenge. P<0,05 for CTLA-4 vs. IgG in both cryo and RF figures. One out of three representative experiments is shown (n=5-11). (B) Control experiment showing that CTLA-4 treatment by itself is insufficient to eradicate the primary tumor or re-challenges. Mice with established B16OVA tumors (5-7mm) were injected with 200 μg anti-CTLA-4 antibodies at day 10, 13 or 16 after tumor inoculation (solid line, arrows) or PBS (dotted line). Next, tumor growth was monitored in time (left panel). CTLA-4 treatment or PBS given 40 days prior to a B16OVA challenge ($15x10^3$ cells) did not affect survival of the mice (right panel). (C) At day 10 after ablation, a mix of LN and spleen cells was obtained from mice treated as indicated. T cells were harvested from spleen and LN and restimulated with irradiated, IFN-γ-treated B16-OVA cells and IL-2 for 10 days, followed by staining with OVA tetramers (K^b) and anti-CD8b. Depicted numbers represent the percentages of tetramer-positive cells within the CD8b+ population. (D) T cells from the same bulk cultures were used for restimulation with B16OVA cells. Supernatant from these cultures was harvested 24h later and analyzed for IFN-γ content by standard ELISA methods. Shown are means with sd from triplicates, *= P<0,05 vs. IgG. Experiments shown in figures (B-D) were repeated twice with comparable results.

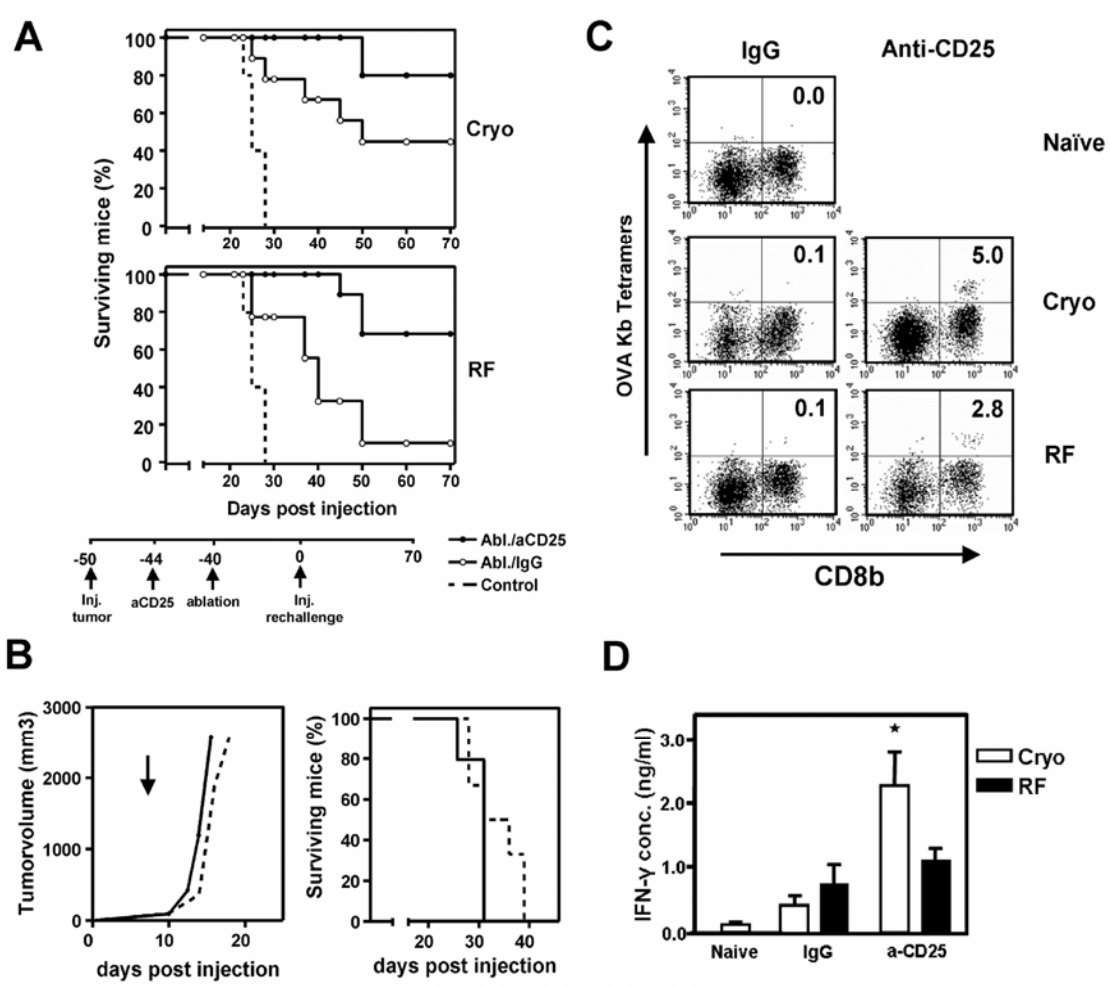

Fig. 7: Depletion of regulatory T cells improves therapeutic outcome of ablation. 4 days prior to ablation of B16-OVA tumors, mice were injected i.p. with 200 μg anti-CD25 antibodies or control IgG. (A) 40 days following ablation of tumor-bearing mice receiving antibodies, a tumor rechallenge was performed as described before. Figures depict survival curves demonstrating growth reduction/protection after ablation plus Treg depletion. As a control, tumor growth was monitored by injection of the same tumor dose into naïve mice (dotted lines). T=0 corresponds to the time of injection of the tumor re-challenge. P<0,05 for aCD25 vs. IgG in both cryo and RF figures. One out of three representative experiments is shown (n=5-9). (B) Control experiment showing that Treg depletion by itself is insufficient to eradicate the primary tumor or re-challenges. Mice with established B16OVA tumors (5-7mm) were injected with 200 μg anti-CD25 antibodies (solid line, arrow) or PBS (dotted line). Next, tumor growth was monitored in time (left panel). Treg depletion or PBS given 40 days prior to a B16OVA challenge ($15x10^3$ cells) did not affect survival of the mice (right panel). (C) At day 10 after ablation, a mix of LN and spleen cells was obtained from mice treated as indicated. T cells were harvested from spleen and LN and restimulated with irradiated, IFN-γ-treated B16-OVA cells and IL-2 for 10 days, followed by staining with OVA tetramers (K^b) and anti-CD8b. Depicted numbers represent the percentages of tetramer-positive cells within the CD8b+ population. (D) T cells from the same bulk cultures were used for restimulation with B16OVA cells. Supernatant from these cultures was harvested 24h later and analyzed for IFN-γ content by standard ELISA methods. Shown are means with sd from triplicates, *= P<0,05 vs. IgG. Experiments shown in figures (B-D), were repeated twice with comparable results.

Discussion

Ex vivo generated mature dendritic cells (DC) have been shown to evoke tumor-specific responses in cancer patients[10,26]. DC vaccination is, however, time consuming and expensive, and in many cases the anti-tumor response falls short in strength to cure patients with established tumors. Herein, we report that tumor-debris created by radiofrequency and cryo ablation comprises an effective antigen source for DC. Moreover, we show that tumor ablation could be efficiently combined with immune modulating approaches. This creates an effective '*in situ* DC-vaccine' capable of inducing protection against lethal tumor re-challenges.

In situ tumor destruction with cryo, radiofrequency, or laser ablation has received increasing attention as a treatment modality for focal cancer[19,22,27-29]. However, little is known regarding the induction of immune responses after *in situ* tumor destruction or the fate of the generated tumor-debris. In our experiments we applied a mouse B16 tumor model, in which *ex vivo* generated DC vaccines are mostly only effective in a *prophylactic* setting. Applying two types of ablation techniques, we now demonstrated that tumor-debris remaining *in situ* after tumor destruction creates an effective antigen depot for the induction of *therapeutic* anti-tumor immunity by DC. Using two distinct exogenous antigens (OVA and KLH) and two different approaches (radioactive and fluorescent labeling) to monitor the fate of antigens, we showed that the majority of antigens remained at the ablated site and that very little antigen spreading was observed except to the draining LN. Within the draining LN, a large percentage of DC acquired antigen as soon as one day and for at least three days following ablation. The *in vivo* loading of DC upon cryo ablation was significantly more efficient than with radiofrequency ablation (Fig. 2, 3). The exact nature of this difference remains to be elucidated but is likely related to the kind of antigens that are created by the ablation and/or to the endogenous signals that are produced upon tumor destruction.

Our results do not provide answers on *how* exactly DC acquire their antigens. It is, for instance, not known whether DC travel to the tumor and take up the antigens locally, or that the antigen floats to the lymph node via lymphatics, where lymph node resident DC engulf this material. According to a recent study demonstrating LN-DC that accumulated antigen deposited in subcutaneous tissue[30], both options might be occurring at the same time. The authors showed that antigen was first detected in LN-residing DC, followed by a second wave of antigen positive DC that migrated from the periphery into the LN. Both waves were required for efficient immune response induction and were dependent on the presence of the challenge site. Our finding that both at day 1 and day 3 after ablation antigen-loaded DC could be discerned from the LN suggests that similar dynamics take place in our model. In this context, it is interesting to note that antigen loading in tumor bearing control mice seemed to decline in these 3 days (see also Fig. 2*d*). Furthermore, we observed that antigens from the tumor depot preferentially accumulate in DC, but not in B-cells or macrophages (not shown). The basis for the observed antigen accumulation in DC remains to be studied in more detail, but is likely related to their strategic location within the LN and their ability to retain antigens within their endocytotic compartment, whereas macrophages rapidly degrade antigens in their lysosomes.

The primary goal of conventional DC-based vaccination is to obtain tumor antigen-loaded DC in the draining lymph nodes that are properly activated so that they do initiate immune responses. When comparing antigen positive DC loaded by ablation with antigen positive DC from a conventional DC vaccine we could demonstrate that far more DC containing antigen were present in the draining lymph nodes when radiofrequency or cryo ablation was performed (Fig. 3). Moreover, both ablation procedures were able to significantly increase the absolute number of DC per LN, whereas vaccination was less able to do so (Fig. 4). Analysis of the maturation state of antigen-loaded and unloaded DC in naïve, tumor-bearing and tumor-ablated mice revealed two interesting phenomena. First, DC that contained antigen expressed significantly higher levels of co-stimulatory molecules than antigen negative DC. These data are in line with *in vitro* data indicating that antigen uptake can affect DC activation[31]. Second, ablation resulted in a significant further increase in co-stimulatory molecule expression on antigen positive, but not antigen negative DC. We note that neither antibodies present after positive MACS sorting, nor TLR-ligands often present in OVA-batches[32] did bias the CD80 staining on DC, as DC purified by negative selection showed similar results and OVA-Alexa did not mature DC *in vitro* (not shown). The exact nature of these ablation-dependent signals need further clarification, but may well represent cytokines or other endogenous mediators released after ablation[32-35]. It has, for instance, been shown that heat shock proteins and uric acid are present in cell debris, which both influence DC and other parts of the immune system[36,37]. Importantly, the observed ablation-induced increase in the number and maturation state of antigen loaded DC is apparently not sufficient to induce complete tumor protection of mice.

Combination of ablation with *in vivo* immune modulation by either blockade of CTLA-4 signaling or depletion of regulatory T cells was shown to have beneficial effects in our rechallenge model. In both cases it provided a further delay in tumor growth compared to ablation alone. Importantly, only when CTLA-4 blockade or regulatory T cell depletion were performed together with ablation, significant amounts of active OVA specific T cells could be observed. It will be interesting to investigate the nature of the signals resulting in DC maturation and subsequent T cell expansion, and the possible effects of CTLA-4 blockade or Treg depletion on this. Moreover, possible future research can aim on combination of CTLA-4 blockade and Treg depletion. It was demonstrated in a murine tumor model that this combination had a striking synergistic effect on tumor immunity[7].

Collectively, our data show that *in vivo* tumor destruction in combination with systemic immune modulation creates a unique and potent, '*in situ* DC-vaccine'. Although radiofrequency ablation seems to be less efficient in loading DC compared to cryo ablation, both techniques can be efficiently combined with immune modulation. The fact that both ablative treatments as well as both the immune interventions are currently applied in cancer patients, makes this promising '*in vivo* DC-vaccine' readily applicable in clinical settings.

Materials and Methods

Animals

9-11 weeks old female C57BL/6n mice were purchased from Charles River Wiga (Sulzfeld, Germany). Animals were held under specified pathogen-free conditions in the Central Animal Laboratory (Nijmegen, the Netherlands). All experiments were performed according to the guidelines for animal care of the Nijmegen Animal Experiments Committee.

Tumors

Mice were injected subcutaneously at the right femur with $500x10^3$ cells of the OVA-transfected murine melanoma cell line B16F10 (B16-OVA, clone MO5), which was kindly provided by dr. Kenneth Rock[38], or wt B16F10. Cells were cultured and injected as described before[24]. Tumor volumes were scored every three days with the formula (AxB^2) x 0.4, in which A is the largest and B is the shortest dimension. Tumors were selected for ablation when their diameter measured between 5 to 7mm (d9-10) and only if the tumor was relatively round (>98% of mice).

Radiofrequency ablation

Animals were anaesthetized by isoflurane inhalation and properly shaved at the tumor area and on the contra-lateral flank. After placement and proper attachment of the contra lateral side onto an electricity-conducting pad (grounding pad), the tumor area was disinfected with alcohol. A radiofrequency ablation needle with active tip of 8 mm (SMK-15, Cotop, Amsterdam, the Netherlands) was inserted s.c. and placed in the middle of the tumor. After placement of the radiofrequency ablation needle, impedance could be evaluated on the radiofrequency lesion generator system (Model RFG-3B, Radionics, Burlington, MA, USA). Next, treatment was started by delivering radiofrequency energy. During a treatment cycle of +/- 80 sec., temperature could be monitored by means of a thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of 75-80°C could be reached.

Cryo ablation

Animals were properly shaven and anaesthetized by isoflurane inhalation. The tumor area was disinfected with alcohol and subsequently wetted with distilled water. The tip of the liquid nitrogen cryo ablation system (CS76, Frigitronics, Shelton, CT) was placed onto the tumor and after proper freeze attachment, treatment was started. During two treatment cycles of +/-70 seconds the tumor and a small strip around it were frozen to less than -100°C. Treatment was considered successful when the whole tumor appeared frozen macroscopically.

Re-challenge model

Forty days after ablation of B16-OVA tumors, mice were challenged by subcutaneous injection at the contra-lateral femur of either $15x10^3$ B16-OVA cells or $15x10^3$ EL4 cells (numbers defined by titration). Injections were performed in 100 μl PBS. Mice were sacrificed when tumors reached a volume of ± 850 mm^3.

^{111}Indium conjugation and antigen monitoring

KLH (Calbiochem, Darmstadt, Germany) (10 mg/ml) was conjugated with 240 µg cDTPA (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 0.1 M $NaHCO_3$, pH 8.2, during 30 min. Unconjugated DTPA was removed by dialysis against 0.1 M Na-citrate buffer, pH 5.0. The KLH-DTPA conjugate (1.5 mg) was incubated with 1.5 mCi $^{111}InCl_3$ (Mallinckrodt, Petten, The Netherlands) in 1.2 ml 0.1 M Na-citrate buffer, pH 5.0, during 30 min. Similar procedures were followed for the ^{111}In-OVA conjugate. Radiochemical purity of each preparation (>95%) was determined by instant thin-layer chromatography (ITLC, Gelman Sciences Inc., MI). For antigen monitoring experiments, mice received intra-tumoral injections of OVA/KLH labeled with ^{111}In (^{111}In-OVA, ^{111}In-KLH) or ovalbumin conjugated to Alexa-488 (OVA-Alexa-488) (Molecular Probes, Leiden, the Netherlands). Conjugates (20 µg (=20µCi)) were injected directly before ablation in 20 µl PBS. At various time points after injection of the protein conjugates, mice were anesthetized and scintigraphic images were acquired using a γ-camera with ^{111}In collimator (Siemens Orbiter, Siemens Inc. Hoffmann Estate, IL) as described previously[15]. For biodistribution studies mice were killed at different time points after injection of the protein conjugates. Liver, kidney, spleen, draining LN (r. sup. ing.) and non-draining LN (l. sup. ing.) were collected, weighed and counted in a γ-counter (1480 Wizard, Wallac Oy, Finland). Injection standards were taken for physical decay correction. In DC sorting experiments, mice received ^{111}In-KLH i.t., and at different time points post ablation CD11c+ DC were sorted as described below and counted in the γ-counter.

Magnetic bead cell sorting and flow cytometric analysis

For antigen uptake experiments (^{111}In-KLH and OVA-Alexa), draining LN's from 5-8 mice were pooled and after crushing, dissociation in DNAse/collagenase/EDTA, and passage through nylon mesh[39], cells were counted and sorted by standard MACS isolation with a MACS Midi column (Miltenyi Biotec). Positive selection of DC was done using CD11c beads (clone N418, Miltenyi Biotec, B.Gladbach, Germany), whereas negative selection/enrichment was done on the CD90 T cell marker (Thy1.2, 30.H12, Miltenyi Biotec). Sorts were verified by CD3e or CD11c (HL3) staining (not shown). Subsequently, cells were stained and analyzed on a FACS-Calibur™ system (BD) with the CELLQuest software. Stainings were performed using the following mAbs: CD11c-APC (HL3), CD8b-FITC (53-5.8), CD3e-PE (17-A2), biotinylated CD80 (1G10), and streptavidin-PE. All antibodies were purchased from BD Pharmingen (Alphen a/d Rijn, the Netherlands).

DC culture from GFP-transgenic mice

GFP-expressing DC were cultured and injected as described elsewhere[25]. Briefly, bone marrow was collected from GFP-transgenic mice and cultured for 7 days in the presence of GM-CSF and IL-4. At day 7, 1 µg/ml LPS was added for 24 h maturation. Next, the non-adherent fraction was harvested, washed and loaded for 1 hr with the K^b-peptide of OVA (SIINFEKL). $1x10^6$ cells were injected subcutaneously or peri-tumorally at the femur. Isolation of LN cells and sorting was identical as described above.

Antibody treatments

In the anti-CTLA-4 treatment mice received anti-CTLA-4 antibody (clone 9H10[5]) at day 0, 3 and 6 after ablation. Treg depletion was performed by injection of anti-CD25 antibodies (clone PC61,[40]) 4 days prior to ablation. Injections (200 µg) were done intra-peritoneal in PBS. In all cases depletion was successful as verified by FACS (not shown).

Tetramer staining and IFN-γ measurement

A T cell culture was obtained from spleen and draining LN's of mice 10 days after ablation of a B16-OVA tumor or from naïve control mice. Stimulation of these cells ($100x10^3$) was performed by addition of irradiated, IFN-γ-treated, B16-OVA cells ($50x10^3$) in IL-2 (10 CU/ml) supplemented culture medium. At day 5 and day 10, cells were collected and cleaned in a density gradient. At day 10 of culture, cells were stained for 15 min at RT by OVA-tetramers (H-$2K^b$) conjugated to APC (Pelimers, Sanquin, Amsterdam, the Netherlands), counterstained with CD8b, and analyzed by FACS. Same bulk cultures were used to collect supernatant 24 hours after stimulation with irradiated B16-OVA cells. Capture and biotinylated detection antibodies directed to mouse IFN-γ were purchased from BD Pharmingen and, using standard ELISA procedures, IFN-γ concentration was measured in 50 µl of supernatant.

Statistical analysis

Data were analyzed for statistical significance by Student's T-test, except for the Kaplan Meier survival curves for which a log rank test was used.

References

1. Banchereau, J. & Palucka, A.K. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* **5**, 296-306 (2005).
2. Adams, S., O'Neill, D.W. & Bhardwaj, N. Recent advances in dendritic cell biology. *J Clin Immunol* **25**, 87-98 (2005).
3. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**, 133-46 (2003).
4. Townsend, S.E. & Allison, J.P. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science* **259**, 368-70 (1993).
5. Egen, J.G., Kuhns, M.S. & Allison, J.P. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* **3**, 611-8 (2002).
6. Chen, L. et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell* **71**, 1093-102 (1992).
7. Sutmuller, R.P. et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* **194**, 823-32 (2001).
8. Sakaguchi, S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* **6**, 345-52 (2005).
9. Banchereau, J. et al. Immunobiology of dendritic cells. *Annu Rev Immunol* **18**, 767-811 (2000).
10. Figdor, C.G., de Vries, I.J., Lesterhuis, W.J. & Melief, C.J. Dendritic cell immunotherapy: mapping the way. *Nat Med* **10**, 475-80 (2004).
11. De Vries, I.J. et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res* **63**, 12-7 (2003).
12. Coulie, P.G. & van der Bruggen, P. T cell responses of vaccinated cancer patients. *Curr Opin Immunol* **15**, 131-7 (2003).
13. Schuler, G., Schuler-Thurner, B. & Steinman, R.M. The use of dendritic cells in cancer immunotherapy. *Curr Opin Immunol* **15**, 138-47 (2003).
14. Steinman, R.M. & Mellman, I. Immunotherapy: bewitched, bothered, and bewildered no more. *Science* **305**, 197-200 (2004).
15. Eggert, A.A. et al. Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res* **59**, 3340-5 (1999).
16. Josien, R. et al. TRANCE, a tumor necrosis factor family member, enhances the longevity and adjuvant properties of dendritic cells *in vivo*. *J Exp Med* **191**, 495-502 (2000).
17. Steinman, R.M. & Pope, M. Exploiting dendritic cells to improve vaccine efficacy. *J Clin Invest* **109**, 1519-26 (2002).
18. Furumoto, K., Soares, L., Engleman, E.G. & Merad, M. Induction of potent antitumor immunity by *in situ* targeting of intratumoral DCs. *J Clin Invest* **113**, 774-83 (2004).

19. Curley, S.A. Radiofrequency ablation of malignant liver tumors. *Ann Surg Oncol* **10**, 338-47 (2003).
20. Raj, G.V., Reddan, D.J., Hoey, M.B. & Polascik, T.J. Management of small renal tumors with radiofrequency ablation. *Urology* **61**, 23-9 (2003).
21. Garcea, G., Lloyd, T.D., Aylott, C., Maddern, G. & Berry, D.P. The emergent role of focal liver ablation techniques in the treatment of primary and secondary liver tumours. *Eur J Cancer* **39**, 2150-64 (2003).
22. Ruers, T. & Bleichrodt, R.P. Treatment of liver metastases, an update on the possibilities and results. *Eur J Cancer* **38**, 1023-33 (2002).
23. Zagoria, R.J., Chen, M.Y., Kavanagh, P.V. & Torti, F.M. Radiofrequency ablation of lung metastases from renal cell carcinoma. *J Urol* **166**, 1827-8 (2001).
24. den Brok, M.H. et al. *In situ* tumor ablation creates an antigen source for the generation of antitumor immunity. *Cancer Res* **64**, 4024-9 (2004).
25. Eggert, A.A. et al. Analysis of dendritic cell trafficking using EGFP-transgenic mice. *Immunol Lett* **89**, 17-24 (2003).
26. Rosenberg, S.A., Yang, J.C. & Restifo, N.P. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* **10**, 909-15 (2004).
27. Ruers, T.J., Joosten, J., Jager, G.J. & Wobbes, T. Long-term results of treating hepatic colorectal metastases with cryosurgery. *Br J Surg* **88**, 844-9 (2001).
28. Veth, R., Schreuder, B., van Beem, H., Pruszczynski, M. & de Rooy, J. Cryosurgery in aggressive, benign, and low-grade malignant bone tumours. *Lancet Oncol* **6**, 25-34 (2005).
29. Erce, C. & Parks, R.W. Interstitial ablative techniques for hepatic tumours. *Br J Surg* **90**, 272-89 (2003).
30. Itano, A.A. et al. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* **19**, 47-57 (2003).
31. Randolph, G.J., Beaulieu, S., Lebecque, S., Steinman, R.M. & Muller, W.A. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* **282**, 480-3 (1998).
32. Pasare, C. & Medzhitov, R. Toll-dependent control mechanisms of CD4 T cell activation. *Immunity* **21**, 733-41 (2004).
33. Sporri, R. & Reis e Sousa, C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* **6**, 163-70 (2005).
34. de Jong, K.P. et al. Serum response of hepatocyte growth factor, insulin-like growth factor-I, interleukin-6, and acute phase proteins in patients with colorectal liver metastases treated with partial hepatectomy or cryosurgery. *J Hepatol* **34**, 422-7 (2001).
35. Huang, A. et al. Phase I study of percutaneous cryotherapy for colorectal liver metastasis. *Br J Surg* **89**, 303-10 (2002).
36. Shi, Y., Evans, J.E. & Rock, K.L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-21 (2003).

37. Somersan, S. et al. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* **167**, 4844-52 (2001).
38. Falo, L.D., Jr., Kovacsovics-Bankowski, M., Thompson, K. & Rock, K.L. Targeting antigen into the phagocytic pathway *in vivo* induces protective tumour immunity. *Nat Med* **1**, 649-53 (1995).
39. Vremec, D., Pooley, J., Hochrein, H., Wu, L. & Shortman, K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* **164**, 2978-86 (2000).
40. Onizuka, S. et al. Tumor rejection by *in vivo* administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* **59**, 3128-33 (1999).

Chapter 5

Synergy between *in situ* tumor destruction and TLR9 stimulation results in a highly effective *in vivo* dendritic cell vaccine

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Submitted

Abstract

Dendritic cells (DC) are professional antigen presenting cells that play a pivotal role in the induction of immunity. *Ex vivo* generated, tumor antigen-loaded mature DC are currently exploited as cancer vaccines in clinical studies. However, antigen loading and maturation of DC directly *in vivo* would greatly facilitate the application of DC-based vaccines. We have previously shown that *in situ* tumor destruction by ablative treatments efficiently delivers antigens for the *in vivo* induction of anti-tumor immunity. In this paper we demonstrate that although 20% of the draining lymph node DC acquire antigen after *in situ* cryo ablation, only partial protection against a subsequent tumor re-challenge is observed. However, we also demonstrate that a combination treatment of cryo ablation plus TLR9 stimulation via CpG-ODN is far more effective in the eradication of local and systemic tumors than either treatment modality alone. Analysis of the underlying mechanism revealed that *in situ* tumor ablation synergizes with TLR9 stimulation to induce DC maturation and efficient cross-presentation in tumor-bearing mice, leading to superior DC function *in vivo*. Therefore, *in situ* tumor destruction in combination with CpG-ODN administration creates a unique, '*in situ* DC-vaccine' that is readily applicable in the clinic.

Introduction

Dendritic cells (DC) are the most potent antigen presenting cells of the immune system[1]. Immature DC reside in peripheral tissues where they take up and process antigens from their surroundings. In a stimulatory environment, like in an infection, immature DC undergo maturation and acquire the capacity to cross-present exogenous antigens in MHC class I [2,3]. Particularly, the Toll-Like Receptor (TLR) proteins initiate the DC maturation process upon recognition of conserved pathogen-associated molecular patterns, like LPS or unmethylated CpG oligodeoxynucleotides (CpG-ODN)[4-6]. Maturation is accompanied by migration of the DC to the draining lymph node (LN), where they subsequently present antigens to immune cells to induce immunity. DC that did not perceive an activating environment do not mature and induce tolerance rather than immunity[7].

Since DC are critical in inducing effective immune responses, *ex vivo* generated DC are currently applied to stimulate anti-tumor immunity in clinical trials[1,8-11]. Although tumor-specific responses have been obtained with tumor antigen-loaded DC-based vaccines, many questions regarding effective tumor antigens and DC migration remain unanswered[8,12]. Moreover, *ex vivo* generation of DC vaccines is time-consuming and costly. *In vivo* loading and maturation of DC would therefore greatly improve the applicability of DC vaccination.

Recently, an antibody directed against the mouse DC antigen DEC-205 was shown to target OVA antigens preferentially to DC *in vivo*[13]. An alternative approach to create an *in situ* DC vaccine applied retrovirus-mediated expression of the chemokine CCL20 in tumors to increase the number of intra-tumoral DC[14]. As previously shown for *ex vivo* generated DC vaccines[15], both studies confirmed that maturation of *in vivo* loaded DC by either agonistic anti-CD40 antibodies or the TLR9-ligand CpG-ODN was essential to induce a potent immune response.

Tumor-debris left in the body after *in situ* tumor destruction is suggested to be a potential tumor antigen source for DC *in vivo*, and would provide a direct way of *in situ* DC-targeting, without the need for retroviral infection or construction of recombinant proteins. Tumor ablative treatments, like cryo or radiofrequency ablation, are successfully used in clinical settings to destruct different types of tumors[16-20]. Tumor ablation has been associated with the occurrence of immune activation, especially via the induction of inflammatory cytokines[21,22]. Nevertheless, patients treated with an ablative regimen generally develop systemic recurrences as a consequence of the outgrowth of distant micro-metastases, implying that in general no protective immune response is induced. Indeed, we recently demonstrated in a new mouse model for *in situ* tumor ablation that only weak anti-tumor immune responses are induced following tumor ablation alone[23]. Adoptive transfer experiments however showed that the immunity induced is tumor specific and T cell-dependent[23].

Here, we demonstrate that when ablation of established B16 tumors (5-7mm) is combined with CpG-ODN administration, potent anti-tumor immune responses are induced. Results show that following ablation, an antigen depot is created from which 20% of DC found in the draining LN internalized tumor antigens. Moreover, the data indicate that

tumor ablation synergizes with CpG-ODN administration to not only enhance the number and maturation state of LN-DC, but also to increase cross-presentation[2], leading to the efficient induction of CD8+ T cells. *In situ* tumor destruction together with DC activation by CpG-ODN constitutes a powerful '*in situ* DC-vaccine' that is readily applicable in the clinic without prior knowledge of tumor antigens.

Results

Cryo ablation creates an antigen depot essential for the induced anti-tumor immunity

To determine whether the induction of protective immunity following *in situ* tumor-destruction was dependent on the presence of the antigen depot, established B16OVA tumors (5-7mm) were cryo ablated. Successfully ablated mice (70% remains recurrence-free) were re-challenged with either B16OVA or non-related EL4 thymoma cells according to the time schedule in Fig. 1*a*. In part of the mice, the ablated tumor was excised directly following ablation. In line with previous data (our unpublished results), cryo ablation of B16OVA tumors resulted in partial protection against a lethal B16OVA re-challenge (Fig. 1*b*), but had no effect on the outgrowth of EL4 tumors (Fig. 1*c*). Excision of cryo ablated B16OVA tumors directly after ablation completely abrogated the observed anti-tumor effect (Fig. 1*b*). These data demonstrate that the tumor-specific immune response induced following cryo ablation is critically dependent on the presence of the ablated tumor material.

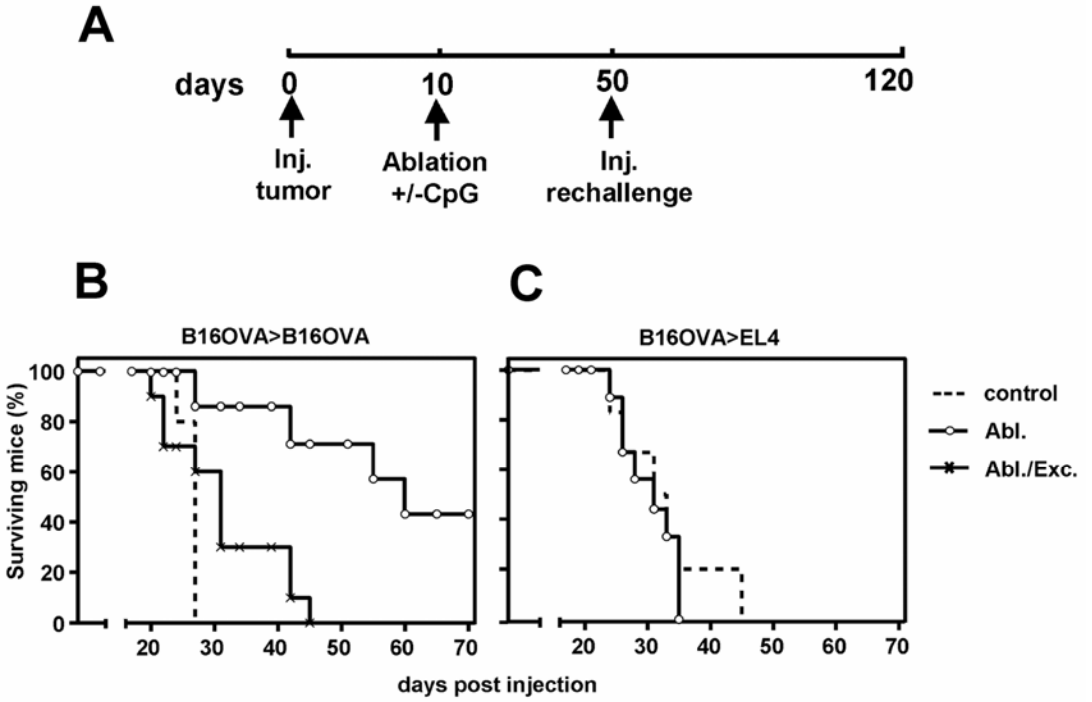


Fig. 1: Basal immunity is dependent on depot formation. (A) Time schedule outlining the different treatments as used in the experiments. Ten days after inoculation, the B16OVA melanomas (5-7mm) were cryo ablated. (B-C) Directly after ablation, the tumor was excised (**x**) or left *in situ* (○). Forty days later, tumor-free mice were re-challenged with B16OVA (B) or the non-related EL4 tumor (C). The survival of mice is depicted in which T=0 corresponds to the time of injection of the tumor re-challenge. As a control, tumor growth was monitored by injection of the same tumor cell dose into naïve mice (dotted lines). P<0,005 for ablation vs. excision. One out of two representative experiments is shown (n=5-10).

Enhanced anti-tumor immunity upon ablation with CpG-ODN administration.

The results from figure 1 also emphasize that the developed immunity to tumor rechallenges is sub-optimal. To explore whether the TLR9 ligand CpG-ODN 1668, could result in enhancement of the anti-tumor response, CpG-ODN was administered as a single

peri-tumoral injection directly following ablation. We observed that CpG-ODN injection alone, without ablation, did not have any effect on either the outgrowth of the primary tumor (Fig. 2*c*) nor lethal tumor re-challenges 40 days later (Fig. 2*d*). Interestingly, the combination of ablation of the tumor plus CpG-ODN administration resulted in complete protection against the B16OVA re-challenge 40 days later (Fig. 2*a)*. These data thus demonstrate that the combination of *in situ* tumor destruction and CpG-ODN administration is superior in inducing anti-tumor immunity relative to either treatment alone.

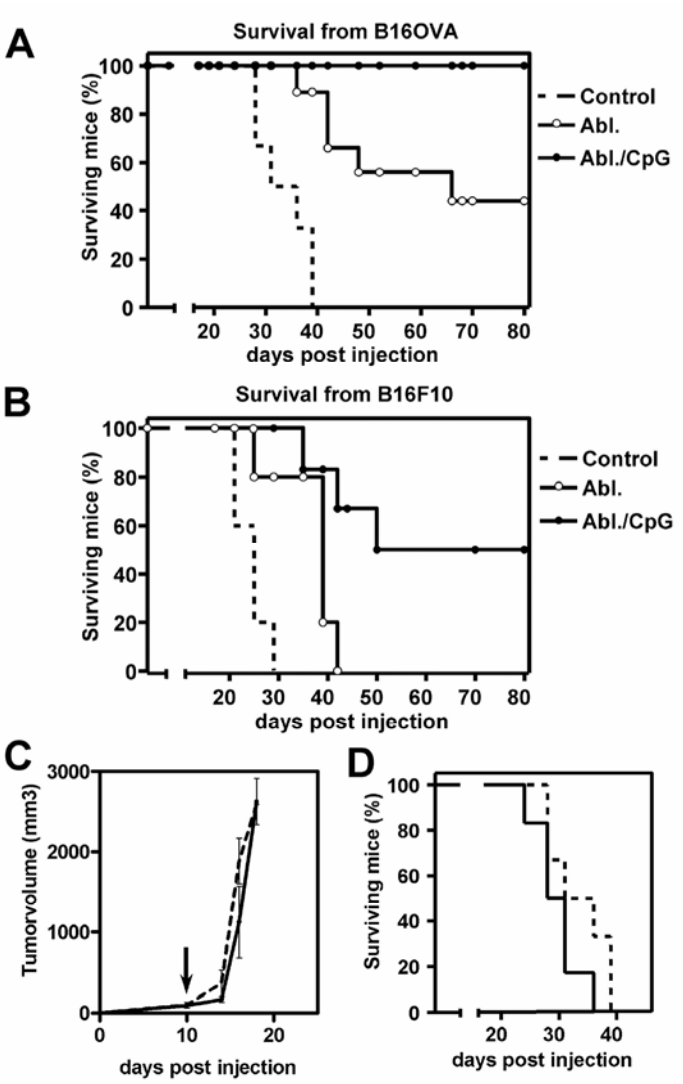


Fig. 2: Potent anti-tumor immunity following combined ablation/CpG-ODN treatment. Mice with established B16OVA melanomas (5-7mm) were cryo ablated. One hour after ablation, mice received 100 μg CpG-ODN (●) or PBS (○) peri-tumorally. Forty days later, a re-challenge with $15x10^3$ B16OVA cells was given s.c. in the contra-lateral leg. (A) Survival curve demonstrating complete protection of tumor outgrowth after ablation with CpG-ODN. *P*<0,005 for both lines vs. control. One out of three

(B) Survival curve of tumor-free mice from A, after secondary re-challenges. After rejection of the first B16OVA re-challenge, mice were re-challenged with $10x10^3$ wild-type B16F10 cells inoculated s.c. on the left flank, and $15x10^3$ B16OVA cells on the right. Depicted is survival from the B16F10 tumors (*n*=5-6 per group) in which *P*<0,005 for Abl./CpG vs. control. All B16OVA tumors were rejected again (not shown). As a control, tumor growth was monitored by injection of the same tumor dose into naïve mice (dotted line). T=0 corresponds to the time of injection of the tumor re-challenge. One out of two representative experiments is shown.

(C-D) Control experiment showing that CpG-ODN by itself is insufficient to eradicate the primary tumor or re-challenges. Established B16OVA tumors (5-7mm) were injected with 100 μg CpG-ODN (solid line, arrow) or PBS (dotted line). Tumor growth was monitored in time (C). S.c. injection of 100 μg CpG-ODN (solid line) or PBS (dotted line), 40 days prior to a B16OVA challenge ($15x10^3$ cells) did not affect survival of the mice (D).

To study immunity to different antigens than the immuno-dominant OVA protein, the protected mice from figure 2*a* received a second set of rechallenges, including wild-type B16F10 tumors. These mice completely rejected the re-challenge with B16OVA (not shown) but, more interestingly, were also partly protected against the poorly immunogenic, parental B16F10 tumor cells (Fig. 2*b*). Importantly, this occurred only when mice received co-treatment with CpG-ODN. This suggests that the combination treatment induces a potent memory response which is not only directed against the immuno-dominant epitopes but also to other (unknown) antigens expressed by the parental tumor.

Cryo ablation leads to efficient in vivo antigen acquisition by LN CD11c(+) DC and synergizes with CpG-ODN in in vivo DC maturation.

As we have recently found that following tumor ablation antigens are almost solely present in the CD11c(+) fraction at one and even three days after ablation (our unpublished results), we set out to explore the effect of *in situ* tumor destruction plus CpG-ODN administration on DC. These effects could provide a possible mechanism responsible for the potent induction of anti-tumor immunity. In order to visualize uptake of antigens by flowcytometry, chicken egg ovalbumin conjugated to the fluorophore Alexa-488 (OVA-Alexa) was injected intra-tumoral (i.t.) prior to ablation. As shown in Fig. 3*b*, over 20% of all CD11c(+) DC became OVA-Alexa(+) after cryo ablation, whereas much less antigen uptake was observed in non ablated mice (<5%). Co-administration of CpG-ODN did not significantly effect the loading of DC in the draining LN.

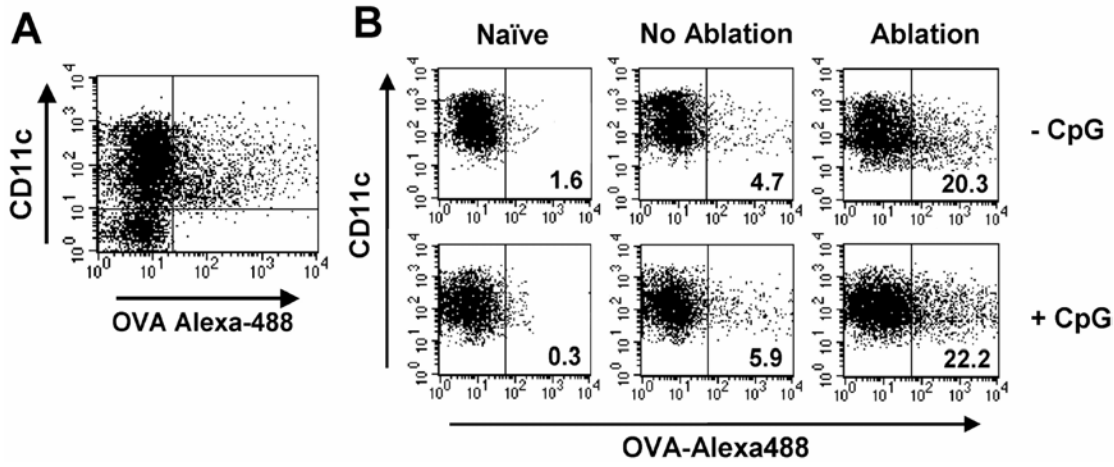


Fig. 3: OVA-Alexa488 uptake following combined ablation/CpG-ODN treatment. (A-B) FACS analysis of CD11c(+) DC isolated from pooled LN suspensions of naïve, tumor-bearing or tumor-ablated mice (n=6 per group). Mice received 20 μg ovalbumin conjugated to Alexa-488 (OVA-Alexa488) i.t. just prior to the time point of ablation. One hour later, 100 μg CpG-ODN was injected peri-tumorally as indicated. Two days after the indicated treatments the number of OVA-Alexa(-) and OVA-Alexa(+) DC was determined. Fig. 3*a* shows a typical FACS plot after a CD11c positive beads sort. OVA-Alexa488(+) cells were solely present in the CD11c(+) fraction as shown by the CD11c(-) cells in the positive as well as the negative bead fractions. In Fig. 3*b* the CD11c(-) population is gated out. Values shown in panel B are percentages of OVA-Alexa(+) cells within the CD11c(+) fraction.

Next, we studied the effect of cryo ablation and CpG-ODN on DC maturation. Hereto, OVA-Alexa(+) or OVA-Alexa(-) DC were analyzed for expression of the maturation markers CD80 and CD86. Phenotypical analysis of OVA-Alexa(+) DC showed a 3-fold increase in CD80 expression relative to OVA-Alexa(-) DC in tumor-bearing and naïve mice (MFI's 1007, 332 and 315 resp.) (Fig. 4*a* and *b*). Following ablation, CD80 expression further increased on OVA-Alexa(+) DC but had no effect on OVA-Alexa(-) DC (MFI 1374 and 369 resp.). This finding indicates that DC that acquired OVA-Alexa, preferentially up-regulate CD80, which is further enhanced by cryo ablation.

CpG-ODN injection alone also resulted in a significant increase in CD80 expression on OVA-Alexa(-) DC in both control and tumor-bearing mice (MFI 315 to 536, 332 to 605) and in a further increase on OVA-Alexa(+) DC from tumor-bearing mice (MFI 1007 to 1342) (Fig. 4*b*). When CpG-ODN injection was combined with ablation, an additive maturation of OVA-Alexa(-) DC (MFI 369 to 999) was observed. Most surprisingly, CD80 expression on OVA-Alexa(+) DC increased tremendously relative to DC from tumor-bearing mice (MFI 1374 to 2682). This effect on DC maturation of OVA-Alexa(+) DC is synergistic when compared to the effects seen when ablation or CpG-ODN are provided separately. Moreover, the observed synergy was not related to eradication of the tumor and its immune suppressive environment, as *in vivo* ablation of *ex vivo* created tumor lysates demonstrated comparable synergy on maturation (Fig. 5). Similar, but somewhat less profound effects were observed for CD86 expression (not shown).

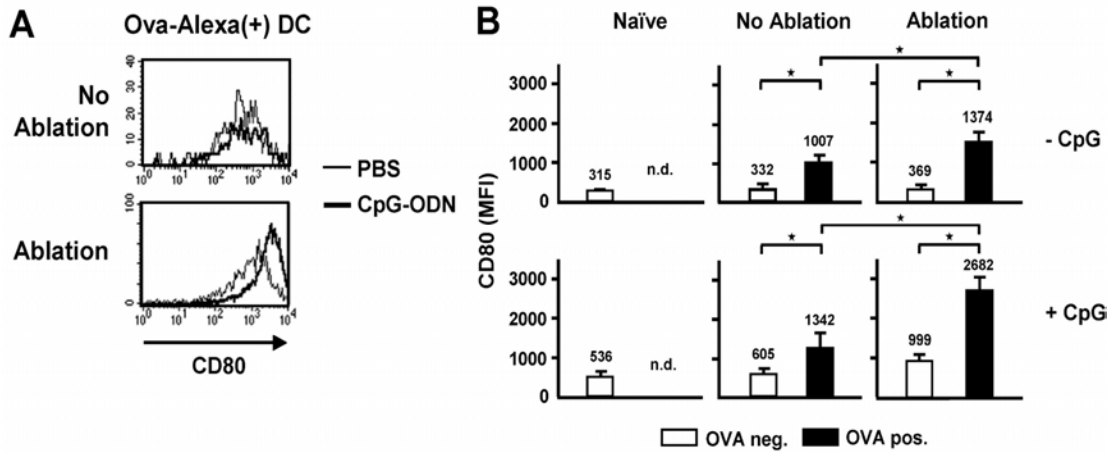


Fig. 4: Synergistic maturation of DC after combined ablation/CpG-ODN treatment. (A-B) FACS analysis of CD11c(+) DC isolated from pooled LN suspensions of tumor-bearing or tumor-ablated mice (n=6 per group). Mice received 20 μg ovalbumin conjugated to Alexa-488 (OVA-Alexa488) i.t. just prior to the time point of ablation. One hour later, 100 μg CpG-ODN was injected peri-tumorally as indicated. Fig. 4*a* shows typical FACS histograms of co-stainings with CD80 on OVA-Alexa(+) DC, presented as bars in Fig. 4*b*. Data indicate that high levels of CD80 expression are only observed when CpG-ODN and ablation are combined. Values in panel B are shown as mean fluorescence intensities with sd from three independent experiments, *=P<0,05.

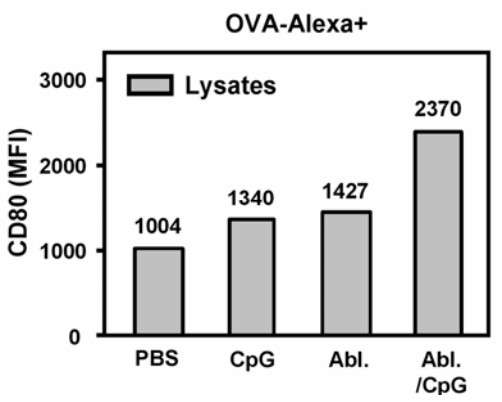


Fig. 5: DC maturation is dependent on ablation. (A) 10x10^6 B16OVA cells were lysed by freeze thaw cycles and injected s.c.. After co-injection of OVA-Alexa and CpG-ODN as indicated, the injection depot was cryo ablated or left untreated. Next, CD80 expression on Alexa-488 positive DC (6 mice pooled per group) was determined. Depicted are MFI's obtained from one of two experiments with comparable results.

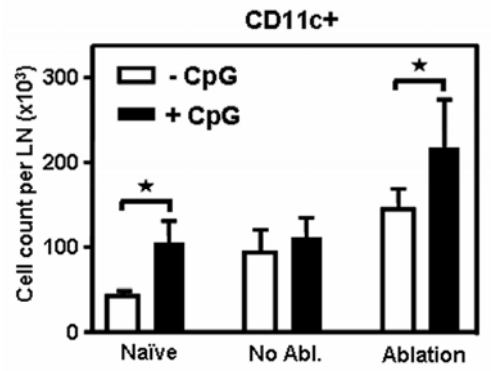


Fig. 6: Effects on total DC counts Shown are absolute CD11c(+) numbers per LN after magnetic bead sorting. Results are shown as means with sd, *=*P*<0,05. Data were obtained from three independent experiments.

Increased DC numbers in draining lymph nodes after cryo ablation plus CpG-ODN administration.

Analysis of the absolute numbers of CD11c(+) DC in the draining LNs following the different treatments demonstrated that both injection of merely CpG-ODN or the presence of a tumor resulted in a two-fold increase in DC numbers relative to naïve mice (Fig. 6). Ablation alone resulted in a 3-fold increase in the numbers of LN-DC, while tumor ablation combined with CpG-ODN treatment was again most effective. This implies that the total number of DC actually loaded with tumor antigen is even higher than can be concluded from the relative numbers.

CpG-ODN following ablation enables cross-presentation of tumor derived antigens

To assess the function of DC following ablation, CpG-ODN administration, or the combination treatment, we determined the ability of the CD11c(+) DC to cross-present antigens to MHC class I and II-restricted OVA-specific T cell hybridomas. Significant activation of MHC class II-restricted DO11.10 cells was readily observed upon co-culturing with CD11c(+) DC isolated from tumor ablated mice (Fig. 7*a*). In contrast, CD11c(+) DC from tumor-bearing mice without ablation did not activate DO11.10, nor did CD11c(-) cells. The administration of CpG-ODN to these mice increased DO11.10 activation only to a small extend. CpG-ODN administration after ablation significantly increased DO11.10 activation by CD11c(+) cells compared to tumor ablation or tumor alone and now also the CD11c(-) cell fraction of these mice displayed DO11.10 activating properties.

Next, we analyzed the cross-presenting capacity of these DC by testing their ability to activate the MHC class I-restricted OVA-specific B3Z T cells. Strikingly, only DC from mice treated with cryo ablation plus CpG-ODN were able to efficiently activate B3Z T cells

(Fig. 7*b*). Little or no activation of B3Z T cells could be discerned after ablation or CpG-ODN administration alone. Since DC from ablated mice that did not receive CpG-ODN treatment did process and present antigens to the MHC class II-restricted DO11.10 T cells, these data demonstrate that efficient cross-presentation after ablation by DC *in vivo* is co-dependent on CpG-ODN.

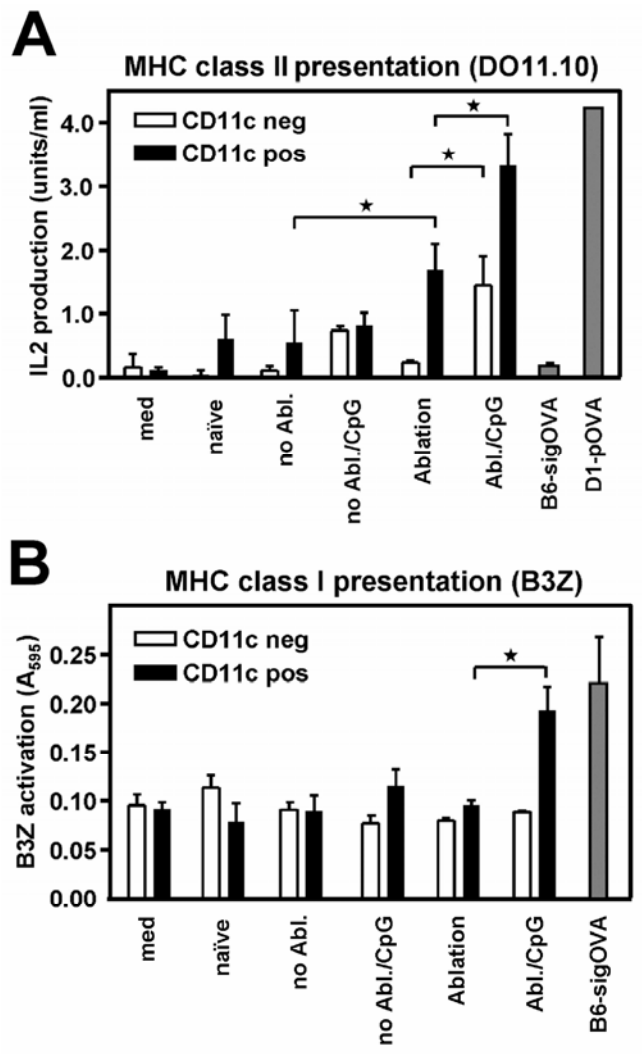
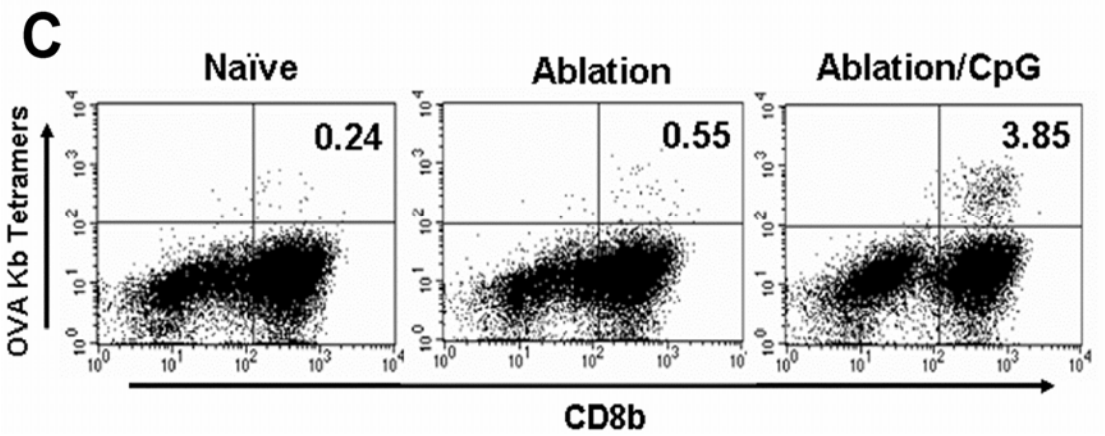


Fig. 7: Efficient cross-presentation following combined ablation/CpG-ODN treatment. (A-B) Two days after ablation of B16OVA tumors with or without co-injection of 100 μg CpG-ODN, CD11(+) DC were sorted from draining LN's (n=7-8). Subsequently, they were cultured overnight with MHC class I or MHC class II, OVA-specific T cell hybridomas (B3Z and DO11.10 resp.). T cell activation was measured by IL-2 production (DO11.10, (A)) or LacZ production (B3Z, (B)). B6-sigOVA is a cell-line presenting high levels of the MHC class I OVA peptide-complex but no OVA class II peptide-complexes on its cell surface. Figures show means with sd from triplicates, *=P<0,05. One experiment out of two is shown. (C) Induction of OVA-specific CTL was determined. At day 10 after ablation, a mix of LN and spleen cells was obtained from mice treated as indicated. T cells were harvested and restimulated with irradiated, IFNγ-treated B16OVA cells and IL-2 for 7 days, followed by staining with OVA tetramers (K^b) and anti-CD8b. Depicted numbers represent the percentages of tetramer-positive cells within the CD8b+ population.



Finally, we analyzed mice with ablated B16OVA tumors (in the absence of exogenously added OVA) for the presence of OVA-specific CD8+ T cells by K^b-tetramer analysis. The data revealed that, consistent with the observed cross-presentation, abundant OVA-specific CD8+ T cells were only present in combined ablation/CpG-ODN treatment (Fig. 7*c*).

Collectively, these data thus indicate that cryo ablation plus CpG-ODN results in synergistically improved DC functions of OVA-Alexa(+) DC, as well as a large increase in the total numbers of these DC.

CpG-ODN injection following ablation generates therapeutic anti-tumor immunity

Since ablation is often used in advanced disease, when micro-metastasis are present, the potency of the model was analyzed in a stringent wild-type B16F10 metastasis model. Hereto, B16F10 tumor-bearing mice were injected with $30x10^3$ B16F10 cells in the contra-lateral flank 3 days prior to the ablation/CpG-ODN treatment of the primary B16F10 tumor. Subsequently, the growth of this 'metastasis' was monitored. Interestingly, next to successful elimination of the primary tumor in mice treated with ablation/CpG-ODN, regression of the established contra-lateral B16F10 metastasis was observed in 40% of the mice, and a growth reduction in the others. As expected, cryo ablation or CpG-ODN alone had no or little effect on outgrowth of the metastasis and gave 100% tumor take (Fig. 8*a*).

In the course of the experiments described above, we made the intriguing observation that CpG-ODN administration improved the success-rate of the B16OVA cryo ablation itself. Whereas 30% of the mice normally developed a local recurrence within 15 days following cryo ablation, ablation plus CpG-ODN treatment completely prevented the outgrowth of local recurrences (Fig. 8*b*).

These results thus demonstrate that ablation directly followed by CpG-ODN administration has a fast local effect in addition to the induction of a broad protective memory response.

In conclusion, these data indicate that *in situ* tumor destruction plus TLR9 stimulation leads to a more potent local and systemic anti-tumor response than either treatment modality alone. This treatment regimen allows for direct antigen-loading and maturation of DC *in vivo* without delivery of defined tumor antigens.

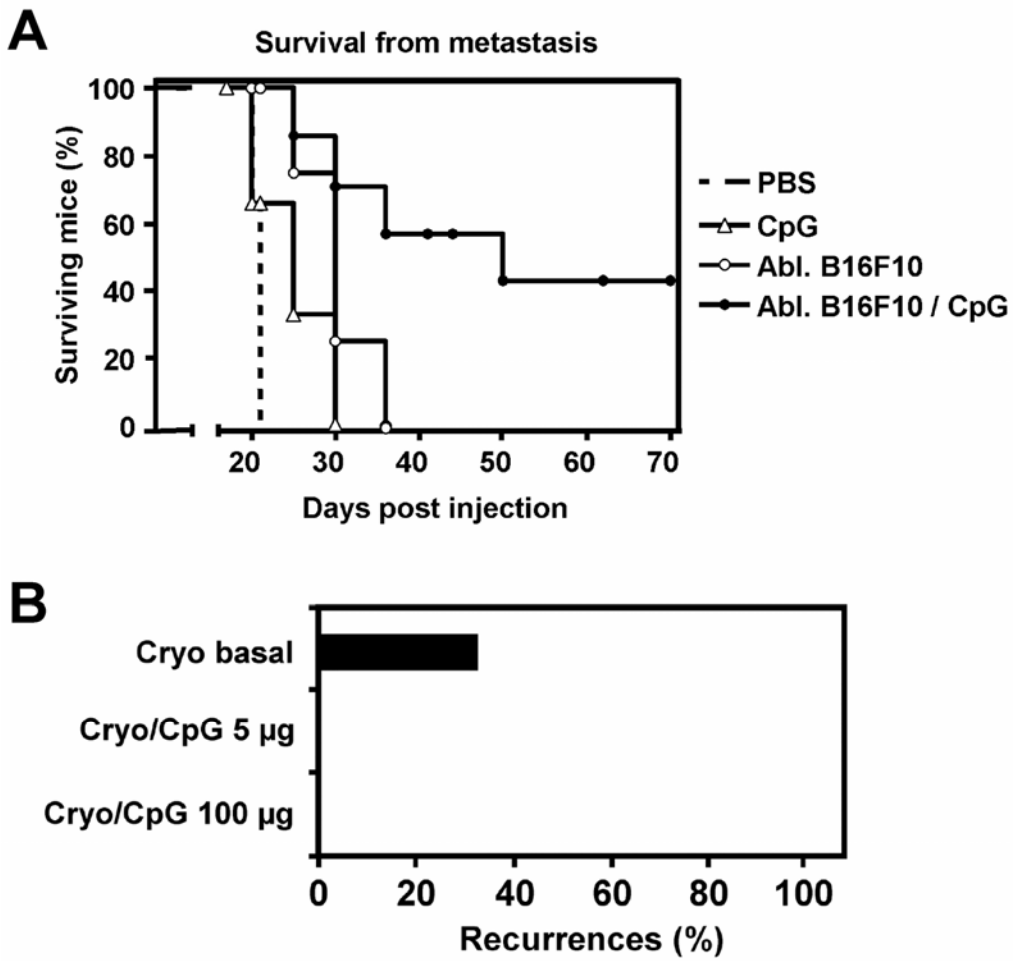


Fig. 8: CpG-ODN improves therapeutic outcome of ablation. (A) Stringent wild-type B16 metastasis model. Three days before ablation of a wild-type B16F10 tumor with (●) or without (○) co-administration of 100 µg CpG-ODN, $30x10^3$ B16F10 cells were inoculated contra-laterally of the primary tumor. After ablation was performed, growth of the metastasis was monitored. Tumor take was monitored by injection of the same tumor dose into naïve mice (dotted line). S.c. injection of CpG-ODN contra-lateral of the metastasis was performed as a control (Δ). T=0 corresponds to the time of injection of the metastasis. $P<0{,}005$ for ablation/CpG vs. ablation alone. One out of two representative experiments is shown (n=5-12 per group). (B) After ablation of B16OVA tumors, mice were monitored for tumor recurrence at the site of ablation. Shown are mice that received only ablation or ablation with 5 or 100 µg CpG-ODN peri-tumorally. Values are obtained from several experiments resulting in 20-60 mice per group.

Discussion

In recent years, *ex vivo* generated mature dendritic cells (DC) have been shown to evoke tumor-specific responses in cancer patients[24-26]. DC vaccination is, however, time consuming and expensive, and in many cases the anti-tumor response falls short in strength to cure patients with established tumors. Herein, we report that tumor-debris created by ablation of established tumors comprises an effective antigen source for DC *in vivo*. Moreover, we show that tumor ablation synergizes with the TLR9-ligand CpG-ODN to augment DC function and cross-priming *in vivo*. This creates an effective '*in situ* DC-vaccine' capable of inducing protection against lethal tumor re-challenges as well as regression of pre-existing metastases.

In situ tumor destruction with cryo, radiofrequency, or laser ablation has received increasing attention as a treatment modality for focal cancer[16-20,27-29]. However, little is known regarding the induction of immune responses after *in situ* tumor destruction or the fate of tumor-debris. Applying a mouse B16 tumor model for cryo ablation, we recently demonstrated that tumor-debris remaining *in situ* after tumor destruction creates an effective antigen depot for DC *in vivo*. Using intra-tumoral injection of ^{111}indium-labeled KLH proteins prior to ablation, we could monitor the fate of tumor-debris and demonstrate that tumor derived antigens were efficiently taken to the draining lymph node. We showed that within the draining LN, up to 20% of DC contained antigen as soon as one day and for at least three days following ablation (our unpublished results). The current analysis of the immune response induced following tumor ablation revealed that removal of the antigen depot directly after ablation completely abrogated the development of specific immunity. Although a tumor-specific immune response could be observed when leaving the tumor-debris *in situ*, mice were poorly protected against a subsequent tumor challenge. These data indicate that, despite efficient loading of DC takes place, immune activation following tumor ablation is sub-optimal.

In order to increase anti-tumor immunity following ablation, we explored stimulation of TLR9 by CpG-ODN either alone or in combination with ablation. Peri-tumoral injection of CpG-ODN has previously been shown to elicit coordinated T cell responses and induction of anti-tumor immunity in relatively immunogenic mouse models[30]. In more challenging settings with large tumor masses and less immunogenic tumors the efficacy of CpG-ODN alone was less profound. Using different tumor models we now demonstrate that ablation plus CpG-ODN treatment of 5-7mm B16OVA tumors resulted in complete protection against a lethal B16OVA tumor re-challenge, and co-incided with the presence of OVA-specific CD8+ T cells. As neither CpG-ODN, nor ablation alone was able to protect all mice, we conclude that both CpG-ODN and ablation both are essential in the induction of immunity. Recent mouse studies also indicated that CpG-ODN induced tumor growth delay and improved survival when combined with radiotherapy[31] or chemotherapy[32], although the mechanism responsible for these effects remained to be determined.

Interestingly, the combination treatment of B16OVA tumors was also able to protect 50% of the mice against outgrowth of the poorly immunogenic parental B16F10 tumor, indicating the simultaneous induction of responses against multiple epitopes. Moreover, local CpG-ODN stimulation also improved the efficacy of the ablation itself, most

likely by activation of the innate immune system. When applied to a wild-type B16F10 metastasis model, ablation plus CpG-ODN treatment induced significant regression of pre-existing B16F10 metastases (Fig. 8). In addition, we also made the striking observation during the course of our studies that local recurrence percentages at the ablated site were reduced from 30% to 0% when CpG-ODN was administered following ablation (Fig. 8).

To unravel the mechanism responsible for the induction of potent anti-tumor immunity following combination treatment *in vivo*, we focussed on the effects on DC. Analysis of the maturation state of antigen-loaded and non-loaded DC in naïve, tumor-bearing, tumor-ablated and CpG-ODN-treated mice revealed several interesting phenomena. First, DC that contained antigen expressed significantly higher levels of co-stimulatory molecules than antigen negative DC. These data are in line with *in vitro* data indicating that antigen uptake can affect DC activation[33]. Secondly, ablation resulted in a significant further increase in co-stimulatory molecule expression on antigen positive, but not antigen negative DC. Thirdly, CpG-ODN administration alone resulted in an increase in co-stimulatory molecule expression on antigen positive DC, but also on antigen negative DC. Importantly, the observed increase in the number and maturation state of antigen-loaded DC, induced by CpG-ODN or ablation alone, is apparently not sufficient to induce complete tumor protection of mice.

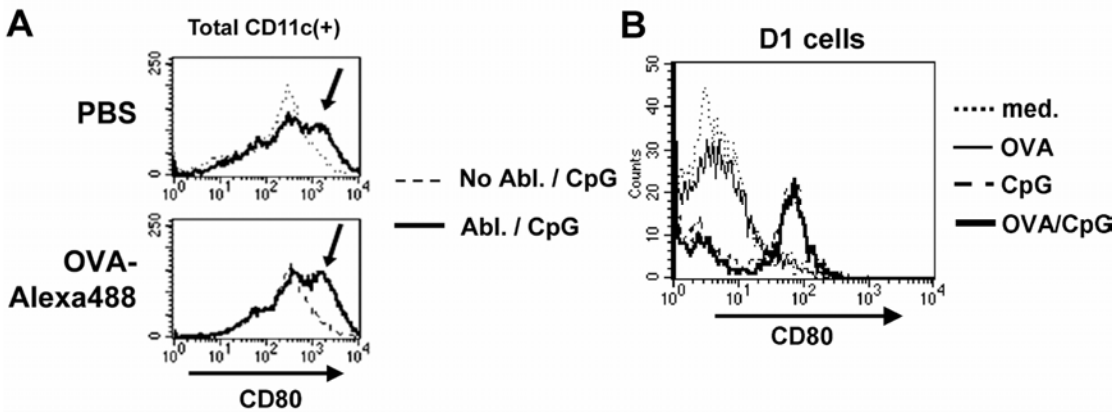


Fig. 9: (a) Exogenous and endogenous tumor antigens lead to identical DC maturation. At day two after ablation with or without co-injection of 100 μg CpG-ODN, FACS analysis was done on sorted DC from LN's. Prior to ablation, 20 μg ovalbumin conjugated to Alexa-488 (OVA-Alexa488) or PBS was injected i.t.. Histograms show a comparable induction of the CD80-high DC (arrows), independent of OVA administration. In the lower panel the CD80-high cells corresponded to the OVA-Alexa488(+) cells. **(b) OVA-Alexa-488 contains no substantial amounts of activating TLR-ligands.** D1 dendritic cells were incubated o/n with medium, 500 μg/ml OVA-Alexa-488, 1 μg/ml CpG-ODN or both. Next, CD80 expression was analyzed. Other batches of impure OVA did show maturation (not shown).

The finding that antigen uptake-dependent DC maturation was comparable with or without exogenously added OVA-Alexa (see also Fig. 9*a*) provides strong support for these model antigens truly mimicking endogenous tumor antigens. In addition to this, OVA-specific T cells were readily observed following ablation plus CpG-ODN administration in a setting where no additional OVA was administered (Fig. 7*c*). We also note that neither

antibodies used during positive MACS sorting, nor TLR-ligands often present in OVA-batches[34] did bias the CD80/CD86 staining on DC, as DC purified by negative selection showed similar results and OVA-Alexa did not mature DC *in vitro* (not shown and Fig. 9*b*).

Strikingly, CpG-ODN administration in combination with tumor ablation resulted in a tremendous increase in co-stimulatory molecule expression (Fig. 4*b*). These data are indicative for a synergistic interplay between signals induced by ablation and those resulting from TLR9 triggering. The exact nature of these ablation-dependent signals needs further clarification, but may well represent cytokines released after ablation[35-37]. Alternatively, endogenous TLR-ligands like stress-induced factors might be involved in the synergistic maturation of DC observed[38-40]. An additional explanation for this synergistic DC maturation could be the destruction of the tumor itself. Ablation of the tumor would eliminate tumor-induced factors preventing full responsiveness to CpG-ODN[15]. However, since synergistic DC maturation was comparable when ablation with CpG-ODN was performed on intact tumors or on injected B16OVA tumor lysates, in our model the negative influence of the tumor-milieu on DC function is limited (Fig. 5).

The combined ablation and CpG-ODN treatment not only affected the phenotype of LN-DC but also their capacity to cross-present antigens and activate T cells. Although ablation alone was able to generate DC that presented antigens in MHC class II, CpG-ODN co-administration was essential to obtain MHC class I presentation and CD8 cytotoxic T cell activation. The role of CpG-ODN in cross-presentation by DC *in vitro* has recently been reported[41,42], but had not been formally shown *in vivo*. Our data thus provide for the first time direct evidence for the crucial role of CpG-ODN in cross-presentation of antigens by DC *in vivo*.

An alternative approach to target antigen to DC *in vivo* explored antibodies directed against the mouse DC antigen DEC-205, which were shown to target OVA antigens preferentially to DC *in vivo*[13]. Others applied retrovirus-mediated expression of the chemokine CCL20 in tumors to increase the number of intra-tumoral DC[14]. As previously shown for *ex vivo* generated DC vaccines, both studies confirmed that maturation of *in vivo* loaded DC by either agonistic anti-CD40 antibodies or the TLR9-ligand CpG-ODN was essential to induce a potent immune response. Our results demonstrated that the tumor-debris left in the body after *in situ* tumor destruction forms a potent tumor antigen source for DC *in vivo*, and provides a much more direct way of *in situ* DC-targeting, without the need for retroviral infection or construction of recombinant proteins.

Collectively, these data show that *in vivo* tumor destruction in combination with CpG-ODN administration creates a unique and potent, '*in situ* DC-vaccine'. The fact that both treatment modalities are currently applied separately in cancer patients, makes that this promising '*in vivo* DC-vaccine' is readily applicable in clinical settings.

Materials and Methods

Animals

9-11 weeks old female C57BL/6n mice were purchased from Charles River Wiga (Sulzfeld, Germany). Animals were held under specified pathogen-free conditions in the Central Animal Laboratory (Nijmegen, the Netherlands). All experiments were performed according to the guidelines for animal care of the Nijmegen Animal Experiments Committee.

Tumors

Mice were injected subcutaneously at the right femur with $500x10^3$ cells of the OVA-transfected murine melanoma cell line B16F10 (B16OVA, clone MO5), which was kindly provided by dr. Kenneth Rock[43], or wt B16F10. Cells were cultured and injected as described before[23]. Tumor volumes were scored every three days with the formula (AxB^2) x 0.4, in which A is the largest and B is the shortest dimension. Tumors were selected for ablation when their diameter measured between 5 to 7mm (d9-10) and only if the tumor was relatively round (>98%).

Cryo ablation and in vivo procedures

Animals were properly shaven and anaesthetized by isoflurane inhalation. The tumor area was disinfected with alcohol and subsequently wetted with distilled water. The tip of the liquid nitrogen cryo ablation system (CS76, Frigitronics, Shelton, CT) was placed onto the tumor and after proper freeze attachment, treatment was started. During two treatment cycles of +/-70 seconds the tumor and a small strip around it was frozen to less than -100ºC. Treatment was considered successful when the whole tumor appeared frozen macroscopically. In Fig. 1*b*, the ablated tumor was excised afterwards, after which the skin was seamed by clamps. For antigen monitoring experiments, mice received intra-tumoral injections of ovalbumin conjugated to Alexa-488 (OVA-Alexa-488) (Molecular Probes, Leiden, the Netherlands). Conjugates (20 μg) were injected directly before ablation in 20 μl PBS. CpG-ODN 1668 (5'-TCCATGACGTTCCTGATGCT-3') (total backbone phosphorothioated) (Sigma Genosys, Haverhill, UK) was used for *in vivo* immune activation. 100 μg CpG-ODN in 30 μl PBS was injected 1 hour after ablation, divided over three injections in the peri-tumoral area. In Fig. 5, $10x10^6$ B16OVA cells were lysed by freeze thaw cycles and injected s.c. with OVA-Alexa, after which similar ablative treatment was performed as described above.

Magnetic bead cell sorting and flow cytometric analysis

For antigen uptake experiments, draining LN's from 5-8 mice were pooled and after crushing, dissociation in DNAse/collagenase/EDTA, and passage through nylon mesh[44], cells were counted and sorted by standard MACS isolation with a MACS Midi column. Positive selection of DC was done using CD11c beads (clone N418, Miltenyi Biotec, B.Gladbach, Germany), whereas negative selection/enrichment was done on the CD90 T cell marker (Thy1.2, 30.H12, Miltenyi Biotec). Sorts were verified by CD3e or CD11c (HL3) staining (not shown). Subsequently, cells were stained and analyzed on a FACS-Calibur™

system (BD) with the CELLQuest software. Staining was performed using the following mAbs: CD11c-APC (HL3), CD8b-FITC (53-5.8), CD3e-PE (17-A2), biotinylated CD80 (1G10), biotinylated CD86 (GL-1) and streptavidin-PE. All antibodies were purchased from BD Pharmingen (Alphen a/d Rijn, the Netherlands).

Antigen presentation to B3Z and DO11.10

The B3Z T cell hybridoma contains *lacZ* that is induced upon engagement of its TCR that recognizes OVA peptide (257-264) in the context of $H2K^b$ [45], whereas the DO11.10 T cell hybridoma, produces IL-2 upon engagement of its TCR that recognizes OVA-peptide (323-339) bound to $I\text{-}A^d$ (but cross-reacts to $I\text{-}A^b$). β-Galactosidase activity of the B3Z T cells ($100x10^3$/well) after overnight incubation with LN-derived, CD11c sorted DC ($100x10^3$/well) was determined by incubating with 0.15 mM chlorophenolred-β-d-galactopyranoside (Calbiochem), 9 mM $MgCl_2$, 0.125% Nonidet P-40 and 100 mM β-mercaptoethanol in PBS for 4 h at 37°C, and OD_{595} was determined. Same procedures were performed with the DO11.10 cells, but here IL-2 production was determined using standard ELISA procedures. As control the B6MEC transfected with murine B7.1, $H\text{-}2K^b$, and a construct expressing an ER targeting signal sequence, followed by the $OVA_{257–264}$ CTL epitope SIINFEKL (B6-B7.1-sigOVA) was used[46].

Tetramer stainings

A T cell culture was obtained from spleen and draining LN's of mice 10 days after ablation of a B16OVA tumor or from naïve control mice. Stimulation of these cells ($100x10^3$) was performed by addition of irradiated, IFN-γ-treated, B16OVA cells ($50x10^3$) in IL-2 (10 CU/ml) supplemented culture medium. At day 5 and day 10, cells were collected and cleaned in a density gradient. At day 10 of culture, cells were stained for 15 min at RT by OVA-tetramers ($H\text{-}2K^b$) conjugated to APC, which were a kind gift of S.H. van der Burg (LUMC, Leiden, The Netherlands) and counterstained for CD8b.2.

Re-challenge and metastasis model

Forty days after ablation of B16OVA tumors, mice were challenged by subcutaneous injection at the contra-lateral femur of either $15x10^3$ B16OVA cells or $15x10^3$ EL4 cells (numbers defined by titration). Some mice that rejected the first re-challenge received a second set of re-challenges with $15x10^3$ B16OVA cells and $10x10^3$ B16F10, inoculated on day 120 (end of Fig. 1*a*) on the right and left flank. Injections were performed in 100 μl PBS. Mice were sacrificed when tumors reached a volume of ± 850 mm^3. In the metastasis model, $30x10^3$ B16F10 cells were injected contra-laterally of the matching tumor to ablate. Three days later, cryo ablation was performed on the primary tumor and 100 μg CpG-ODN was administered peri-tumoral. Next, the growth of the metastasis was monitored.

Statistical analysis

All data were analyzed for statistical significance by Student's T-test, except for the Kaplan Meier survival curves for which a log rank test was used.

References

1. Banchereau, J. & Palucka, A.K. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* **5**, 296-306 (2005).
2. Bevan, M.J. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**, 1283-8 (1976).
3. Reis e Sousa, C. Dendritic cells as sensors of infection. *Immunity* **14**, 495-8 (2001).
4. Akira, S. & Takeda, K. Toll-like receptor signalling. *Nat Rev Immunol* **4**, 499-511 (2004).
5. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* **5**, 987-95 (2004).
6. Krieg, A.M. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* **20**, 709-60 (2002).
7. Steinman, R.M. & Nussenzweig, M.C. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* **99**, 351-8 (2002).
8. Figdor, C.G., de Vries, I.J., Lesterhuis, W.J. & Melief, C.J. Dendritic cell immunotherapy: mapping the way. *Nat Med* **10**, 475-80 (2004).
9. De Vries, I.J. et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res* **63**, 12-7 (2003).
10. Coulie, P.G. & van der Bruggen, P. T cell responses of vaccinated cancer patients. *Curr Opin Immunol* **15**, 131-7 (2003).
11. Schuler, G., Schuler-Thurner, B. & Steinman, R.M. The use of dendritic cells in cancer immunotherapy. *Curr Opin Immunol* **15**, 138-47 (2003).
12. Steinman, R.M. & Mellman, I. Immunotherapy: bewitched, bothered, and bewildered no more. *Science* **305**, 197-200 (2004).
13. Bonifaz, L.C. et al. *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* **199**, 815-24 (2004).
14. Furumoto, K., Soares, L., Engleman, E.G. & Merad, M. Induction of potent antitumor immunity by *in situ* targeting of intratumoral DCs. *J Clin Invest* **113**, 774-83 (2004).
15. Vicari, A.P. et al. Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. *J Exp Med* **196**, 541-9 (2002).
16. Curley, S.A. Radiofrequency ablation of malignant liver tumors. *Ann Surg Oncol* **10**, 338-47 (2003).
17. Raj, G.V., Reddan, D.J., Hoey, M.B. & Polascik, T.J. Management of small renal tumors with radiofrequency ablation. *Urology* **61**, 23-9 (2003).
18. Garcea, G., Lloyd, T.D., Aylott, C., Maddern, G. & Berry, D.P. The emergent role of focal liver ablation techniques in the treatment of primary and secondary liver tumours. *Eur J Cancer* **39**, 2150-64 (2003).

19. Ruers, T. & Bleichrodt, R.P. Treatment of liver metastases, an update on the possibilities and results. *Eur J Cancer* **38**, 1023-33 (2002).
20. Zagoria, R.J., Chen, M.Y., Kavanagh, P.V. & Torti, F.M. Radiofrequency ablation of lung metastases from renal cell carcinoma. *J Urol* **166**, 1827-8 (2001).
21. Soanes, W.A., Ablin, R.J. & Gonder, M.J. Remission of metastatic lesions following cryosurgery in prostatic cancer: immunologic considerations. *J Urol* **104**, 154-9 (1970).
22. Sanchez-Ortiz, R.F., Tannir, N., Ahrar, K. & Wood, C.G. Spontaneous regression of pulmonary metastases from renal cell carcinoma after radiofrequency ablation of primary tumor: an *in situ* tumor vaccine? *J Urol* **170**, 178-9 (2003).
23. den Brok, M.H. et al. *In situ* tumor ablation creates an antigen source for the generation of antitumor immunity. *Cancer Res* **64**, 4024-9 (2004).
24. Nestle, F.O. et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* **4**, 328-32 (1998).
25. Jonuleit, H. et al. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T cell responses in humans following intranodal injection. *Int J Cancer* **93**, 243-51 (2001).
26. Su, Z. et al. Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. *Cancer Res* **63**, 2127-33 (2003).
27. Ruers, T.J., Joosten, J., Jager, G.J. & Wobbes, T. Long-term results of treating hepatic colorectal metastases with cryosurgery. *Br J Surg* **88**, 844-9 (2001).
28. Erce, C. & Parks, R.W. Interstitial ablative techniques for hepatic tumours. *Br J Surg* **90**, 272-89 (2003).
29. Veth, R., Schreuder, B., van Beem, H., Pruszczynski, M. & de Rooy, J. Cryosurgery in aggressive, benign, and low-grade malignant bone tumours. *Lancet Oncol* **6**, 25-34 (2005).
30. Heckelsmiller, K. et al. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. *J Immunol* **169**, 3892-9 (2002).
31. Milas, L. et al. CpG oligodeoxynucleotide enhances tumor response to radiation. *Cancer Res* **64**, 5074-7 (2004).
32. Weigel, B.J., Rodeberg, D.A., Krieg, A.M. & Blazar, B.R. CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma. *Clin Cancer Res* **9**, 3105-14 (2003).
33. Randolph, G.J., Beaulieu, S., Lebecque, S., Steinman, R.M. & Muller, W.A. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science* **282**, 480-3 (1998).
34. Pasare, C. & Medzhitov, R. Toll-dependent control mechanisms of CD4 T cell activation. *Immunity* **21**, 733-41 (2004).
35. Sporri, R. & Reis e Sousa, C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* **6**, 163-70 (2005).

36. de Jong, K.P. et al. Serum response of hepatocyte growth factor, insulin-like growth factor-I, interleukin-6, and acute phase proteins in patients with colorectal liver metastases treated with partial hepatectomy or cryosurgery. *J Hepatol* **34**, 422-7 (2001).
37. Huang, A. et al. Phase I study of percutaneous cryotherapy for colorectal liver metastasis. *Br J Surg* **89**, 303-10 (2002).
38. Shi, Y., Evans, J.E. & Rock, K.L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-21 (2003).
39. Skoberne, M., Beignon, A.S. & Bhardwaj, N. Danger signals: a time and space continuum. *Trends Mol Med* **10**, 251-7 (2004).
40. Somersan, S. et al. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* **167**, 4844-52 (2001).
41. Datta, S.K. et al. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J Immunol* **170**, 4102-10 (2003).
42. Maurer, T. et al. CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. *Eur J Immunol* **32**, 2356-64 (2002).
43. Falo, L.D., Jr., Kovacsovics-Bankowski, M., Thompson, K. & Rock, K.L. Targeting antigen into the phagocytic pathway *in vivo* induces protective tumour immunity. *Nat Med* **1**, 649-53 (1995).
44. Vremec, D., Pooley, J., Hochrein, H., Wu, L. & Shortman, K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* **164**, 2978-86 (2000).
45. Karttunen, J., Sanderson, S. & Shastri, N. Detection of rare antigen-presenting cells by the lacZ T cell activation assay suggests an expression cloning strategy for T cell antigens. *Proc Natl Acad Sci U S A* **89**, 6020-4 (1992).
46. Schoenberger, S.P. et al. Efficient direct priming of tumor-specific cytotoxic T lymphocyte *in vivo* by an engineered APC. *Cancer Res* **58**, 3094-100 (1998).

Chapter 6

Toll-like Receptor 2 controls expansion and function of regulatory T cells

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Abstract

Regulatory T cells (Treg) play a central role in the suppression of immune reactions and prevention of autoimmune responses harmful to the host. During acute infection however, Treg might hinder effector T cell activity directed towards the elimination of the pathogenic challenge. Pathogen recognition receptors from the Toll-like receptor (TLR) family expressed by innate immune cells are crucial for the generation of effective immunity. We have recently shown the $CD4^+CD25^+$ Treg subset in TLR2-/- mice to be significantly reduced compared with wildtype littermate control mice, indicating a link between Treg and TLR2. Here we report that the TLR2-ligand Pam_3Cys, but not LPS (TLR4) or CpG-ODN(TLR9), directly acts on purified Treg in a MyD88-dependent fashion. Moreover, when combined with TCR-stimulation, TLR2-triggering augmented Treg proliferation *in vitro* and *in vivo* and resulted in a temporal loss of the suppressive Treg phenotype *in vitro* by directly affecting the Treg themselves. Importantly, in TLR2-/- recipient mice, adoptively transferred wildtype Treg were neutralized by systemic administration of TLR2-ligand during the acute phase of a *Candida albicans* infection resulting in a 100-fold reduced *Candida* outgrowth. This demonstrates that also *in vivo* TLR2 controls the function of Treg and establishes a direct link between TLRs and the control of immune responses through regulatory T cells.

Introduction

It is now evident that the rediscovered regulatory/suppressor T cells (Treg) play a dominant role in our immune system (reviewed in [1,2]). Two types of Treg can be distinguished, $CD4^+CD25^+$ intrinsic Treg, that are already present in the naïve individual, and those induced in the periphery. T cell receptor (TCR) triggering renders intrinsic Treg able to suppress both $CD4^+$ and $CD8^+$ lymphocytes[3,4] via a process that is cell-cell-contact dependent, though the exact mechanism is not yet identified. Several studies have shown that their depletion results in autoimmune syndromes like thyroiditis, gastritis, insulin-dependent diabetes mellitus, colitis and arthritis[5-8]. On the other hand, active Treg hinder the induction of immune responses against pathogens and tumors[9-11], emphasizing the importance of a tight control of these regulators themselves. Importantly, IL-2[3], IL-6[12] and strength of the TCR signal[13] have been reported to release effector T cells from Treg mediated suppression.

Besides these control mechanisms regulating the sensitivity of effector T cells to suppression, still many questions remain regarding regulatory mechanisms acting at the level of the Treg themselves. Treg are able to proliferate in normal un-manipulated mice[14]. In addition, the finding that CD80/CD86-deficient mice have significantly decreased numbers of Treg indicates a role for co-stimulation in Treg homeostasis[15]. Furthermore, mature DC have been reported to induce Treg expansion[16]. In line with this, TLR-triggered DC were recently shown to induce Treg proliferation by a cooperative action of IL-1 and IL-6[17]. Together, there is accumulating evidence that Treg anergy can be overcome when the right stimulatory environment is provided, showing that Treg play a dynamic role in the control of immune responses.

One family of receptors involved in immune-regulation is the Toll-like receptor (TLR) family, a class of receptors that recognizes pathogen-associated molecular patterns (PAMPs) or endogenous inflammation-associated molecules[18]. So far, thirteen receptors have been identified with different or overlapping ligands. TLR1/2 heterodimers for example can recognize bacterial lipoproteins, TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria and TLR9 recognizes bacterial DNA (unmethylated CG-motifs). TLRs are abundantly expressed on innate immune cells, like macrophages and DCs, and TLR9-induced IL-6 production by DC releases effector T cells from Treg mediated suppression[12]. Recently, TLRs were also found on T cells including Treg[19]. Therefore, we hypothesized that some TLR-ligands might directly affect the Treg. We have previously shown that Treg numbers in the circulation of TLR2-/- mice, but not TLR4-/- mice, are significantly reduced compared to their wildtype littermate controls[10]. Moreover, we and others have shown that the immune response against the fungal pathogen *Candida albicans* is affected by Treg[9,10]. Using highly purified intrinsic Treg and conventional $CD4^+$ T cells, we now show that TLR2-triggering on the intrinsic Treg themselves results in proliferation of these regulatory T cells *in vitro* and *in vivo*. Moreover, in presence of the TLR2-ligand Pam_3Cys the suppressive phenotype of the regulatory T cells is temporarily abrogated *in vitro* and *in vivo*, thereby enhancing the anti-fungal response in an acute infection model. After removal of the TLR2-trigger the Treg fully regained their suppressive capabilities. These findings demonstrate that in addition to their effects on antigen

presenting cells (APC), TLR-ligands can directly act on the intrinsic regulatory T cell resulting in dynamic modulation of the immune response.

Results

TLR2-signalling modulates CD4$^+$ CD25$^+$ T cell levels in vivo.

Recently, we demonstrated decreased numbers of circulating CD4$^+$CD25$^+$ regulatory T cells (Treg) in blood of TLR2-/- mice, but not of TLR4-/- mice[10]. These findings suggest a role for TLR2-signaling in Treg homeostasis and/or function. As TLR2-signaling is critically dependent on the adaptor molecule MyD88[18], we now determined the relative number of regulatory T cells present in blood and spleen of MyD88 deficient mice and their wildtype littermate controls.

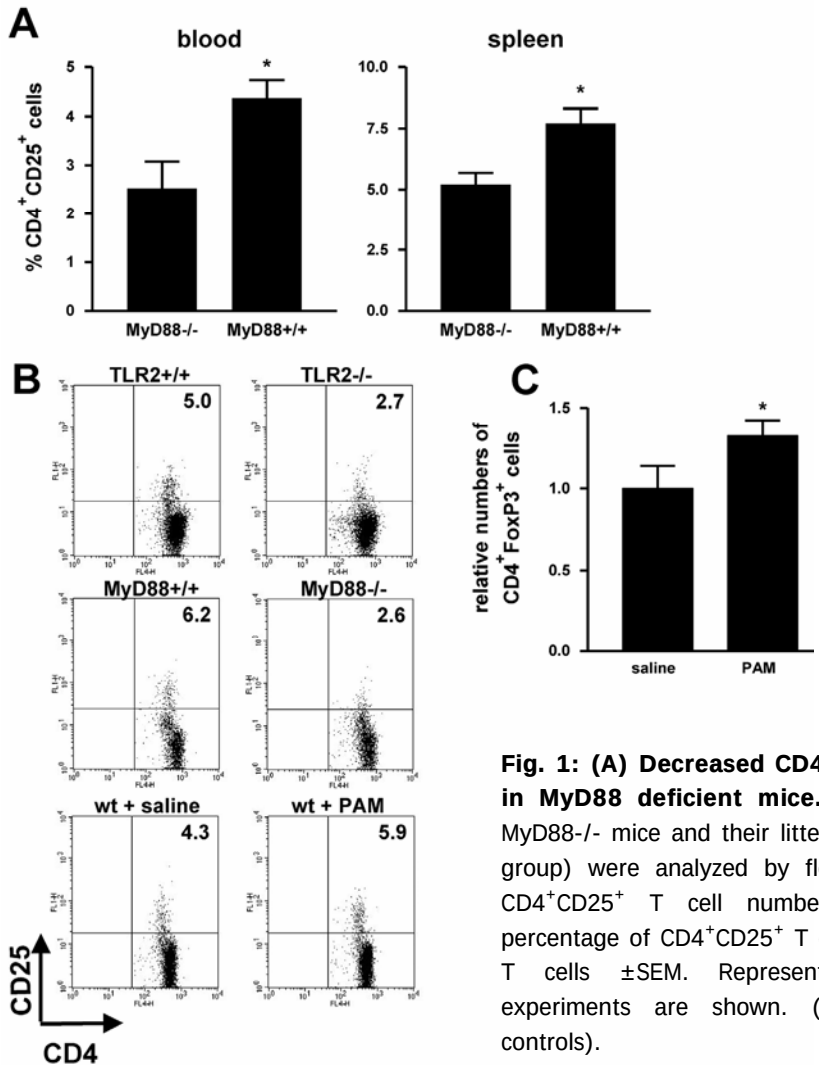

Fig. 1: (A) Decreased CD4$^+$CD25$^+$ T cell numbers in MyD88 deficient mice. Blood and spleens from MyD88-/- mice and their littermate +/+ controls (4 per group) were analyzed by flow cytometry for relative CD4$^+$CD25$^+$ T cell numbers. Data indicate mean percentage of CD4$^+$CD25$^+$ T cell numbers of total CD4$^+$ T cells ±SEM. Representative results of three experiments are shown. (*=P<0.02 with wildtype controls).

(B-C) TLR2-signaling modulates CD4+CD25+ T cell numbers. (B) Blood from TLR2-/-, MyD88-/- and their wildtype littermate controls as well as from PAM-challenged mice was analyzed by flow cytometry for CD4 and CD25 expressing cells. Values in the upper-right quadrant indicate the percentage CD4$^+$CD25$^+$ T cells from the total number of CD4$^+$ T cells. Data are representative of three independent experiments with four mice per group. (C) PAM induces an increase in FoxP3$^+$ cells in the periphery. Mice (four per group) were challenged i.p. with 20 µg PAM and two days later the percentage of CD4$^+$FoxP3$^+$ T cell of the total CD4$^+$ T cells in the blood was determined by flowcytometry. Data indicate the average relative increase with saline control set at 1. *=P<0.05 for saline control compared with PAM treated group)

As shown in Fig. 1*a*, MyD88-/- mice, like TLR2-/- mice[10], contained significantly lower numbers of $CD4^+CD25^+$ T cells compared to their wildtype controls. In contrast, the percentage of $CD4^+CD25^-$ conventional Th cells did not differ between MyD88 and control mice (15.9 ± 1.2 and 17.6 ± 1.5 respectively) as well as TLR2-/- mice and their controls (17.7 ± 2.1 and 18.3 ± 1.9 respectively). In Fig. 1*b* and *c*, representative $CD4^+CD25^+$ T cell stainings from individual TLR2-/- and MyD88-/- mice and their wildtype (WT) littermate controls are shown. The decreased Treg numbers in both the TLR2- and MyD88-knockout mice indicate that a lack of TLR2-signaling is responsible for the observed decrease of $CD4^+CD25^+$ T cell numbers *in vivo*.
These results thus demonstrate a relation between the TLR2/MyD88-signaling pathway and Treg numbers *in vivo*.

TLR-triggering in the presence of APC modulates Treg in vitro.
TLR2 is expressed by cells of the innate immune system, including APC, as well as by Treg cells[19]. TLR-triggering compounds are known to promote APC activation[18], resulting in the production of cytokines affecting T cell function[12]. Alternatively, some TLR-ligands might directly act on the Treg cells themselves. To address the role of TLRs in Treg function, we analyzed the effect of different TLR-ligands on Treg proliferation *in vitro*. As expected, Treg cells cultured in the presence of irradiated unstimulated APC and soluble anti-CD3 did not display proliferation (Fig. 2*a*), in accordance with their anergic state[20]. Addition of various TLR-ligands to these APC/Treg cocultures significantly enhanced their proliferative capacity resulting in increased numbers of Treg (Fig. 2*a*). This observation is in line with a recent report describing inflammatory cytokine production by TLR-triggered DC results in increased proliferation of the Treg[17]. The effect of TLR-ligands on Treg proliferation is also reflected by the up-regulation of the T cell activation marker CD25. As shown in Fig. 2*b*, addition of TLR-ligands LPS (TLR4), Pam_3Cys-SKKKK (PAM, TLR2), or CpG-ODN(TLR9) all resulted in a significant increase in CD25 expression on Treg. However, the increase in CD25 expression was most pronounced upon addition of PAM. Thus, these data show that in the presence of APC, TLR-ligands induce Treg proliferation and CD25 up-regulation.

TLR2-triggering in the absence of APC modulates Treg in vitro.
To investigate the direct effects of these TLR-ligands on intrinsic regulatory T cells and conventional $CD4^+CD25^-$ Th cells, highly pure (>98%) Treg and Th cells were incubated with the TLR-ligands plus anti-CD3 antibodies and IL-2 but, importantly, in the absence of APC. Interestingly, only the addition of PAM, but not purified LPS or CpG, resulted in profoundly increased expression of T cell activation markers CD25 (Fig. 2*c*, and Fig. 3*a*) and CD69 (not shown) on Treg. Only limited effects of TLR-stimulation were observed for conventional Th cells.
In contrast to highly purified LPS (TLR4-ligand), the synthetic TLR2-ligands Pam_3Cys (TLR1/2) and MALP-2 (TLR2/6) as well as the natural TLR2 ligands in Peptidoglycan, commercial (non-pure) LPS and heat-killed *Candida* (all containing TLR2-ligands) induced CD25 up-regulation, indicating that besides synthetic TLR2-ligands, also natural TLR2-ligands directly affect Treg (Fig. 3*b*).

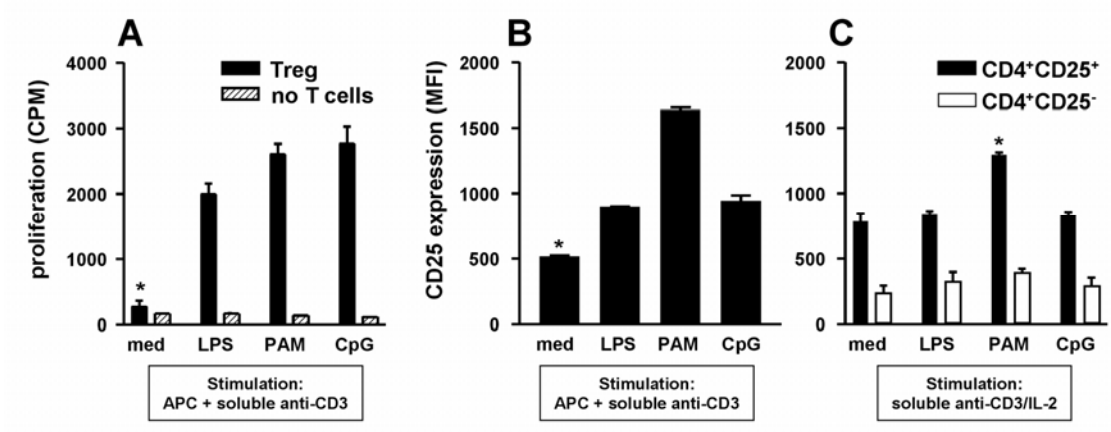

Fig. 2: *In vitro* TLR2 signalling results in Treg proliferation. Below each graph, the specific T cell stimulation is indicated. (A) Proliferation of regulatory T cells in the presence of irradiated APC, anti-CD3 and TLR-ligands. Irradiated APC and anti-CD3 (medium control, med) were cultured for three days with or without 10^4 purified Treg and with or without the addition of TLR-ligands: purified LPS (TLR4), PAM (TLR2) or CpG-ODN(TLR9). Values indicate average counts per minute (CPM) of triplicate wells ± SD. (*= P< 0.02 for medium control compared to TLR-ligands) (B-C) CD25 expression by purified $CD4^+CD25^{+/-}$ T cells. (B) Treg were cultured for three days in the presence of irradiated APC and anti-CD3 (medium control) or with addition of purified LPS, PAM or CpG. CD25 expression was measured by flow cytometry. Values indicate average mean fluorescence intensity (MFI) of anti-CD25-FITC stained $CD4^+$cells of triplicates ± SD. *=P<0.02 for medium control compared to TLR-ligands. (C) T cell activation in the absence of APC. Purified $CD4^+CD25^+$ Treg or $CD4^+CD25^-$ conventional Th cells were cultured with IL-2 and soluble anti-CD3 (medium control) or with the addition of purified LPS, PAM or CpG-ODN (no APC present). After three days, CD25 expression was measured by flow cytometry. Values indicate average MFI of triplicates ± SD. One representative experiment out of three is shown. *=P<0.02 PAM vs medium control.

Of note, differences between the basal levels of CD25 expression of Treg stimulated with either anti-CD3/APC or anti-CD3/IL-2 (Fig. 2*b* versus 2*c*) can be explained by the different amounts of (co)-stimulatory signals the Treg receive with each different stimulation approach. Both approaches use anti-CD3 but differ in the use of APC versus IL-2. Yet, in the absence of APC, TLR2-ligand PAM results in increased expression of T cell activation markers on Treg.

TLR2-signalling by Treg themselves induces expression of CD25.

To exclude that the effects caused by PAM were the result of a contamination in the synthetic PAM preparation, we tested Treg purified from TLR2- and MyD88-deficient mice. We found that only WT Treg cells responded to PAM with an increase in CD25 expression, whereas no effect was observed for TLR2-deficient and MyD88-deficient Treg (Fig. 4*a*), indicating that PAM acts through both TLR2- and MyD88-dependent signaling pathways.

To further exclude that a small amount of contaminating cells within the FACS-sorted Treg preparations are responsible for the observed effects, we used $CD4^+CD25^+$ T cells from TLR2-/- mice that we found unable to respond to PAM (Fig. 4*a*). To mimic a cellular contamination, increasing amounts of wildtype syngeneic APC were added to FACS-sorted

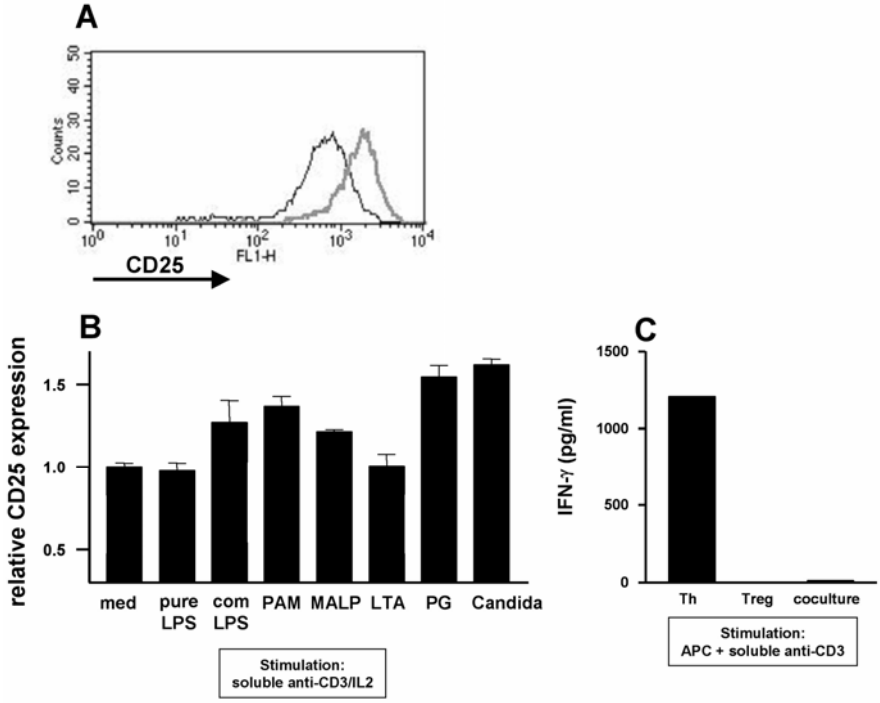

Fig. 3: (A) Example of CD25 expression by freshly isolated regulatory T cells incubated for three days in medium with anti-CD3/IL-2 (thin line) or supplemented with TLR2 ligand PAM (thick gray line). (B) Effect of different TLR-ligands on Treg activation. PAM-expanded Treg cells were cultured with anti-CD3, IL2 and the indicated TLR-ligand (10 µg/ml purified LPS, 10 µg/ml commercial LPS, 2 µg/ml PAM, 2µg/ml MALP-2, 10µg/ml LTA, 10µg/ml Peptidoglycan (PG), and 2 x10^5 heat-killed *Candida*/ml). The cells were incubated for three days and subsequently CD25 expression was analyzed by flow cytometry. Representative results from two experiments are shown. (C) *In vitro* suppression assay: PAM-expanded Treg were rested for at least six days in the absence of TLR-ligands and subsequently cocultured for three days with 10^4 fresh naïve $CD4^+$ T cells, irradiated APC and anti-CD3. After three days, IFN-γ production was measured in the supernatant using the murine inflammation CBA kit.

TLR2-/- Treg in the presence of PAM/anti-CD3. The results (Fig. 4*b*) showed that almost equal numbers of wildtype APC were needed to increase CD25 expression on the TLR2-deficient Treg. This shows that in our experiments with highly pure WT Treg (Figure 2*c* and 4*a*) PAM must directly act on the Treg.

These data thus indicate that the TLR2-ligand PAM, but not TLR4 or TLR9 ligands, is able to directly trigger regulatory T cells in a MyD88-dependent manner.

TLR2 signals induce Treg expansion in vitro.

In an attempt to establish long-lived Treg cultures, TLR-ligands were added to a culture of purified Treg, feeder cells and supplemented with soluble anti-CD3 and IL-2. In a primary stimulation, the addition of PAM, LPS or CpG-ODN increased the proliferation of the regulatory T cells (Fig. 2*b*). In multiple experiments however, the addition of LPS or CpG-ODNwas not sufficient to obtain viable regulatory T cell lines (not shown). In contrast, co-culturing the Treg in the presence of PAM resulted repeatedly in the generation of a pure $CD4^+CD25^+$ T cell line. Analysis of proliferation of CFSE-labeled freshly isolated wildtype

and TLR2-/- derived Treg showed that wildtype but not TLR2-/- Treg responded to stimulation with anti-CD3 and TLR2-ligand (Fig. 4*c*).

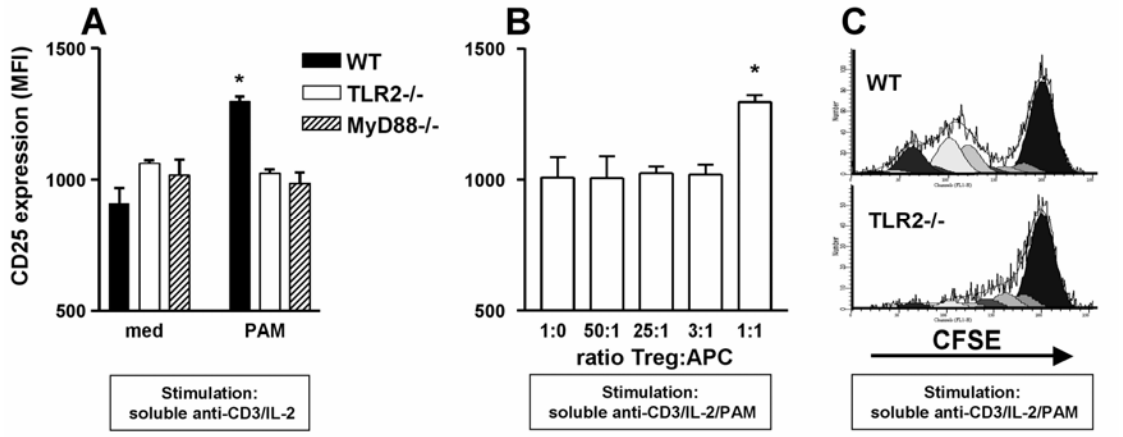

Fig. 4: PAM induces CD25 expression through TLR2-signaling. Below each graph, the specific T cell stimulation is indicated. (A) TLR2 and MyD88 expression is required for PAM-mediated increase of CD25 expression. Purified WT, TLR2-/- and MyD88-/- $CD4^+CD25^+$ T cells were cultured for three days with anti-CD3, IL-2 (medium control) or with the addition of PAM. Subsequently, the cells were harvested and CD25 expression was analyzed by flow cytometry. Values indicate average MFI from triplicate wells ± SD. (*= P< 0.02 with medium control). A representative result of three experiments is shown. (B) High numbers of wildtype APC are required to increase CD25 expression on TLR2-/- Treg cells. Purified $CD4^+CD25^+$ T cells from TLR2 -/- mice were incubated for three days with the increasing amounts of wildtype APC plus TLR2-ligand PAM and anti-CD3 and subsequently CD25 expression was analyzed by flow cytometry. Values indicate average MFI from triplicates ± SD (*= P< 0.05 with medium control). (C) Proliferation of CFSE-labeled freshly isolated wildtype and TLR2-/- Treg after stimulation with soluble anti-CD3, IL-2 and PAM. After four days proliferation resulting in a decrease of fluorescent signal in the daughter cells, was analyzed by flow cytometry and ModFit analysis software. Representative results of two experiments are shown.

The phenotypic characteristics of these PAM cultured Treg were consistent with the reported intrinsic Treg markers, including CD4, CD25, CTLA-4, GITR, CD103[1,21] and the Treg specific transcription factor FoxP3[22-24] (Figure 5). Of note, TLR2-/- Treg that were treated *in vitro* with the same PAM-based expansion protocol did not proliferate (see Figure 4*c*), but did express similar amounts of CD4, CD25, CTLA-4, GITR, CD103 and FoxP3 (data not shown). Importantly, wildtype Treg expressed low but significant amounts of TLR2 mRNA (Figure 5*b*) as well as protein (Figure 5*c*), which further strengthens our hypothesis of TLR2-mediated control of Treg function.

Cooperation between TLR2- and TCR-signalling results in Treg expansion.

To address the effects of combined TLR2- and TCR-signaling in more detail, we analyzed the expression of the activation marker CD25 on *in vitro* expanded Treg in time. Our results show that TLR2-triggering of Treg cooperated with anti-CD3-mediated TCR stimulation resulting in maximal increased CD25 expression (Fig. 6*a*) as compared with either stimulation alone. This shows that these cells remained responsive towards TLR2

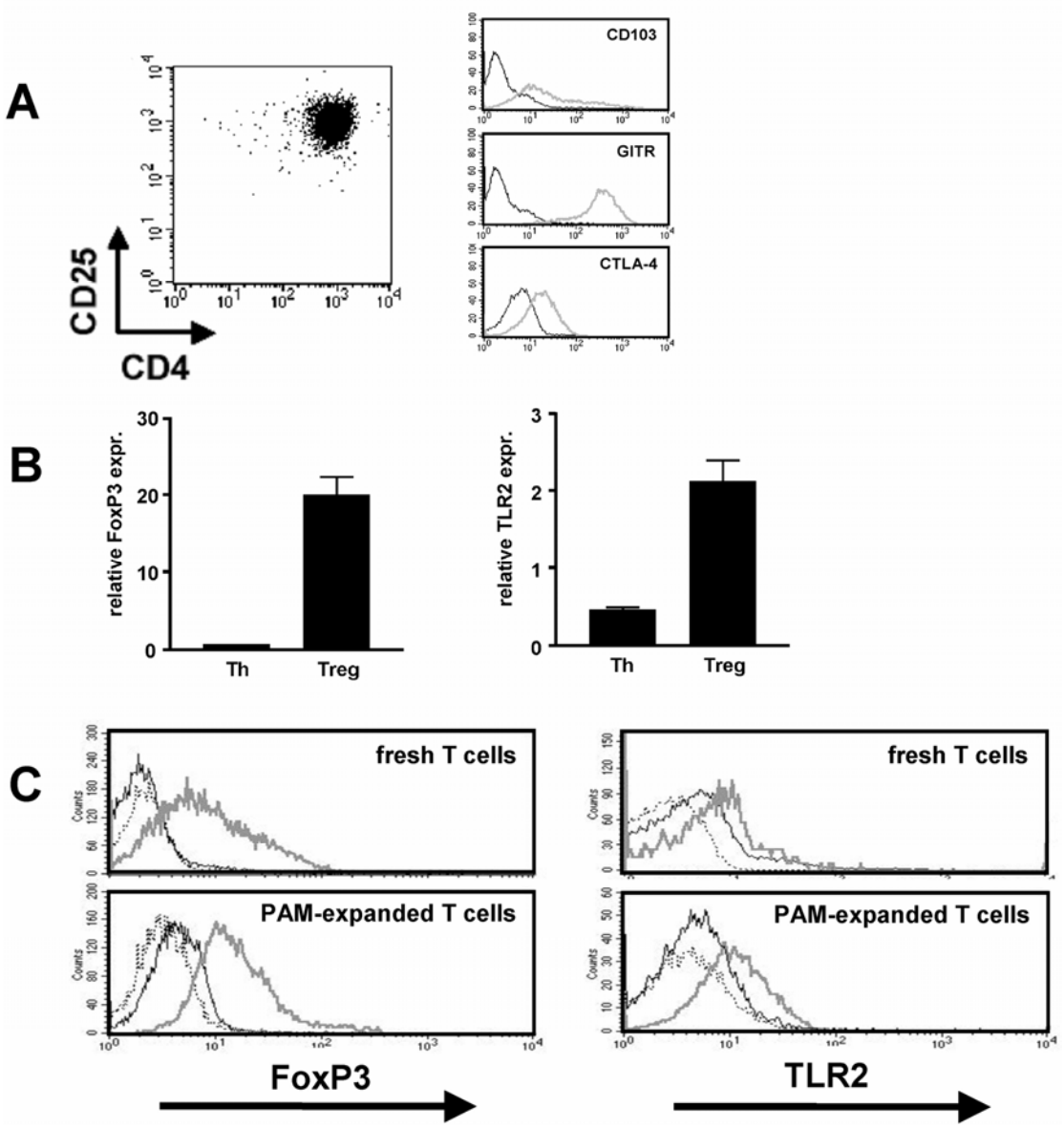

Fig. 5: Phenotype of PAM-expanded Treg. Expression of intrinsic Treg specific markers on PAM-expanded resting (7 days after stimulation with PAM) regulatory T cells was analyzed by flow cytometry and quantitative PCR. (A) The PAM-expanded Treg expressed the markers CD4, CD25, GITR, CTLA-4 and CD103 (indicated by the thick gray lines, corresponding isotype controls are indicated with a thin black line). CTLA-4 was detected by standard intracellular staining procedure. (B) Expression of FoxP3 (left panel) and TLR2 (right panel) mRNA by resting PAM-expanded Treg and conventional $CD25^-$ T helper cells was determined by quantitative PCR. The quantitative PCR results are indicated as mean relative mRNA expression from 3 replicate measurements (shown as arbitrary units relative to PBGD) ± SD. (C) Expression of FoxP3 (left panel) and TLR2 (right panel) protein determined by flow cytometry on resting PAM-expanded T cells: Treg (indicated with gray lines) and conventional T helper cells (indicated with black lines), as well as freshly isolated T cells: $CD4^+CD25^+$ Treg (gray lines) and $CD4^+CD25^-$ Th cells (black lines). Corresponding isotype controls are indicated by the dotted lines. Representative results from two experiments are shown.

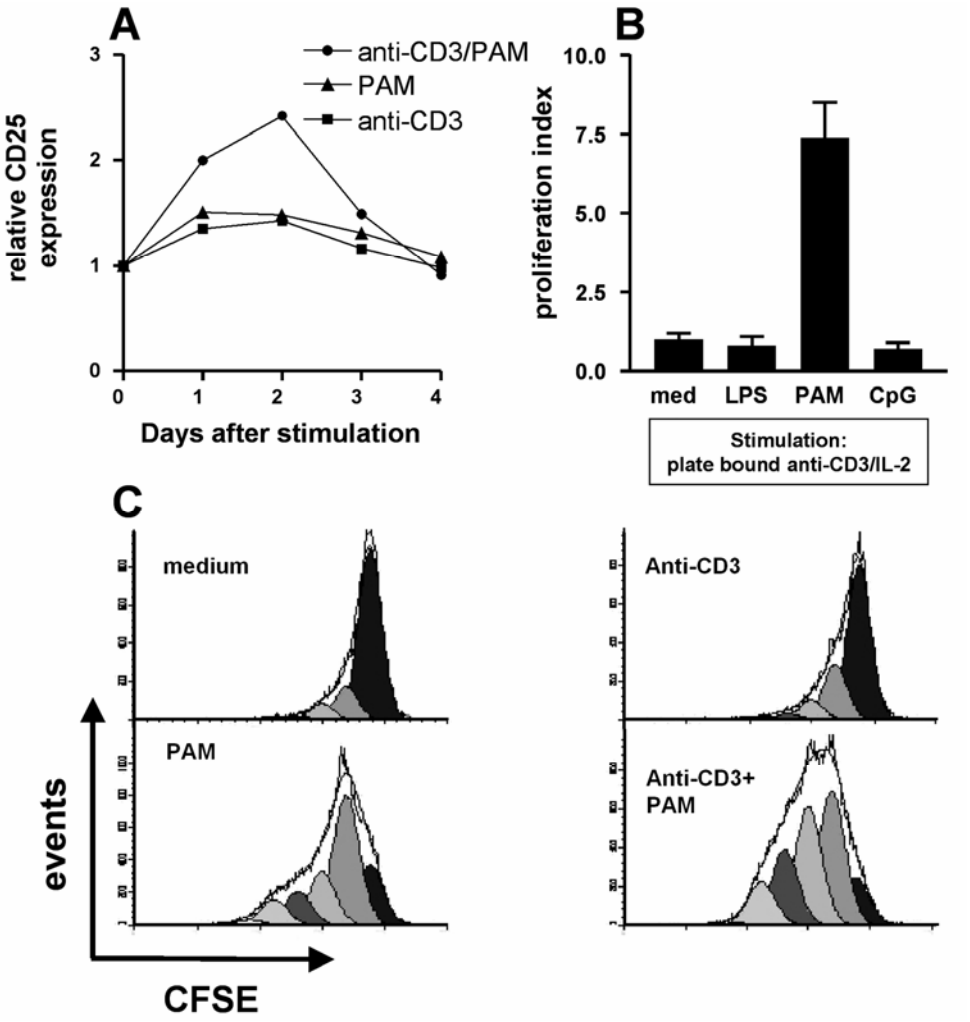

Fig. 6: Proliferation of PAM-expanded regulatory T cells. (A) TLR2- and TCR-signals cooperate to increase CD25 expression on Treg. The Treg cells were incubated with either PAM, anti-CD3 or the combination of both in IL-2 supplemented medium. CD25 expression was analyzed daily by flow cytometry and indicated as relative MFI to the medium control. (B) Proliferation of Treg is induced by TLR2-signaling. PAM-cultured Treg were stimulated on anti-CD3 coated plates with IL-2 (medium control) or with addition of the indicated TLR-ligands. After three days, proliferation was measured by [^{3}H]thymidine incorporation and shown as average CPM of triplicates relative to medium control ± SD. (C) Proliferation of CFSE-labeled PAM-expanded Treg. The labeled Treg were cultured for three days in the presence of IL-2 supplemented medium (med) or with the indicated stimulus (PAM and/or anti-CD3). Proliferation, resulting in a decrease of fluorescent signal in the daughter cells, was monitored by flow cytometry and (since *in vitro* cultured T cell lines display a more broad signal after CFSE labeling compared with freshly isolated T cells) analyzed using ModFit software. Representative results from three experiments are shown.

stimulation and that optimal Treg activation requires both TCR and TLR2 signaling. This is further demonstrated by the observation that the addition of PAM in combination with a strong TCR signal (applying plate bound anti-CD3) induced proliferation of the Treg, this in contrast to TLR4 or TLR9 ligands (Fig. 6*b*)**.** The proliferation of Treg induced by TLR2-triggering and/or TCR stimulation was further visualized by their CFSE dilution profile. Illustrative for their anergic state, comparing medium control treated Treg with anti-CD3 stimulation, TCR signaling alone did not induce proliferation in these cells (Fig. 6*c*). However, the addition of TLR2-ligand alone induced some proliferation of the Treg as observed by the decrease in CFSE signal. However, maximal proliferation of the Treg was observed when both TCR- and TLR2-stimulations were applied. Of note, although PAM increased the proliferation of regulatory T cells up to 10 times, Treg proliferation remained low compared to the proliferation observed for conventional T cells.

TLR2-expanded Treg remain suppressive.

Importantly, to address if the expanded Treg were still capable of suppressing conventional T cell responses, we performed *in vitro* suppression assays. The PAM-expanded Treg were rested for at least five days in the absence of PAM and subsequently co-cultured with freshly isolated $CD4^+CD25^-$ conventional T cells. Analysis of the T cell response after three days showed that the Treg efficiently suppressed the proliferation (Fig. 8*a*) as well as IFN-γ production (Fig. 3*c*) of freshly isolated conventional T cells (Th). Conventional $CD25^-$ Th cells that were expanded using the same TLR2-ligand-based culture protocol did not exert any suppressive effects (Fig. 8*a*). In addition, the supernatant of anti-CD3 activated Treg did not transfer any suppressive effects, nor did we detect any cytokine production by these Treg (using the mouse inflammation Cytometric Bead Array (not shown)). Moreover, when placed behind a semi-permeable membrane the PAM-expanded Treg failed to suppress Th-proliferation confirming that these Treg mediate suppression via cell-contact (Fig. 7).

To address the functional quality of the PAM-expanded Treg we compared titrated amounts of freshly isolated wildtype and TLR2-/- Treg with the *in vitro* expanded PAM-Treg in an *in vitro* suppression assay. From the effective suppression of fresh Th cells by both wildtype and TLR2-/- Treg we can conclude that there is no qualitative difference between wildtype and TLR2-/- Treg (Fig. 8*b*). Moreover, the PAM-expanded wildtype Treg were at least as efficient as the freshly isolated Treg. Collectively, these data indicate that TLR2-triggering results in the expansion of regulatory T cells that remain fully functional in the absence of TLR2-ligand.

TLR2-triggering on Treg temporarily abrogates suppression in vitro

To address the functional consequences of TLR-triggering on regulatory T cells, we performed suppression assays in the presence or absence of TLR2-ligand PAM. To proof that any effects of TLR2-triggering on suppression are dependent on TLR2-signaling by the Treg itself and not by conventional T cells or APC, we performed a suppression assay with both APC and conventional $CD4^+$ T cells isolated from TLR2-/- mice but with freshly isolated wildtype (TLR2+/+) regulatory T cells. This setup ensures that TLR2 is solely expressed by the Treg subset and that all PAM-induced effects are caused via TLR2-

signaling by the Treg. The results show that PAM induced some proliferation in the WT Treg, although proliferation of the Treg remained approximately 15-fold lower as compared to the TLR2-/- Th cells (Fig. 9*a*). As expected, PAM had no effect on TLR2-/- Th cell proliferation. In Treg/Th cocultures, WT Treg efficiently suppressed the proliferation of TLR2-/- conventional Th cells. In contrast, addition of TLR2-ligand to the co-culture completely abrogated suppression as observed by the restored TLR2-/- Th proliferation (Fig. 9*a*). To exclusively monitor proliferation of the Th subset in Treg/Th co-culture suppression assays, we used CFSE-labeled Th cells from TLR2-deficient mice and wildtype Treg in a suppression assay similar as described above. From the CFSE dilution profile (Fig. 9*b*, the percentage of cells that proliferated >3 times is indicated) we can conclude that WT Treg inhibit the Th proliferation in the co-culture (to a similar extent as reported before for this kind of analysis[17]). The suppressive effect is however abrogated upon the addition of PAM. As expected, PAM had no effect on stimulated TLR2-/- Th cells. Moreover, when WT Treg were pretreated overnight with anti-CD3 and PAM, extensively washed and subsequently added to TLR2-/- Th cells in a co-culture suppression assay, their suppressive ability was also abrogated (data not shown). Therefore, these results demonstrate that PAM-mediated TLR2-signaling on Treg themselves is responsible for the observed neutralization of their suppressive effect.

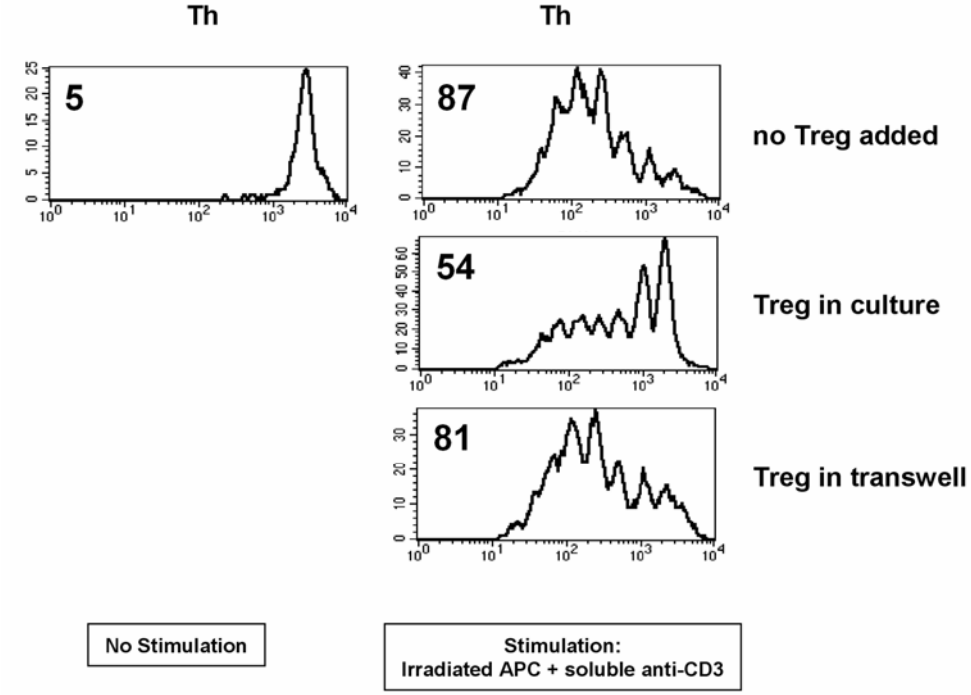

Fig. 7: PAM-Treg suppression is cell-contact dependent. Transwell suppression assay: 10^6 Freshly isolated CFSE labeled $CD4^+$ Th cells were incubated with 10^6 irradiated APC and 1µg/ml anti-CD3 per well of a 24-wells plate. If indicated 10^6 PAM expanded Treg and 10^6 irradiated APC were added in the culture with the fresh Th cells or in the upper-chamber of the transwell (pore size 0.4µm). After four days of culture, CFSE fluorescence was measured by flowcytometry. The value indicates the percentage of cells in the proliferative fraction.

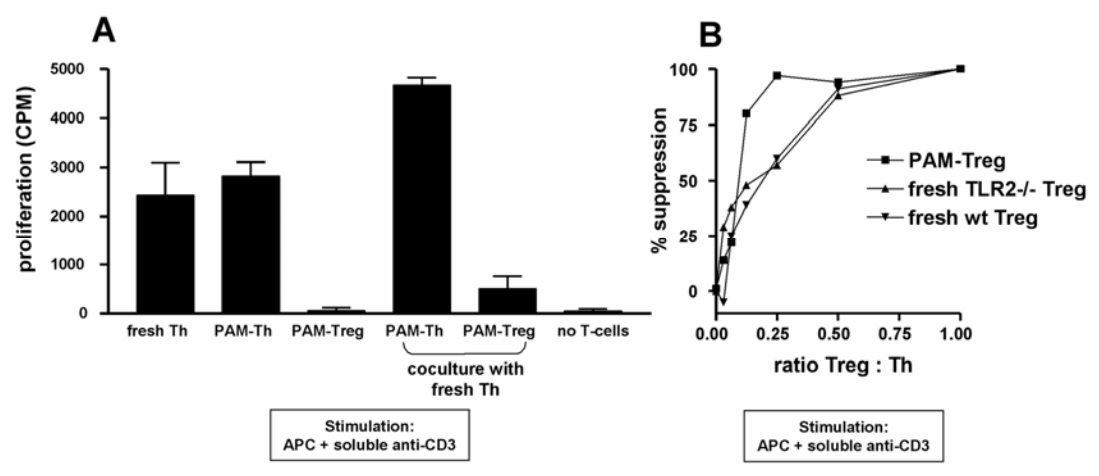

Fig. 8: PAM-expanded Treg remain suppressive. (A) *In vitro* suppression assay: PAM-expanded Treg or control conventional Th cells (0.5 x 10^4) were rested for at least five days in the absence of TLR-ligands and subsequ ently cocultured for three days with 10^4 fresh naïve $CD4^+$ T cells, irradiated APC and anti-CD3. After three days, proliferation was measured and indicated as average CPM from triplicates ± SD. Representative results from three experiments are shown. (B) Comparison of suppressive capacity of freshly isolated wildtype and TLR2-/- Treg with PAM-expanded Treg. Fresh naïve $CD4^+$ Th-cells (2 x 10^4) were cocultured with titrated numbers of Treg. After three days, proliferation was measured by [^{3}H]thymidine incorporation. Relative suppression was calculated with proliferation in the absence of Treg (fresh $CD4^+$ Th cells only) set at zero and proliferation at the Treg/Th ratio of 1 at 100%. Suppression/proliferation was measured from the average CPM from triplicate wells.

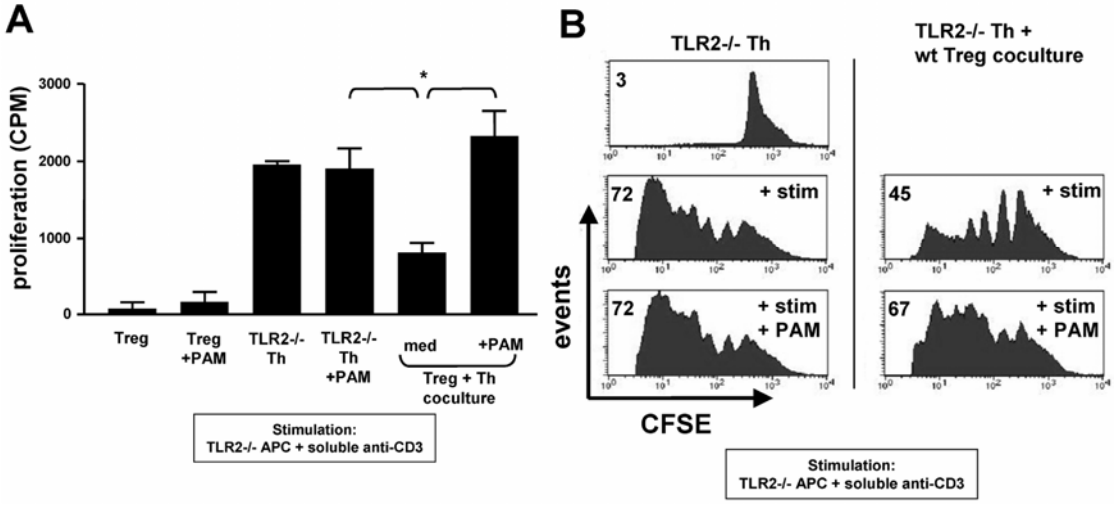

Fig. 9: TLR2 controls Treg suppressor function *in vitro*. (A) To analyze the direct effects of TLR2-triggering on Treg suppressor function *in vitro*, 10^4 TLR2-/- conventional T cells (Th) and 0.5 x 10^4 freshly isolated wildtype $CD4^+CD25^+$ Treg cells were (co)-cultured for three days. Soluble anti-CD3 and irradiated TLR2-/- APC were used to stimulate the T cells, ensuring that TLR2 was solely expressed by the Treg. If indicated, PAM was added at the start of the coculture. Data indicate average proliferation from triplicates ± SD. (B) CFSE-labeled TLR2-/- Th (10^5) were cocultured for four days with 0.5 x 10^5 WT Treg as described in (A). CFSE fluorescence intensity was measured by flow cytometry. Analysis was performed on all the $CFSE^+$ cells, using an exclusionary gate for the Treg subset (CFSE-negative $CD25^{high}$). The percentage of cells that divided >3 times is indicated. Representative results from two independent experiments are shown. (*= P< 0.05)

TLR2 induces Treg expansion in vivo

Interestingly, we observed that systemic PAM administration to wildtype mice resulted in an increase in $CD4^+FoxP3^+$ T cell numbers. This can be explained by a direct (see above) or indirect effect (for example via APC) of the TLR2-ligand. To address the *in vivo* significance of the direct effects of TLR2-triggering on Treg expansion, TLR2-/- mice were reconstituted with freshly isolated and fluorescent (CFSE) labeled OT-II transgenic Treg. This setup ensures that the infused Treg are the only TLR2-ligand responsive cells in these mice. The OT-II transgenic Treg express a TCR ($V\alpha2^+$) specific for a chicken Ovalbumin-derived peptide (OVA-peptide) in the context of the murine MHC class II $I\text{-}A^b$. The OT-II-Treg reconstituted TLR2-/- mice were subsequently challenged with either PAM or OVA-peptide alone, or with the combination of PAM and OVA-peptide. Our results show that no significant proliferation of the infused Treg was induced by either OVA-peptide or PAM alone (Fig. 10*a*). In contrast, when PAM and OVA-peptide were combined a significant part of the infused Treg had proliferated (75% versus 12% in the PAM alone treated mice,). We confirmed by flowcytometry that the proliferating T cells remained FoxP3-positive (data not shown). Thus TCR- and TLR2-signals cooperate to induce proliferation of Treg *in vivo* by directly affecting the Treg themselves.

TLR2 modulates Treg function in vivo

To asses if direct TLR2-signaling of Treg *in vivo* can result in a modulation of Treg function, we used an acute fungal (*Candida albicans*) infection model in which the kidney is the major fungal target organ[10]. We have previously shown that Treg inhibit the anti-fungal immune response as depletion of Treg prior to a *Candida* challenge resulted in decreased *Candida* outgrowth from the kidney[10] and increased IFN-γ production by splenocytes[10]. Applying this infection model in TLR2-/- mice reconstituted with 4×10^6 syngeneic wildtype Treg, we ensured that all effects of PAM-administration must be caused by TLR2-triggering of the infused Treg, since these are the only TLR2-expressing cells in this system. The WT-Treg reconstituted mice were challenged with an intravenous injection of live *Candida* and we monitored *Candida* outgrowth in the presence or absence of TLR2-ligand administration. The results show that the WT Treg-reconstituted TLR2-/- mice exhibited a 2-log increase in *Candida* outgrowth compared to the non-reconstituted TLR2-/- controls (Fig. 10*b*), indicating that the infused Treg are potent inhibitors of the anti-*Candida* immune response. Strikingly, administration of TLR2-ligand to the WT Treg-reconstituted mice restored the level of *Candida* outgrowth to the level of the non-reconstituted TLR2-/- mice (Fig. 10*b*)**,** indicating that the TLR2-trigger abrogated the suppressive effects of the infused Treg *in vivo*. The administration of TLR2-ligand alone to the TLR2-/- mice did not affect the *Candida* outgrowth (not shown). In addition, the administration of PAM-expanded conventional Th cells (4×10^6, plus/minus PAM) did not affect the *Candida* outgrowth in the TLR2-/- mice (Fig. 10*c*). This shows that Treg but not conventional Th cells are able to inhibit the immune response against *C. Albicans*. Moreover, IFN-γ production by *ex vivo Candida*-stimulated splenocytes was analyzed. *Candida* stimulated splenocytes of the Treg-infused TLR2-/- mice produced significantly less IFN-γ compared to TLR2-/- controls (Fig. 10*d*). Moreover, this suppression of IFN-γ production was absent in splenocytes from mice that received both Treg and TLR2-ligands. Of note, TLR2-ligand

alone had no effect on cytokine production by TLR2-/- splenocytes *ex vivo* (not shown). The above-described results demonstrate that TLR2-triggering abrogated the suppressive capacity of the infused Treg *in vivo*. Since the infused Treg are the only TLR2 expressing cells in this *in vivo* system, the abrogation of suppression can only be explained by a direct effect of the TLR2-ligand on the infused TLR2+/+ Treg in the TLR2-/- mice.

Collectively, our combined results show that the suppressive function of the regulatory T cells themselves is directly controlled by the pathogen associated molecular pattern receptor TLR2. In the presence of TLR2-ligand, Treg expansion and a temporal loss of suppression is observed. After removal of the TLR2-ligand, the expanded Treg regain their suppressive capabilities.

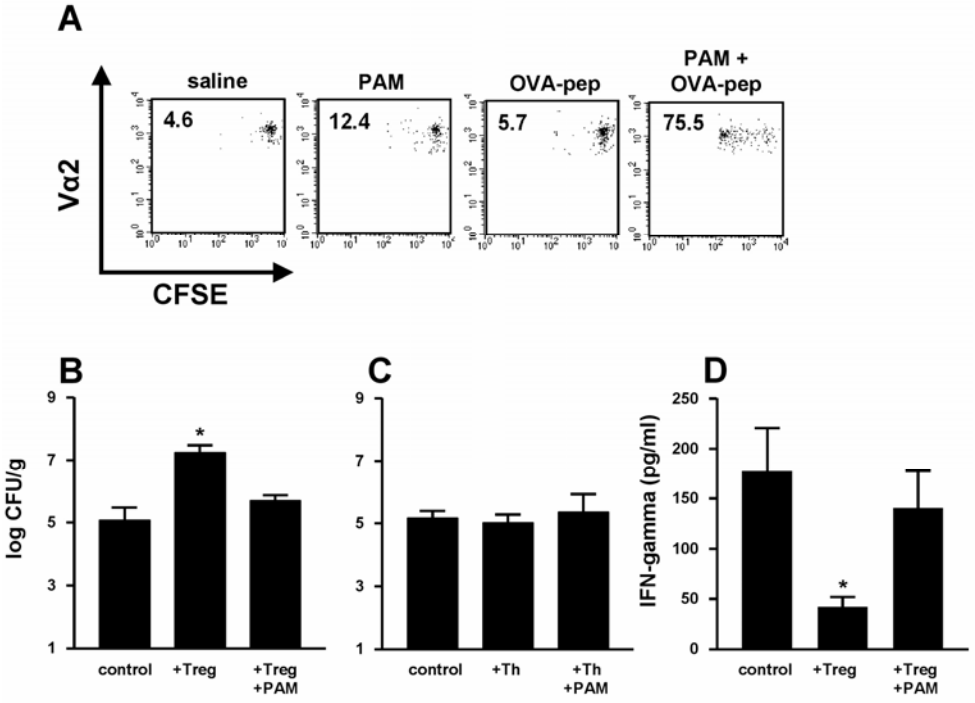

Fig. 10: TLR2 controls Treg suppressor function *in vivo*. (A) TLR2- and TCR-triggering cooperate to induce Treg expansion *in vivo*. TLR2-/- mice were reconstituted with 2 x 10^6 freshly isolated and CFSE labeled OT-II-transgenic Treg (TCR of OT-II transgenic T cells is Vα2 and specific for the Ovalbumin derived peptide presented in I-A^b). The reconstituted mice were subsequently challenged i.p. with either PAM (20µg/mouse) or OVA-peptide (10µg/mouse) alone or with the combination of PAM and OVA-peptide. After four days, splenocytes were isolated and analyzed by flowcytometry for CFSE-fluorescent signal of the infused cells. The cells shown are gated for the $CD4^+$, $Vα2^+$, $CFSE^+$ cells and Propidium-Iodide positive (death) cells wee excluded from the analysis. The value indicates the percentage of cells within the proliferative fraction (>one division). (B/C) TLR2-triggering abrogates Treg mediated suppression of anti-*Candida albicans* immunity *in vivo*. TLR2-/- mice (5 per group) were reconstituted with 4 x 10^6 wildtype PAM-expanded Treg (B) or conventional Th cells (C) and challenged intravenously with 10^5 live *Candida* cells one day later (day 0). If indicated, mice received an intraperitoneal injection of 100 µl saline (controls) or 20 µg PAM/100 µl saline on day –1, 1, 3, and 5. Seven days after the challenge, *Candida* outgrowth (CFU/gram tissue ± SEM) from kidneys was monitored. (D) *Ex vivo* IFN-γ production (± SEM) by *Candida* stimulated splenocytes was measured as described in the Material and Methods section. Representative results of two independent experiments are shown (*= P< 0.05 with TLR2-/- control).

Discussion

The identification of $CD25^{high}$, $CD4^+$ T cells as T cells bearing a suppressive phenotype has renewed the interest in the regulatory T cell [5]. These Treg are now emerging as major regulators of our immune system. Lack of Treg results in various autoimmune syndromes. On the other hand, Treg mediated suppression might hinder the development of effective immune responses, which are crucial for the elimination of tumors and infections. Therefore, the regulators themselves need to be well controlled. We now demonstrate for the first time that TLR2 triggering by pathogen associated molecular patterns (PAMPs) present on or secreted by infectious organisms can directly modulate Treg function.

Our initial observation that $CD4^+CD25^+$ T cell numbers were decreased in TLR2-deficient but not in TLR4-deficient mice, led us to investigate the role of TLR2 signaling on $CD4^+CD25^+$ T cell functionality in more detail. Analysis of blood and spleen of MyD88-deficient mice revealed that in addition to TLR2, also MyD88 is required for normal $CD4^+CD25^+$ T cell levels *in vivo* (Fig. 1). Moreover, massive TLR2-triggering by intravenous injection of TLR2-ligand PAM had the opposite effect and resulted in a significant proliferation and increase of $CD4^+CD25^+$ T cell numbers (Fig. 1).

The *in vivo* increase of $CD4^+CD25^+$ T cells by TLR2-ligand PAM might be caused by an indirect effect via APC or by direct TLR2-triggering of regulatory T cells. *In vitro* we found that all TLR-ligands tested increased Treg proliferation in the presence of irradiated APC and anti-CD3 (Fig. 2). However, our results also demonstrate that the TLR2-ligand PAM, but not purified LPS or CpG, is able to induce proliferation of Treg cells in the absence of APC, by directly affecting the Treg themselves. Next to PAM, also MALP-2, Peptidoglycan and heat-killed *Candida* resulted in Treg activation, emphasizing that besides synthetic ligands also natural TLR2-ligands can modulate Treg. Using the TLR2-ligand PAM we were able to establish long lived (up to 5 months), proliferating regulatory T cell lines. Although the exact mechanism remains to be elucidated, the finding that TLR2-triggering of Treg strongly enhances CD25 expression implies that increased sensitivity to IL-2 might be involved. The *in vitro* expanded Treg expressed CD4, CD25, GITR, CTLA-4, CD103 and FoxP3 (Fig. 5), all typical regulatory T cell markers[1,21]. These suppressor T cell lines further expressed significant amounts of the PAM receptor TLR2 (Fig. 5), but also remained sensitive for TLR2 stimulation further emphasizing the direct interacting between TLR2-ligands and Tregs (Fig. 6). Furthermore, they fully maintained their suppressive capacities following withdrawal of TLR2 ligands (Fig. 8). Interestingly, TLR2-/- Treg expressed the same surface markers as wildtype Treg and were equally efficient in the suppression of naïve T cell responses (Fig. 8). This indicates that TLR2 is not involved in the suppression process itself. We additionally showed that the effects of TLR2-triggering on Tregs temporarily abrogates their suppressive capabilities as well (Fig. 9). We hypothesize that TLR2 induces proliferation of the Treg during which there is a temporal loss of the suppressive phenotype.

The physiological relevance of our findings was assessed by infusion of TLR2+/+ Treg into TLR2-/- mice allowing for the analysis of direct TLR2-effects on Treg function *in vivo*. Importantly, the combination of TCR-and TLR2-signals induced strong proliferation of Treg *in vivo* (Fig. 10*a*), confirming that TLR2 and TCR signals cooperate in the proliferation

of Treg. Furthermore, in line with our *in vitro* observations, infused WT Treg inhibited anti-fungal immune responses in TLR2-/- mice, whilst co-administration of TLR2-ligands resulted in a loss of the suppressive phenotype of the infused Treg as observed by the restored anti-fungal immunity (Fig. 10*b-d*).

Recently, it was found that IL-2[3] and strong TCR signals[13] release effector T cells from Treg mediated suppression. Furthermore, the presence of the TLR9 ligand CpG-ODNin a suppression assay containing APC resulted in a reduced suppression by the Treg. This is due to TLR9 triggered production of IL-6 by the APC rendering conventional T cells insensitive to Treg mediated suppression[12]. Another report nicely links TLR4 and TLR9 signaling with Treg mediated CD8 tolerance[25]. The authors demonstrate that ligation of TLR4 or TLR9 on DC is required for breaking Treg mediated CD8 tolerance *in vivo*. However, in addition to the effects of TLR-ligands on DC, the results described here show that it is also possible that a TLR-ligand directly affects Treg, resulting in abrogation of suppression and expansion of the Treg subset. A study by Komai-Koma et al.[26] showed that TLR2-triggering also stimulates activated conventional T cells. We were able to confirm their observations on activated conventional $CD4^+$ T cells (data not shown). However, using TLR2-deficient conventional T cells, we demonstrate that it is the regulatory T cell subset (freshly isolated or *in vitro* expanded) that is highly sensitive for TLR2 stimulation, resulting in profound proliferation and reduced suppression by the Treg. The effects of TLR-ligands *in vivo* might thus be more complicated then initially thought, affecting multiple cell types of both the innate and the acquired immune system.

Recently a role for TLR4 on Treg homeostasis was reported[19]. We and others[17,26] however did not find any effects of purified LPS on T cells (conventional or regulatory). We note that in contrast to purified LPS, commercially obtained LPS (containing contaminating TLR2-ligands[27]) induced a significant CD25 up-regulation on Treg. Thus to exclude bacterial contaminations in all our experiments, we only used purified LPS and synthetic TLR2 and TLR9 ligands. Moreover, we confirmed the purity of our TLR2 and TLR4 ligands by measuring their activation capacity on TLR2- and TLR4-deficient DCs (Fig. 11).

An intriguing question that remains to be answered is why TLR2 but not TLR4 is able to directly affect Treg. TLR2 but not TLR4 is found to promote IL-10 production by innate immune cells *in vitro*[28]. The accumulating evidence that TLR2 is also involved in immuno-suppression *in vivo* (reviewed in [29]) renders TLR2 unique from the other TLRs. In this context it is interesting to note a report linking the anti-inflammatory Glucocorticoids with TLR2 expression[30]. These observations strengthen the idea that TLR2, besides an activating function, could be involved in tolerance as well by acting directly on APC and Treg. In addition, other TLRs not tested here could act on Treg. It was recently shown that TLR8 can abrogate the suppressor function of human Treg[31]. Although TLR8 did not induce proliferation of human Treg, this report strengthens our findings of TLR2 mediated control of murine Treg. We are now investigating the possibility that different TLR-signals can differentially modulate Treg function.

Our finding of the abrogation of suppression after TLR2-stimulation, is in line with reports indicating that Treg rapidly lose their ability to inhibit proliferation after receiving

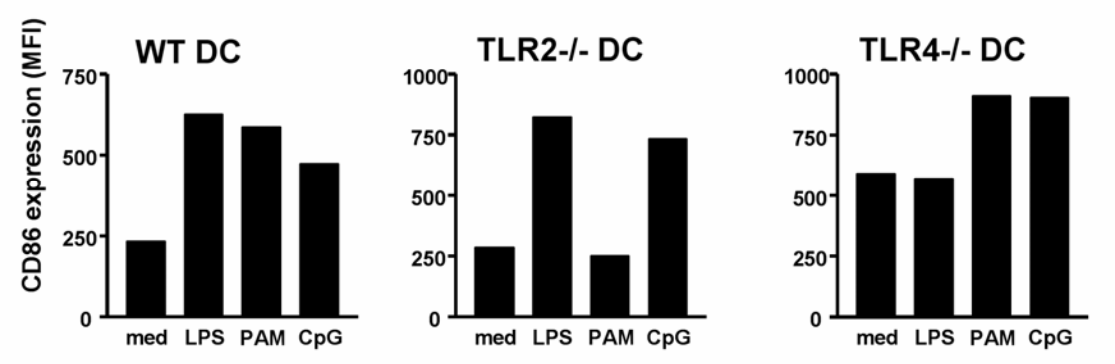

Fig. 11: TLR-ligand induced activation of mouse bone marrow derived DC of wildtype, TLR2-/- and TLR4-/- origin. Day 7 bone marrow derived DC were incubated for 24 hours with the indicated TLR-ligands and subsequently the expression of activation marker CD86 was measured by flow cytometry. A representative result from two experiments is shown.

strong activation signals[13]. In addition, Komai-Koma reported that TLR2 functions as a co-stimulatory molecule for conventional T cells, resulting in increased T cell expansion after TLR2-triggering on the conventional T cells themselves[26]. Furthermore, the finding that TLR8-triggering combined with TCR stimulation on human Treg resulted in reversal of their suppressive capabilities emphasizes the effect of TLR-signaling on Treg mediated suppression[31]. In line with these reports and our own observations, we envisage that TLR2-signaling on murine Tregs contributes to the strength of signals received by the Treg. This forces Treg into the proliferative pathway, which is paralleled by a reversal of suppressive capabilities. Once in a resting state again, the Treg regain their suppressive capacities (see also Fig. 12).

Our observation that PAMPs can directly act on regulatory T cells themselves provides new insights regarding the role of Treg in the induction of immune responses. The results presented here provide evidence for a strict control of regulatory T cells by TLR2-ligands. The current opinion holds that TLR mediated recognition of pathogens results in DC activation and subsequent initiation of T cell responses. We can now add a direct Treg modulating capacity to TLR-ligands. Applying an acute fungal infection model, we unequivocally demonstrate that TLR2-triggering on the Treg themselves abrogates their suppressive activity *in vivo* resulting in increased IFN-γ production and decreased fungal outgrowth. These data imply that TLR2-ligands provided by a microbial invasion during acute infection, mediate Treg expansion and abrogation of Treg mediated suppression, thus allowing a potent immune response to occur. However, post-infection, when the immune system has cleared the pathogen and hence TLR2-ligands are declining, the expanded Treg regain their suppressive activity and could help to restore the immune balance (see also schematic model in Fig. 12). Interestingly, two recent reports suggested that $CD4^+CD25^+$ regulatory T cells control the persistence and memory protective immunity against *Leishmania major* and *C. Albicans*[9,32]. Based on the novel findings described herein,

one could envisage that TLR2-ligands provided by the pathogens[33] could first expand the Treg *in vivo* and abrogate their suppressive phenotype. When only low numbers of the pathogen are present as in the persistent phase of *Leishmania* infection, the Treg regain their suppressive phenotype and could be responsible for pathogen persistence.

Finally, we note that the presence of micro-organisms in the gut might contribute to a constant influx of TLR-ligands that can modulate Treg numbers. In support of this hypothesis is our unpublished observation that germ-free mice have altered Treg numbers as compared to control mice. In addition to microbial derived PAMPs, TLR2-signaling can possibly be induced by endogenous proteins (e.g. Heat Shock Proteins, reviewed by [34]). This would allow the modulation of the Treg compartment in a non-pathogenic, stress induced (anti)-inflammatory environment.

Collectively, our findings have major implications for our understanding of immune regulation by both regulatory T cells and TLRs. Ultimately the knowledge that TLRs can be used to expand and modulate Treg will lead to new methods for Treg based immunotherapy of diseases.

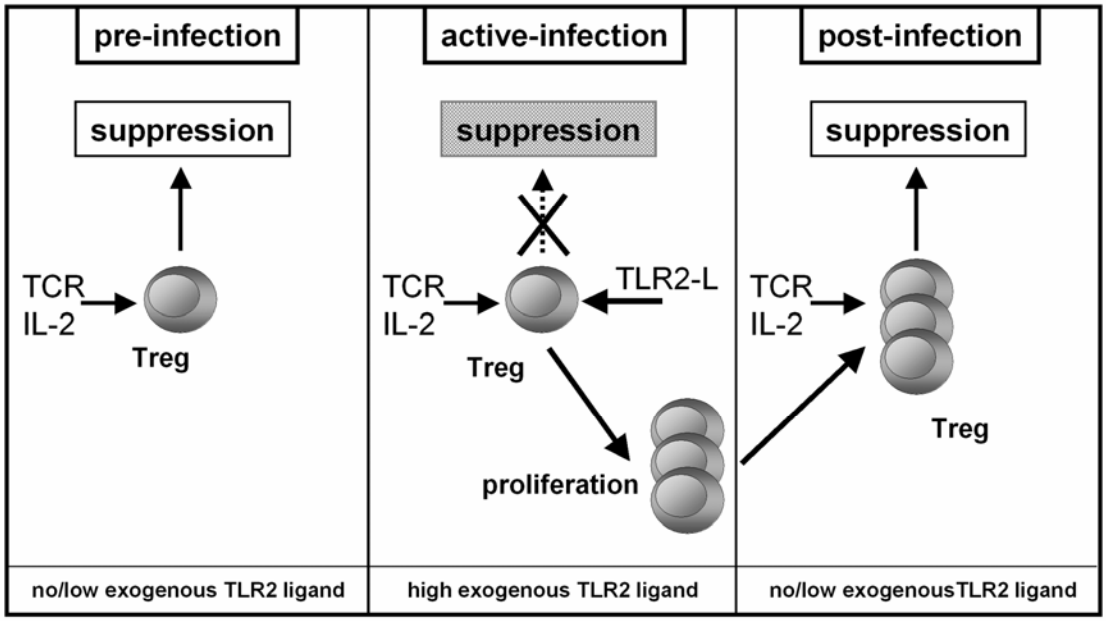

Fig. 12: Model of TLR2-mediated control of regulatory T cell function. In a pre-infection setting, Treg function is mainly regulated by T cell Receptor (TCR) stimulation and IL-2. During an acute infection, pathogen derived TLR2-ligands promote proliferation of Treg paralleled by temporarily abrogated suppression. As a result, the Treg do not suppress the ongoing immune response. Once the pathogen is cleared by the immune system, the source of TLR2-ligands is no longer present and the Treg will regain their suppressive capabilities, thus contributing to the balance between tolerance and immunity.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Wiga (Sulzfeld, Germany). TLR2-/-[35], TLR4-/- and MyD88-/- [36] mice were obtained from S. Akira (Osaka University, Japan). The MyD88-/- mice are backcrossed >8 times on the C57BL/6 background. The OVA-specific TCR transgenic OT II mice (on a C57BL/6 background) were obtained from The Jackson Laboratory. The MyD88-knockout mice were bred in our specified pathogen free animal facility using heterozygous breeding pairs. The wildtype littermates were used as controls for the comparison of Treg numbers. All animal experiments were approved by the Animal Experimental Committee from the St. Radboud University Medical Center and were performed in accordance with institutional and national guidelines.

Antibodies and flow cytometry

Directly labeled monoclonal antibodies used for staining were anti-CD3-PE, anti-CD4-APC, anti-Vα2-PE, anti-CD25-FITC (clone 7D4), anti-rat-PE and all isotype controls were obtained from BD Pharmingen. Anti-mTLR2-PE (clone T2.5) and anti-mFoxP3-PE (clone FJK, staining according to instructions by manufacturer) were obtained from eBioscience, CA, U.S.A., Anti-hamster-APC was obtained from Caltag Laboratories (Burlingame, CA, U.S.A.) In addition, anti-GITR (DTA-1, a kind gift from Prof. S. Sakaguchi, Japan), anti-CTLA-4 (clone 9H10), anti-CD86 and anti-CD103 (BD Pharmingen) were used. Analysis of cell surface markers on lymphocytes was performed using a FACScalibur™ (Becton Dickinson) and CELLQuest™ software. For analysis of relative $CD4^+CD25^+$ T cell numbers *in vivo* 50–100 µl blood was collected in heparin-coated tubes and erythrocytes were lyzed using standard protocols. The remaining lymphocytes were washed and incubated with anti-CD25-FITC and anti-CD4-APC and subsequently analyzed on a flowcytometer

T cell purification and analysis

Spleens from wildtype or KO mice were mashed, filtered and $CD4^+$ T cells were purified using anti-mouse-CD4 Microbeads (MACS, Miltenyi Biotec, Germany) resulting in a 95% pure $CD3^+CD4^+$ T cell population as measured by flow cytometry. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell subsets were obtained by flow cytometry purification of the presorted $CD4^+$ T cells: CD4 cells were stained with APC-conjugated CD4 mAb and FITC-conjugated-CD25. For exclusion of MHC class II^+ cells and B cells, anti-I-A^b (17/227, mouse IgG2a) and PE-conjugated Goat anti-mouse IgG were used. Cell sorting was performed on a Coulter Altra Hypersort cellsorter. The purity of each cell preparation was > 98%. Purified $CD4^+$ T cell subsets were subsequently cultured for three days in complete medium (Iscoves IMDM (Gibco), 9% FCS, 2µM L-glutamine, 50 µM β-Mercapthoethanol, 1% Antibiotic-Antimycotic (Gibco)) with 1 µg/ml anti-CD3 (Pharmingen), 5 Cetus Units (cU) IL-2/ml with or without TLR-ligands. Unless indicated otherwise, TLR-ligands were used at the following concentrations: PAM_3Cys-SKKK (PAM) and MALP-2, both 2 µg/ml (EMC microcollections, Germany), non-purified and purified *E. coli* LPS 10 µg/ml (obtained from Sigma and subsequently purified as described in [27]), Peptidoglycan 10 µg/ml (Sigma-Aldrich), purified

LTA 10 µg/ml (obtained from Gunthard Stübs, University of Konstanz, Germany), heat-killed *Candida albicans* hyphen and conidia 2 x 10^4 CFU per well of 96-well plate (strain UC 820) and CpG-ODN 1668 1 µg/ml (5'-TCCATGACGTTCCTGAATGCT-3') Sigma Genosys, Haverkill, UK). After three days of culture the cells were harvested, stained with APC conjugated anti-CD4 and FITC conjugated anti-CD25 and analyzed. Data indicate MFI (Mean Fluorescence Intensity), presented as the average MFI of three measurements with SD indicated.

Treg culture and suppression assay

Purified $CD4^+CD25^+$ T cells, 10^4 per well of a 96-well plate, were stimulated weekly with 10^5 irradiated APC (CD4-MACS bead depleted splenocytes), 2 µg/ml PAM, 1 µg/ml anti-CD3 (145-2C11, BD Pharmingen) and 20 Cetus units IL-2/ml complete medium. The cells were washed three days after stimulation and maintained in culture medium supplemented with 20 Cetus units IL-2/ml. When necessary, dead cells were removed by ficol density gradient. Cultured Treg cells were used in assays at least 6 days after stimulation. Bone marrow cells were cultured for seven days in complete medium in the presence of 20 ng/ml mIL-4 and mGM-CSF (Prepotech) to obtain bone marrow derived DC (BMDC). Suppression assays: Freshly sorted $CD4^+CD25^-$ naïve T cells (20 x 10^3) and either cultured or freshly isolated Treg cells (5 x 10^3) were mixed and cocultured for three days. If indicated, the T cells were stimulated with TLR-ligands and with either plate bound anti-CD3 (2,5 µg/ml) or soluble anti-CD3 (1 µg/ml) plus 10^5 irradiated APC in complete medium. In a number of coculture experiments, resting TLR2-/- $CD25^-$ conventional T cells were used as responders. These cells were maintained in culture for maximal 6 weeks using weekly restimulation assay with irradiated APC, anti-CD3 (1 µg/ml) and 20 cU IL-2/ml. After three days of coculture, supernatant was collected for cytokine analysis using a mouse inflammation CBA kit (BD biosciences). Proliferation was measured by overnight (20 hours) ^{3}H-thymidine incorporation. In addition, suppression/proliferation was monitored by analysis of CFSE (1 µM) labeled T cells. CFSE-labeled cells (10^5) were cultured with the indicated stimulus for four days and subsequently CFSE fluorescence intensity was measured by flow cytometry. The transwell-suppression assay was performed in a 24-well culture plate (Corning) using 10^6 freshly isolated CFSE labeled $CD4^+$ T cells cocultured with an equal number of Treg cells or with the Treg behind a semi-permeable membrame (0.4µm, Millipore). The T cells were stimulated with 10^6 irradiated APC (also in the upper chamber of the transwell) and 1µg/ml anti-CD3 in a total volume of 1ml culture medium. For monitoring proliferation of *in vitro* expanded Treg by CFSE-fluorescence (*In vitro* expanded T cells displayed a broad CFSE-signal as compared to freshly isolated T cells), we made use of ModFit LT software (Verity Software house, Maine, USA) to illustrate the numbers of daughter generations after four days of proliferation.

Quantitative PCR

Relative mRNA levels were determined using the ABI PRISM 7000 Sequence Detection System and SYBR Green Reagent (Applied Biosystems 4309155). cDNAs were synthesized from 2.0 µg of total RNA using M-LMV reverse transcriptase (Invitrogen 28025-013). PRISM samples contained 1 x SYBR Green Master Mix, 1.5 ul 5 µM primers and 25 ng synthesized

cDNA in a 25 µl volume. Following primers were used; mTLR2 forward (AACCTCAGACAAAGCGTCAAATC), mTLR2 reverse (ACCAAGATCCAGAAGAGCCAAA) (both from Biolegio BV Malden, The Netherlands), mFOXP3 forward (AGGAGAAGCTGGGAGCTATGC), mFOXP3 reverse (GGTGGCTACGATTGCAGCAA), PBGD forward (CCTACCATACTACCTCCTGGCTTTAC), PBDG reverse (TTTGGGTGAAAGACAACAGCAT) (all from Sigma Genosys). Mean relative mRNA expression from 3 replicate measurements was calculated using ABI PRISM 7000 SDS software. Values are expressed as arbitrary units relative to PBGD.

In vivo Treg proliferation

Spleen and inguinal lymphnodes from OT-II transgenic mice were isolated and mashed into single cell suspension. Subsequently, the cells were stained with anti-CD25-FITC and isolated using anti-FITC MACS beads (Miltenyi). Contaminating $CD8^+$ T cells and B cells were removed using negative depletion with Dynal beads. In this way up to 95% pure $CD4^+CD25^+$ T cells were obtained. The OT-II transgenic Treg were CFSE labeled (5µM) and injected intra peritoneal into TLR2-/- mice (2×10^6 per mouse). Four hours later, the mice were challenged i.p. with 20 µg/mouse PAM and/or 10µg/mouse OVA-peptide (ISQAVHAAHAEINEAGR, obtained from Drijfhout, JW, LUMC, Leiden, The Netherlands, peptide was synthesized by standard Fmoc chemistry, and purity (95%) was checked by HPLC). Four days later, the mice were sacrificed, spleens were isolated and the splenocytes were stained with anti-CD4-APC, anti-Vα2-PE and PropidiumIodide and analyzed by flowcytometry. In addition, the cells were stained with anti-FoxP3-PE and CD4-APC to confirm that the CFSE+ cells were still $Foxp3^+$.

C. Albicans infection model

We used a non-lethal *C. albicans* infection model as described[10]. Briefly, 10^5 colony forming units (CFU) of *C. albicans* strain UC 820 were injected iv in mice on day 0. The mice, 5 per group, received if indicated 2×10^6 wildtype *in vitro* expanded Treg or Th cells intravenous in 100 µl saline on day –2 and –1 (4×10^6 in total per mouse). All mice received on days –1, 1, 3 and 5 100 µl saline or 20 µg Pam_3Cys/100µl saline. At day 7, kidneys and livers were aseptically removed, weighed and homogenized in sterile saline in a tissue grinder. The number of viable *Candida* cells was determined by plating serial dilutions on Sabouraud dextrose agar plates. The colonies were counted after 24h at 37 °C and indicated as CFU per gram tissue. The spleens were also isolated from the mice and were used for the analysis of cytokine production in response to heat killed *Candida*. 5×10^6 Splenocytes in 1 ml complete medium were stimulated with 10^7 heat killed *C. albicans* cells. The supernatants were collected after 48h of incubation at 37 °C in 5% CO_2, and measurement of IFN-γ in the supernatant was performed by a commercial ELISA (Biosource, Camarillo, CA; detection limit 16 pg/ml), according to the instructions of the manufacturer.

References

1. Sakaguchi, S. Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses. *Annu Rev Immunol* **22**, 531-562 (2004).
2. Shevach, E.M. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* **2**, 389-400. (2002).
3. Thornton, A.M. & Shevach, E.M. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* **164**, 183-90. (2000).
4. Piccirillo, C.A. & Shevach, E.M. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol* **167**, 1137-40. (2001).
5. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155**, 1151-64. (1995).
6. Itoh, M. et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* **162**, 5317-26. (1999).
7. Shevach, E.M. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol* **18**, 423-49 (2000).
8. Morgan, M.E. et al. CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. *Arthritis Rheum* **48**, 1452-60 (2003).
9. Montagnoli, C. et al. B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to *Candida albicans*. *J Immunol* **169**, 6298-308 (2002).
10. Netea, M.G. et al. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol* **172**, 3712-8 (2004).
11. Sutmuller, R.P. et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J Exp Med* **194**, 823-32. (2001).
12. Pasare, C. & Medzhitov, R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* **299**, 1033-6 (2003).
13. Baecher-Allan, C., Viglietta, V. & Hafler, D.A. Inhibition of Human CD4(+)CD25(+high) Regulatory T Cell Function. *J Immunol* **169**, 6210-7 (2002).
14. Fisson, S. et al. Continuous Activation of Autoreactive CD4+ CD25+ Regulatory T Cells in the Steady State. *J Exp Med* **198**, 737-46 (2003).
15. Salomon, B. et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* **12**, 431-40 (2000).
16. Yamazaki, S. et al. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* **198**, 235-47 (2003).

17. Kubo, T. et al. Regulatory T cell suppression and anergy are differentially regulated by proinflammatory cytokines produced by TLR-activated dendritic cells. *J Immunol* **173**, 7249-58 (2004).
18. Akira, S. & Hemmi, H. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol Lett* **85**, 85-95 (2003).
19. Caramalho, I. et al. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med* **197**, 403-11 (2003).
20. Takahashi, T. et al. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* **10**, 1969-80. (1998).
21. Huehn, J. et al. Developmental Stage, Phenotype, and Migration Distinguish Naive- and Effector/Memory-like CD4+ Regulatory T Cells. *J Exp Med* **199**, 303-13 (2004).
22. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057-61 (2003).
23. Fontenot, J.D., Gavin, M.A. & Rudensky, A.Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**, 330-6 (2003).
24. Khattri, R., Cox, T., Yasayko, S.A. & Ramsdell, F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* **4**, 337-42 (2003).
25. Yang, Y., Huang, C.T., Huang, X. & Pardoll, D.M. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol* **5**, 508-15 (2004).
26. Komai-Koma, M., Jones, L., Ogg, G.S., Xu, D. & Liew, F.Y. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc Natl Acad Sci U S A* **101**, 3029-34 (2004).
27. Hirschfeld, M., Ma, Y., Weis, J.H., Vogel, S.N. & Weis, J.J. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* **165**, 618-22 (2000).
28. Re, F. & Strominger, J.L. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J Immunol* **173**, 7548-55 (2004).
29. Netea, M.G., Van der Meer, J.W. & Kullberg, B.J. Toll-like receptors as an escape mechanism from the host defense. *Trends Microbiol* **12**, 484-8 (2004).
30. Hermoso, M.A., Matsuguchi, T., Smoak, K. & Cidlowski, J.A. Glucocorticoids and tumor necrosis factor alpha cooperatively regulate toll-like receptor 2 gene expression. *Mol Cell Biol* **24**, 4743-56 (2004).
31. Peng, G. et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* **309**, 1380-4 (2005).
32. Belkaid, Y., Piccirillo, C.A., Mendez, S., Shevach, E.M. & Sacks, D.L. CD4(+)CD25(+) regulatory T cells control Leishmania major persistence and immunity. *Nature* **420**, 502-507 (2002).
33. Netea, M.G. et al. The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. *J Infect Dis* **185**, 1483-9 (2002).
34. Kirschning, C.J. & Schumann, R.R. TLR2: cellular sensor for microbial and endogenous molecular patterns. *Curr Top Microbiol Immunol* **270**, 121-44 (2002).

35. Takeuchi, O. et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443-51 (1999).
36. Adachi, O. et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**, 143-50 (1998).

Chapter 7

General Discussion

Adapted from:

Manuscript in preparation

Introduction

In the treatment of cancer, chemotherapy, ablation and radiotherapy are commonly used to destroy tumors *in situ*. This thesis discusses how tumor antigens left inside the body following *in situ* tumor destruction can be processed by the immune system to generate anti-tumor immunity. It also shows that dendritic cells are important in the initiation of this response. These new insights may have significant implications for the implementation of immunotherapy in conventional cancer treatments, as it seems possible to create a synergistic interplay between these modalities. This chapter provides a general overview and discussion about these topics.

Research in the past decennia has led to an enormous progression in the application of immunotherapy. It has become a promising strategy for patients with transplanted organs, auto-immunity or allergy, in which immunotherapy is successfully used to silence destructive responses. In patients with infectious diseases or cancer, immunotherapy has shown to be effective in boosting the immune system. The use of immunotherapy with the intention to fight human cancer has been established since in the 1890's remissions of tumors were observed following administration of bacterial extracts to cancer patients[1]. Since then, numerous studies have been reported using either passive or active immunotherapy to fight a large variety of cancers. Passive immunization comprises strategies like infusion of antigen specific T cells or infusion of antibodies. Active immunization involves infusion of dead tumor cells, cytokine stimulation or dendritic cell vaccination. Although anti-tumor responses have been observed in all of these strategies, clinical research in the past decade has been largely focussed on active immunization with dendritic cell vaccines.

Human as well as animal studies have demonstrated that vaccination with DCs can induce immunity to otherwise weakly immunogenic tumor antigens, and clinical trials applying DC-based vaccines have shown promising results in patients with a variety of tumors[2-4]. Although these positive results have been obtained, it will remain a tremendous task to create specific vaccines for each specific patient. First of all, *ex vivo* generation of DC and the DC vaccine is very labour intensive and costly. Therefore, improvements that will allow *in vivo* loading of endogenous DC would be very helpful. Secondly, our knowledge on specific antigens that should be chosen in each particular cancer is limited. Letting the immune system itself decide against which antigens it will mount an immune response would reduce complexity to a great extend.

In surgical oncology, *in vivo* tumor destruction procedures like radiotherapy or radiofrequency ablation are successfully applied. Upon tumor destruction *in situ*, large amounts of tumor antigens are released. Recent studies performed by us and others suggest that the tumor debris left *in situ* can act as an antigen source for the immune system in general[5-7], and the induction of anti-tumor immunity by DCs in particular[8]. However, to optimally exploit the immunity inducing capacity of the DC that acquired tumor debris, a better understanding of how DCs succeed to induce and modulate immunity *in vivo* is necessary. Chapter 2 of this thesis provides an overview of the current thinking about issues like DC loading, maturation and migration of *ex vivo* produced DC

vaccines[9]. These ideas might, however, also be relevant and useful in the *in vivo* situation. Moreover, our new data suggest that the way DCs handle tumor antigens can be influenced by the type of tumor destruction and carefully timed additional immuno modulation. These novel findings may create possibilities for combination of immuno therapy with surgical tumor destruction, but may also have impact on the future design of *ex vivo*-generated DC vaccines.

In situ tumor destruction: creating an *in vivo* source of tumor antigens

In order to initiate *de novo* antigen-specific immune responses, the immune system must first of all be provided with the appropriate antigens. Antigen presenting cells like DC are able to take up these antigens and process them for presentation on their MHC molecules. Potential tumor antigens are thought to be present in every tumor, which theoretically makes all tumors immunogenic[10]. Among these antigens are 'self' antigens and 'new' antigens. Self antigens are antigens that the immune system has been 'dealing with' for a longer time, and for which tolerance has been developed. 'New' antigens contain epitopes for which the immune system is not tolerant. Examples are tumor residing oncogenic viruses, but also altered or mutated self antigens. On the other hand, the immune system may not be tolerant for all self antigens, as some of the self antigens have only limited expression in the body, making them 'less known' for the immune system. It is commonly said that the immune system is *ignorant* for these antigens[11]. Examples of antigens that contain these *cryptic* or *sequestered* epitopes are proteins that are only expressed in testis tissue or molecules of the MAGE gene family, present in many tumors. The optimal start of an immune response against a tumor will thus theoretically be providing APC with sufficient amounts of the 'new' antigens or 'less known' antigens.

In situ tumor destruction leaves the generated tumor debris inside the body. Since this debris has to be 'cleared' by APCs, it will create a source of antigens to generate anti-tumor immunity. Among these antigens may well be 'new' and 'less known' antigens. Due to the tumor ablation, tumor antigens normally present in low concentrations may now enter immunological presentation pathways, thereby becoming candidates to develop an immune response against. APC, and in particular DCs, determine whether processed antigens elicit an immune response or not[12]. Of importance in this are the signals a DC receives from its surroundings at the time of antigen uptake. In chapter 4 we have shown that upon *in situ* tumor destruction by cryo or radiofrequency ablation, the ablated tumor indeed forms an antigen depot from which DCs in the draining lymph node acquire their antigens. Efficient antigen loading of DC was critically dependent on ablation of the tumor as in tumor-bearing mice far less antigen or antigen-positive DC could be discerned. We used cryo ablation and radiofrequency ablation to destruct tumors, but more types of tumor destructing techniques likely also have the ability to provide the immune system with tumor antigens.

In situ tumor destruction: Some examples

In situ release of antigens can be induced in several ways, e.g. chemotherapy or radiotherapeutic treatment. Although not studied comprehensively, factors like the amount of antigens released, quality of the antigens released and the time-frame of antigen exposure will likely be different in these different strategies. These variations will however affect the loading of APC. In the next part, we will summarize some of the candidate methods possibly effective in releasing antigen *in situ*.

Local tumor ablation

To provide a treatment for cancer that could be used alongside conventional resection of cancer, a variety of tumor ablative techniques has been explored over the past decades. The term 'local tumor ablation' is defined as the direct application of chemical or thermal therapies to a specific focal tumor in an attempt to achieve eradication of the tumor tissue[20]. It is now accepted that tumor ablation is an effective adjunct to resection in achieving complete tumour clearance[21]. It is, for instance, successfully used to ablate small metastases alongside excision of the primary tumor[22]. Ablation furthermore reduces pain and other side effects compared to other treatments, which improves the quality of life for patients[21]. The methods of tumor ablation most commonly used in current practice can be divided into two main groups: chemical ablation and thermal ablation.

Chemical ablation comprises direct intra-tumoral instillation of ethanol (PEI, PAI), acetic acid or a chemotherapeutic compound, which induce coagulation necrosis and cause tumor ablation[23]. Thermal ablation uses an energy source to disrupt tissue integrity and to produce necrosis. Radio frequency ablation is coagulation induction by electromagnetic energy sources with frequencies less than 900 kHz [24]. When frequencies greater than or equal to 900kHz are used, the technique is called microwave ablation[25]. Laser ablation (LITT, laser coagulation therapy) is destruction of tumor tissue via light energy[26,27], whereas cryo ablation uses cold to freeze lesions[28]. An emerging method of tumor destruction is via ultrasound. There are currently two novel methods for applying ultrasound energy: transcutaneous (focussed ultrasound ablation)[29] or direct with a needle-like applicator (direct ultrasound ablation)[30].

Although many have hypothesized about the immunological effects caused by local tumorablation, not much solid evidence is available. Also the differences with respect to the antigens released are not known. Most recent studies combined the ablation of tumors with systemic immune modulation and will be discussed later.

Radiotherapy

In the treatment of solid tumors mostly combinations of surgery, radiotherapy and chemotherapy are used. In the earlier stages of cancer, surgery alone may be curative, but may fail because of inadequate local excision with residual microscopic disease or micrometastases present at the time of diagnosis. Therefore, radiotherapy is a local treatment which is often used after surgery to reduce the chance of local recurrence. In for instance breast and rectal cancer, this combination gives promising results, reducing chances of local relapse. In some solid tumors, like laryngeal cancer, radiotherapy is used

on itself with curative intent[31]. In general, radiotherapy damages the DNA of tumor cells, which makes these cells unable to divide, after which they become apoptotic. A recent study by Teitz-tennenbaum and colleagues using murine tumors demonstrates that when intratumoral injection of DC and radiotherapy were combined, better anti-tumor immunity was observed than when the individual treatments were given[8]. This illustrates that antigens released after radiotherapy may also be useful for the *in situ* induction of anti-tumor immunity.

Chemotherapy

Although chemotherapies all have the same goal: kill tumor cells, not every chemotherapy does this in the same way. Most chemotherapies will induce cell-death by triggering cellular apoptosis signalling, an intrinsic mechanism for cells to shut down their machinery and to eliminate themselves from the body. Apoptotic cells will die quickly and will be recognized and phagocytosed by for instance macrophages and DCs[13]. Chemotherapies like doxorubicin or cyclophosphamide are effective in cancer because cancer cells are more sensitive to the apoptosis signals induced by the drug than normal cells. Other drugs induce non-apoptotic death of tumor cells. This includes necrosis, autophagy (e.g. Temozolomide) or mitotic catastrophe (e.g. Paclitaxel). The different kinds of cell death will lead to different kinds of antigens and, subsequently, different immune responses[14,15].

In support of the hypothesis that chemotherapy can deliver antigens to the immune system, recent studies described enhanced immune responses upon the combination of systemic chemotherapy with the intra-tumoral infusion of DCs[16,17]. Together with studies combining for instance CD40 triggering and chemotherapy, discussed later, this suggests that immunotherapy can indeed be a functional additive treatment to chemotherapy. Although promising, it is likely that not every kind of chemotherapy can be effectively combined with immunotherapy. Many types of chemotherapy will, for instance, eliminate vital parts of the immunesystem, which might be important, besides the DC, in the generation of an effective immune response[18]. Implications of combining chemotherapy with immunotherapy are extensively discussed by Lake et al.[19].

In situ tumor destruction: tumor-derived antigens and dendritic cells

Although *in situ* tumor destruction techniques have been successfully used to generate anti-tumor immunity, many issues still have to be elucidated, including an exact mechanism. And although postulated frequently, even the direct involvement of endogenous DCs has not been demonstrated. Applying a novel mouse model using radiofrequency or cryo ablation of established (5-7mm) murine B16OVA melanomas we set out to determine the immunological consequences of *in situ* tumor destruction and in particular the effects on DC. In chapter 3 we demonstrated that radiofrequency ablation generates antigens for the induction of specific anti-tumor responses. Adoptive transfers further showed that these responses were predominantly T cell-dependent. Subsequently, applying intra-tumoral injection of radioactive or fluorescently labelled ‘tumor antigens’ (KLH and OVA) we were able to show in chapter 4 that, following ablation, tumor-derived

antigens were present inside the draining lymph node, where they appeared to be taken up by DCs. Within the LN, up to 25% of DC contained antigen as soon as one day and for at least three days following ablation. Importantly, the tumor-derived antigens were *only* present in the DC fraction, whereas macrophages etc. were essentially negative. The antigens were not just 'sticking to' the DCs, as confocal microscopy demonstrated structures inside the cytoplasm containing OVA (Figure 1). Interestingly, antigen positive DCs were only present in the tumor draining lymph node and only in case ablation of the tumor was performed. Moreover, DCs were present in much higher numbers compared to a naïve or no-ablation situation. When compared to a conventional DC vaccine (using peptide loaded GFP-DC), ablation resulted in much more antigen-loaded DCs in the draining LN (chapter 4).

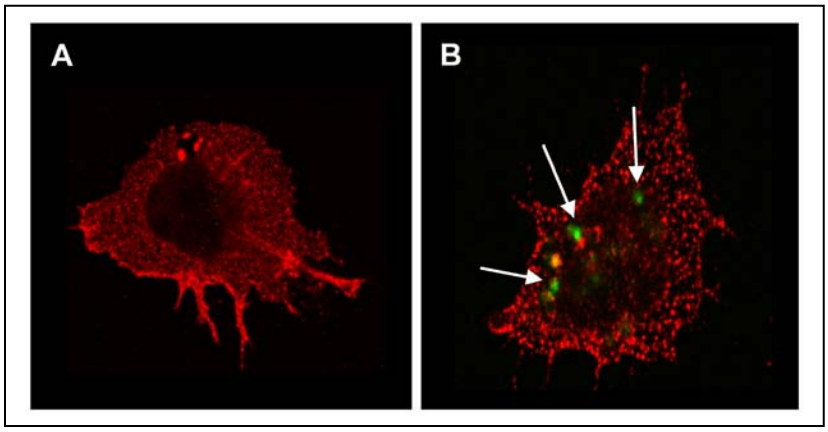

Fig. 1: Dendritic cells take up and internalize tumor-derived antigens. Prior to cryo ablation of established B16-OVA tumors, ovalbumin coupled to the fluorophore Alexa-488 was injected i.t.. Two days later, DCs were isolated from the draining lymph nodes by magnetic bead sorting on CD11c and co-stained for CD86 (red). Pictures show sections at 400x from CLSM Z-scans demonstrating an antigen negative DC (A) and an antigen positive DC (B). Arrows point to the vesicle-like structures containing OVA-Alexa488 (green). Cells from CD11c negative fractions or from mice that did not receive OVA were negative for Alexa-488.

It is currently not known whether antigens are taken up by DCs migrating into the tumor tissue and subsequently to the draining lymph node or that antigen is floating passively towards the draining lymph node where lymph node residing DC take it up. Studies using toxins that block migration may be useful to determine the contribution of DC migration. A recent study demonstrated that LN-DC accumulated antigen deposited in subcutaneous tissue[32]. It showed that after injection antigen was first detected in LN-residing DC, followed by a second wave of antigen positive DC that migrated from the periphery into the LN. Both waves were required for efficient immune response induction and were dependent on the presence of the challenge site. Our finding that both at day 1 and day 3 after ablation antigen-loaded DC could be discerned from the LN suggests that similar dynamics take place following *in situ* tumor ablation. In this context, it is interesting to note that antigen loading on DC in tumor-bearing control mice seemed to decline in these 3 days (chapter 4). Furthermore, antigens from the tumor depot preferentially

accumulated in DC, but not in B-cells or macrophages. The basis for the observed antigen accumulation in DC remains to be studied in more detail, but is likely related to their strategic location within the LN and their ability to retain antigens within their endocytotic compartment, whereas cells like macrophages rapidly degrade antigens in their lysosomes[33].

Irrespective of the strategy selected to load the DCs, it is now evident that activation of both CD4+ and CD8+ T cells is highly beneficial for the induction of anti-tumor immunity. Therefore, loading of both MHC class I *and* MHC class II molecules on the DC is important to boost a strong immune response. Whereas pulsing of DCs with a peptide epitope results in direct loading of MHC class I molecules, efficient presentation of intact tumor antigens or tumor cells relies on the unique capacity of DCs to cross-present exogenous antigens in MHC class I [34]. Importantly, even though in our experiments lymph node residing DC appeared to have taken up large amounts of antigens following ablation, no elevated levels of antigen specific CD8+ T cells could be observed (chapter 4 and 5). Antigen presentation assays demonstrated that MHC class II processing did occur, corresponding to the antigen uptake, but MHC class I processing failed. Also in other studies using tumor destruction alone the amounts of tumor specific CTL were never increased. This suggests that in general, upon tumor destruction, cross-presentation of antigens is limited.

The molecular pathways involved and the factors that trigger cross-presentation of antigens are still poorly understood. Recent studies in mice suggested that cross-presentation is dependent on the intracellular stability of the antigens[35] and is tightly controlled by DC maturation[36,37]. This DC maturation may be accomplished by many factors, as will be discussed in the next sections.

In situ tumor destruction: stimulating immunity by endogenous triggers

Although solid human tumors are frequently infiltrated by DCs, the onset of a proper immune response is nevertheless often hampered. As DC maturation is of crucial importance for immune response induction, the lack of potent DC maturating compounds like microbial PAMPs may well be responsible for this. The necessity of DC maturation to induce potent immune responses has now been well established. Whereas immature DCs have proven to be weak immune stimulators and even can be tolerogenic, mature DCs induce functionally superior CD8+ T cells and concomitant anti-tumor immunity[38]. Accordingly, injection of monocyte-derived immature DC vaccines in cancer patients yielded little T cell-mediated immune responses, whereas following injection of mature DCs these responses were readily detected[39,40].

Inducing potent immune responses by *in vivo* loaded DCs, can thus not go without maturation of these DCs. But what does it take to optimally mature a DC, and, more importantly, how do we actually *define* 'optimal maturation'? Although this issue is still subject to debate, it is generally accepted that DCs mature when they experience immunological 'danger'. In the 'danger hypothesis', Matzinger and others hypothesized that effective immune responses are only induced when antigens are taken up in the context of

'danger'[41]. Moreover, it was stated that 'danger recognition' not only consisted of identifying microbial invasion, but also sensing of tissue disruption and dying cells. When seen in this context, *in situ* tumor destruction by itself should thus not be a null event for DCs.

Apoptosis vs. necrosis

There has been much controversy about the differences in induction of immune responses by apoptotic vs. necrotic cells. Several studies, mainly performed *in vitro*, have found that cell death by necrosis induces APCs to act as potent immune stimulators, whereas apoptotic cells do not cause this effect[42,43]. Since the hypothesis by Matzinger[41] has largely been confirmed, it seems logical that apoptotic death will only induce tolerance. After all, apoptosis is occurring all the time in the steady state turnover of cells in normal healthy tissue, whereas necrosis occurs only in infection. Virally induced apoptosis is emerging as an important exception to this concept. This kind of apoptosis actually promotes the activation of immune responses, which is mediated via expression of caspases in the apoptotic cell and possibly enhanced by intracellular remnants of the viral machinery[44].

From a theoretical point of view, the apoptotic killing by for instance chemotherapy would thus mainly cause tolerance to the released antigens, since there are only very few of these drugs that induce direct necrosis in tumors. The situation after massive apoptotic cell death following chemotherapy or radiotherapy is, however, likely to be more complex. Large parts of the tumor will go into secondary necrosis, which together with endogenous mediators released upon cell death will affect the immunological outcome[15,45].

Endogenous mediators

As discussed, DC activation upon the recognition of 'danger' does not only involve detection of molecules derived from potential pathogens, but also the recognition of endogenous host-derived molecules released by dying cells. It has, for instance, been shown that heat shock proteins and uric acid are present in cellular debris, which both influence DC and other parts of the immune system[15,46,47]. Such endogenous 'danger' signals may, in some cases, act as ligands for PRRs, like TLRs. In recent years, fibronectin A, hyaluran derivatives, inflammatory cytokines, fibrinogen, heat shock proteins, b-defensins and endogenous DNA have all been put forward as putative endogenous TLR ligands[15,45,48]. Some of these studies are still subject to debate, as it is suggested that impure reagents may have biased the outcome.

Endogenous stimuli may be of critical importance for *in situ* tumor destruction procedures to generate an immune response. Upon *in situ* tumor destruction endogenous danger signals are readily released from the damaged tissue/tumor, which may contribute to immune activation. For instance, it has recently been demonstrated in a human study that TNF-α and IL-1β levels were markedly enhanced following radiofrequency ablation or ablation by ethanol instillation[49]. Others found that heat shock protein expression at the tumor margins was increased when tumors were ablated by laser ablation[50]. These mediators may all contribute to immune activation. Indeed, in our research we found that DCs following cryo or radiofrequency ablation were more mature compared to the DCs from tumor-bearing mice (chapter 4 and 5). Also in a recent clinical study using

radiofrequency ablation of hepatocellular carcinoma, it was demonstrated that DCs underwent a transient activation as shown by maturation marker expression[49]. As a contrasting view, one can hypothesize that eradication of the tumor also deletes the suppressive milieu surrounding the tumors, which could be a possible explanation for increased DC maturation after ablation. However, in one of our experiments injection and subsequent ablation of *ex vivo* prepared tumor lysates gave exactly the same results as compared to ablation of intact tumors (chapter 5). This suggests that it is more likely that the maturation increase is ablation-dependent. It still needs more clarification what exactly are the endogenous PRR ligands or cytokines involved in these ablation-dependent effects.

Although there has been controversy about the magnitude of the role endogenous mediators play, it seems more and more likely that there is a synergistic interplay between the endogenous inflammatory mediators and recognition of PAMPs. Recent studies using *ex vivo* produced DCs support this view. For example, human DCs cultured in a combination of IL-1β/TNF-α with type I and II interferons (IFN-α and IFN-γ) and the TLR ligand poly I:C were much more potent IL-12 producers and better inducers of CTLs *in vitro* then either modality alone[51]. Moreover, it was demonstrated that activation of DCs by solely pro-inflammatory cytokines yielded DCs that supported CD4+ T cell clonal expansion, but failed to efficiently direct helper T cell differentiation. In contrast, exposure of these cells to cytokines plus pathogen components generated DCs that did promote T cell help[51,52]. Recent studies suggest that the interplay of multiple TLRs and other pathogen receptors like C-type lectins and nucleotide-binding oligomerization domains (NODs, intracytoplasmic microbial-recognition proteins sensing for specific peptidoglycan muropeptides) may work synergistically[53,54]. Simultaneous stimulation of different pathogen recognition receptors might indeed more closely resemble the natural situation where pathogens containing several molecular triggers will initiate multiple defense mechanisms.

Importantly, although DC maturation is significantly enhanced after the used ablative treatments, only weak immune responses were induced according to our tumor rechallenge models. As shown in chapter 4, when we used cryo ablation of established B16OVA tumors, only 30-40% of the mice were protected from a lethal B16OVA re-challenge. Moreover, although DC maturation following radiofrequency ablation was equal to maturation following cryo ablation, less potent immunity was induced with radiofrequency ablation (20%, chapter 3 and 4). In conclusion: although *in situ* tumor ablation releases mediators that do mature DCs, this maturation may not be sufficient to induce potent immunity. The data also suggest that maturation of DC is not the only factor modulated by tumor ablation. Possibly, changes in Tregs, granulocytes, neutrophils and macrophages also significantly contribute to the generation of the anti-tumor immunity.

Although the weak responses may seem disappointing, it has been demonstrated by us and others that *in situ* tumor destruction can, however, provide a fertile 'breeding-ground' for potential immune responses. Treating the entire process of *in situ* tumor destruction as an -*in vivo* DC vaccine- will give us the enormous benefit that we can use all the known strategies to induce cross-presenting, activated DC already explored in conventional DC vaccines. This view has led us to explore the combination of *in situ* tumor destruction with various local or systemic immune modulations.

In situ tumor destruction: stimulating immunity by exogenous triggers

From *ex vivo* produced anti-tumor vaccines we have learned that modulation of immune responses can take place at the level of antigen processing, but also at the level of T cell induction (chapter 2). Certain ways of maturation of tumor antigen positive DCs may establish better cross-presentation of the antigens taken up, increase migration of these DCs to the draining lymph nodes and subsequently lead to a better presentation to the T cells. Immunological intervention at the interface of interaction between DC and T cell may consecutively skew the functional outcome of antigen presentation. When for instance the relevant co-stimulatory or co-inhibitory molecules are increased or inhibited, more potent activation of T cells will occur. Another target for intervention comprises the regulation of T cell responses. Regulatory T cells, for instance, may down-regulate T cell activity in the periphery and even tolerize them. In this section we will discuss interventions relevant for the stimulation of immunity following *in situ* tumor destruction.

Modulation of DC function and T cell priming

Administration of stimuli containing PAMPs is the most common way to induce DC maturation. Several PRR (TLR, NOD) ligands have been described to mature DC, increase cross-presentation and excretion of cytokines[53,55]. Recently, it was shown that combinations of different ligands could even work together in synergy, altering the responses seen with the individual ligands alone[54].

In combination with *in situ* tumor destruction very few TLR ligands have been tested. We and others have focussed mainly on CpG-ODN, a ligand for TLR-9 (chapter 5). Unmethylated CpG-ODN stimulates DCs to express co-stimulatory molecules, increase antigen presentation and secrete particularly Th1-associated cytokines, such as IL-12[56]. Moreover, the addition of CpG-ODN has shown to overcome the inhibitory effects of immune suppressive factors secreted by B16 melanoma cells on bone marrow derived DCs and to restore their capacity to activate allogeneic T cells[57]. It has been demonstrated that peri-tumoral CpG-ODN initiates strong immune responses mediated by CD8+ T cells and innate effector cells. This results in growth delay and cure of tumors in various settings[58]. The efficacy of CpG-ODN is, however, often negatively correlated with the size of tumors and also seems to be less when the treated tumor is less immunogenic.

However, recently it was shown that CpG-ODN also improved the MHC class I peptide-processing pathway, leading to an increased number of cross-presented epitopes on the cell surface of DCs[36,59]. Since in immunotherapy of cancer it is believed that especially MHC class I presentation is needed for effective eradication of tumor cells, CpG-ODN might therefore be a promising adjuvant for *in situ* tumor destruction. In the experiments presented in chapter 5, *in situ* ablation was able to significantly enhance MHC class II processing and presentation of antigens, while MHC class I presentation was not affected. Interestingly, when we combined *in situ* tumor ablation with CpG-ODN, we were able to show an increased amount of cross-presented antigens on the surface of DCs. Importantly, cross-presentation was absent when no ablation or CpG-ODN injection alone was performed.

The combination treatment not only influenced cross-presentation but also maturation of DCs. When we injected CpG-ODN alone into tumor-bearing or naïve mice, we observed an increase in the expression of maturation markers on isolated DC. However, when we performed this injection directly after ablation of the tumor, the increase was higher than the increase seen with ablation or CpG-ODN alone. This synergistic maturation, together with the increased cross-presentation, suggest a superior advantage over conventional CpG-ODN use. In line with this, only the combination treatment led to T cell induction and subsequent tumor growth delay of non-immunogenic B16 melanomas (Chapter 5). Moreover, in the course of the experiments we did a striking observation: when we normally performed cryo ablation on tumor-bearing mice, we were used to have a constant rate of recurrent tumors at the site of ablation (comparable to clinical situation). Interestingly, when we added CpG-ODN at the time of ablation we noticed an almost complete absence of recurrences. Although the recurrence rates were strongly related to the initial size of the treated tumor, CpG-ODN was able to reduce recurrences for all tested tumor sizes (Figure 2). In support of this, it was shown that CpG-ODN given as a single peri-tumoral injection following *in situ* radiation of murine tumors dramatically enhanced tumor growth delay in sarcoma-bearing mice[5] and in rats with 9L-glioma[60]. Although no relation with APC was studied, the authors found heavy infiltration by inflammatory cells inside the radiated tumor. Similar results were obtained when topotecan, cyclophosphamide or gemcitabine treatment of mice bearing tumors was combined with CpG-ODN treatment[7,61]. It will be very interesting to translate this combi-approach to a clinical setting. However, as TLR expression on DCs is different between species, analysis of different TLR ligands in combination with ablation will be important.

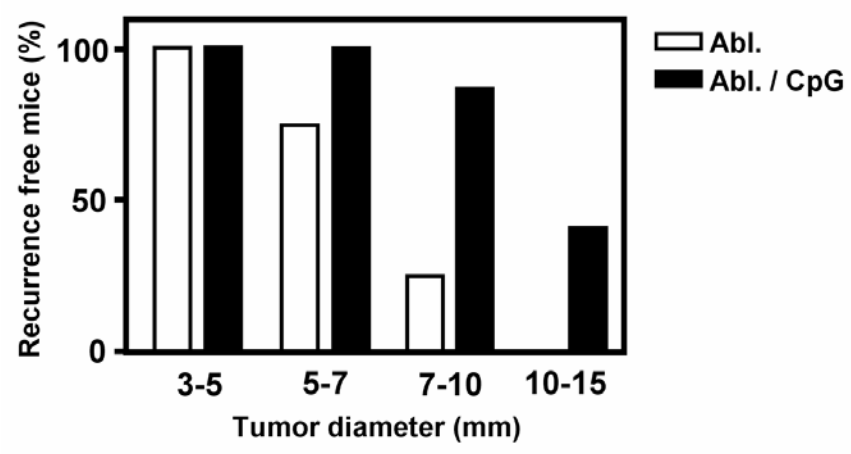

Fig. 2: CpG-ODN improves therapeutic outcome of ablation. At various timepoints after inoculation of B16-OVA tumors, the tumordiameter was examined. Mice were selected for the depicted diameter groups (n=10) after which their tumors were cryo ablated. Next, part of the mice received 100µg CpG-ODN 1668 intratumorally. Shown are the percentages of mice that did not get a recurrent tumor at the ablation site.

A different method to obtain maturation of DC is via CD40 triggering. Besides maturation of DC, CD40 stimulation also enhances the capacity of DC to cross-present antigens to T cells[59]. In addition, these tumor-specific CTL do leave the lymph node more easily, thereby entering the circulation[62]. Van Mierlo and co-workers showed that CD40 triggering via systemic anti-CD40 antibodies is indeed able to cure mice from tumors when applied intratumorally[59]. Via isolation of CD11c+ cells from lymph nodes they were able to show increased DC maturation, which enhanced T-cell priming and expansion. Similar as with CpG-ODN, CD40 triggering is less effective when delivered to hosts bearing very large non-immunogenic tumors. It may, however, be a promising alternative to combine with *in situ* tumor destruction. In this context, Nowak and co-workers demonstrated that induction of tumor cell apoptosis by gemcitabine in combination with CD40 triggering increased tumor antigen cross-presentation, which was able to prime specific T cells[63]. Antibody triggering of other stimulatory members of the tumor-necrosis factor receptor (TNFR) family: OX40, 4-1BB, CD27, CD30 has similar effects as CD40 triggering, when studied in conventional tumor models (reviewed by Croft et al.[64]). It will be extremely interesting to see if future studies can demonstrate a synergistic effect of these antibodies in combination with *in situ* tumor destruction.

In addition to the stimulatory pathways discussed, also blockade of inhibitory receptors, e.g. CTLA-4, has been applied successfully to induce tumor rejection[65]. In the experiments described in chapter 3 and 4, we successfully applied a blocking antibody to CTLA-4 after radiofrequency or cryo ablation of established tumors. By tetramer analysis we could demonstrate that tumor specific T cells were only observed following the combined ablation/antibody treatment. These results were recently confirmed by Demaria and co-workers who demonstrated that CTLA-4 blockade could be efficiently combined with radio-therapeutic treatment of murine tumors[66].

Regulating regulatory T cells

Tumors can escape immune recognition and their subsequent elimination by creating a state of immunological unresponsiveness or self-tolerance to tumor antigens (reviewed by Zou et al.[67]). Moreover, in tumor-associated lymph nodes and peripheral tissues, T cells expressing a CD4+CD25+ regulatory phenotype (Regulatory T cells, Tregs) can be found. Tregs have been shown to be of critical importance in regulation of T cell dependent immune responses and in creating the immunological tolerance to self and non-self[68]. These regulatory T cells are able to suppress T cell and DC function in a cell contact dependent manner and represent one of the feedback mechanisms used by the immune system to limit excessive immune activation. To enhance the effectiveness of *ex vivo* produced DC vaccines, many have combined anti-tumor vaccination with regulatory T cell depletion. This depletion was mostly established via depleting antibodies directed against the Treg marker CD25. Indeed, depletion of $CD25^{+}CD4^{+}$ T cells combined with DC vaccination before a subsequent tumor challenge provided effective anti-tumor immunity in otherwise non-responding mice[68].

We reasoned that *in situ* tumor ablation could function as an *in situ* DC vaccine, which would enable combination with regulatory T cell depletion. The data in chapter 4 showed us that when the depleting antibodies were given 4 days prior to tumor ablation by

cryo or radiofrequency ablation, mice were increasingly protected to a subsequent tumor rechallenge. After ten days, more specific T cells could be found, which also produced more IFN-γ.

Recently, Ercolini and co-workers demonstrated that chemotherapy (cyclophosphamide) was also able to significantly affect regulatory T cell populations[69]. Interestingly, they found that a tumor specific vaccine was only then effective when combined with the T reg-reducing chemotherapy. In line with this finding, it was demonstrated in patients receiving lympho-depleting chemotherapy (cyclophosphamide with fludarabine) that subsequent transfer of tumor-directed T cells was more effective as compared to when no chemotherapy was given[70]. Although no causal relation was investigated, it was suggested that the abrogation of the suppression by Tregs could be a cause for enhanced responsiveness to the T cell transfer.

To exploit the full benefit of DCs in *in situ* DC vaccination, it is important to learn more *about in vivo* inhibitory processes that prevent the immune system to become fully activated. Controlling DC activation, regulatory T cells and immune inhibitory molecules on the cells of the immune system will be rewarding strategies to combine with tumor destruction.

Directions to an effectively co-ordinated immune response

Many of the strategies advocated above, may have effects on other parts of the immune system then already described. When applied in combination with *in situ* tumor destruction, these effects could be crucially important in determining the functional outcome. It would be of particular interest if we could manage to effectively co-ordinate the anti-tumor response to be more effective by combining different strategies.

Future research will have to determine if other TLR ligands have similar effects in combination with *in situ* tumor destruction as described for CpG-ODN. Possible candidates may be Pam_3cys (TLR2), Poly I:C (TLR3), LPS (TLR4) or TLR 7/8 ligands like in use for treatment of warts: Imiquimod or R848[71]. Moreover, different TLRs may work in synergy when stimulated. It was recently shown in human and mouse DCs that TLR3 and TLR4 triggering combined positively with TLR7, TLR8 and TLR9 stimulation[54]. It will be very interesting to see if these particular combinations are also effective in relation to *in situ* tumor destruction.

Furthermore, it might be rewarding to consider the effects of different TLR stimuli on other parts of the immune system. For instance, it was recently demonstrated that a TLR8 ligand was effective in temporarily ceasing the suppressive capacities of Tregs by direct triggering of these cells[72]. The authors successfully applied adoptive transfer of TLR8-stimulated Tregs into tumor bearing mice to enhance anti-tumor immunity. Recently, we demonstrated that the TLR2 ligand Pam_3cys was also able to abrogate Treg-mediated suppression of T cell expansion (chapter 6). Consistently, this enhanced responses in an *in vivo* infection model. Intra-tumoral injection of different TLR ligands following *in situ* tumor destruction may thus be aimed at enhancing DC activation, but also at Treg suppression. Targeting DCs as well as Tregs may result in an increasingly effective anti-tumor response.

TLR stimulation may also act simultaneously on NK cells and various subsets of DC. Activated NK cells have shown to promote DCs that have captured antigen, and *vice versa*, DCs have shown to promote proliferation of NK cells and increase their cytotoxic activity[73,74]. Recently it was demonstrated that in the presence of IL12, human NK cells also respond to double-stranded RNA or unmethylated CpG-DNA[75]. The authors claim that IL-12 or IFN-α produced by DC will first activate NK cells that in turn eliminate DCs that failed to take up antigen or did not express HLA class I molecules to levels required for resistance against NK-mediated killing. In addition, NK cell-derived IFN-γ might induce up-regulation of TLR expression by DCs. Another recent study demonstrated an even more complex interplay between myeloid DC, plasmacytoid DC and NKT cells in response to TLR stimulation. It showed that plasmacytoid DC can regulate effector activity of antigen-stimulated NKT cells in a cell contact-dependent manner. In their model, myeloid DC appeared to be responsible for the presentation of the cognate NKT cell antigen *alpha-galactosylceramide* (alpha-GalCer), whereas plasmacytoid DC appeared to be the most sensitive for CpG-ODN stimulation. In turn, the IFN-α and a cell-contact-mediated signal from the plasmacytoid DC enabled full activation of the NKT cells. Currently not much is known about the activation status of NK or NKT cells in response to tumor destruction. Therefore, it will be very interesting to examine the effects of intra-tumoral administration of exogenous alpha-GalCer (combined with CpG-ODN) following *in situ* tumor destruction.

The above-mentioned studies demonstrate that co-ordinating an immune response following *in situ* tumor destruction is extremely complex. For this, we will need extensive basic knowledge about the intricate processes taking place in both the innate and the adaptive immune system in response to immune modulation.

In situ tumor destruction: an *in vivo* anti-tumor DC vaccine

Based on the data presented in this thesis we believe that there may be certain advantages of combining immunotherapy and *in situ* tumor destruction over other combinations, which we illustrated in Figure 3.

First, in the past decades immunotherapy has demonstrated to generate efficient immune responses, which can efficiently attack tumor cells. However, in immunotherapy the rapid growth of tumors will often exceed the capacity of the immune system to clear all tumor cells. Combination with destruction of large tumor burdens or debulking *in situ* will therefore give an enormous advantage over immunotherapy alone.

Secondly, in clinical studies using immunotherapy there is in general a specific need to know the right antigen to 'fit' the patient's tumor. When antigens are loaded onto DCs via *in situ* tumor destruction there is no need to define or purify the best tumor antigens anymore. DCs will pick the most immunodominant epitopes by themselves. Almost all the studies using *in situ* tumor destruction described an increased availability of tumor antigens, which makes it likely that in theory every human tumor can be used to deliver the right tumor antigens. Immunotherapy should be used to boost subsequent immunity to these antigens.

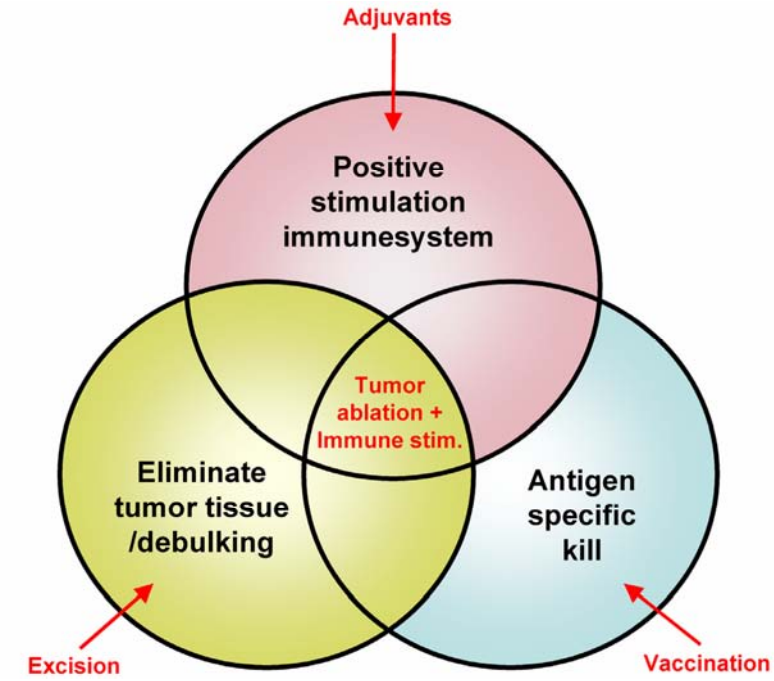

Fig. 3: Advantages of combining immunotherapy and *in situ* tumor destruction. The circles depict three important goals in anti-cancer treatments. Yellow (left) circle: The elimination of large volumes of tumor tissue, by for instance excision, is a very straightforward, but important goal in surgical oncology. Although it can clear the body from many tumorcells, it usually does not lead to antigen specific immune responses. Antigen-specific kill of tumor cells (Blue, right circle), like for instance obtained with DC vaccination, enables the immune system to eliminate previously macroscopically unnoticed tumorcells/metastases. On itself, vaccination often does not produce sufficiently strong immune responses to clear the entire tumor burden. Active downregulation of immune responses by tumorcells or for instance regulatory T cells might well be responsible for this. For this reason, effective 'guidance' of the immune response is needed. This can be done by exogenous or endogenous stimulation. Most desirable, however, are efficient combinations of those two groups. This will result in a powerfull stimulation of immune responses (Purple, upper circle). Clinical research has shown us that the strongest anti-cancer treatments can be found in the overlapping regions of the different circles. We believe that *in situ* tumorablation with immune modulation like with CpG-ODN deserves a place in the centre of the figure, although we are convinced that more treatment modalities will fit in there.

A third lesson learned from animal models is that one should carefully choose the right type of tumor destruction to combine with immunomodulation. As we have discussed in this chapter, *in situ* tumor destruction can have imperative effects on the immune system. We demonstrated that ablation on itself is inducing maturation of DCs and a low level of immunological protection. But importantly, the combination with CpG-ODN worked synergistically, as compared to the individual treatments. It will be also interesting to investigate if other destructive techniques like for instance radiation therapy have similar beneficial effects on DCs in combination with immunological interventions. Moreover, it will be essential to know the effects on the immune system in general.

Immunotherapy applying tumor-antigen-loaded DCs has proven to be feasible and effective in some patients, but a better understanding of how DCs succeed to induce and

modulate immunity is necessary to optimally exploit DCs in these vaccines. We believe that, besides that combination of *in situ* tumor destruction with immune modulation is a promising approach for treatment of cancer, it also comprises a valuable tool to learn more about DC behaviour *in vivo*. This knowledge may be vital for future vaccine design applying *ex vivo* generated DC. One can envisage many variables that can be optimized, including the mode of DC preparation, the subtype, maturation status of DCs, the route of administration and the mode of antigen loading. Understanding the complex interactions between the processing of the *in vivo* provided antigens and maturation stimuli, including their time constraints, will be of critical importance to induce optimal cross-presentation and subsequent CD8 T cell responses.

The challenge in the next years will be to encourage surgeons and immunologists to 'stay in touch'. The technically difficult protocols should not prevent them from initiating clinical trials. Proving efficacy in this kind of studies, by carefully monitoring the immune responses, will ultimately lead to a combination therapy of incontestable efficacy, and provide a well-tolerated cancer treatment for use alongside the conventional treatment modalities.

References

1. Nagorsen, D., Marincola, F.M. & Kaiser, H.E. Bacteria-related spontaneous and therapeutic remission of human malignancies. *In Vivo* **16**, 551-6 (2002).
2. Schreurs, M.W., Eggert, A.A., Punt, C.J., Figdor, C.G. & Adema, G.J. Dendritic cell-based vaccines: from mouse models to clinical cancer immunotherapy. *Crit Rev Oncog* **11**, 1-17 (2000).
3. Figdor, C.G., de Vries, I.J., Lesterhuis, W.J. & Melief, C.J. Dendritic cell immunotherapy: mapping the way. *Nat Med* **10**, 475-80 (2004).
4. Rosenberg, S.A., Yang, J.C. & Restifo, N.P. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* **10**, 909-15 (2004).
5. Milas, L. et al. CpG oligodeoxynucleotide enhances tumor response to radiation. *Cancer Res* **64**, 5074-7 (2004).
6. den Brok, M.H. et al. In situ tumor ablation creates an antigen source for the generation of antitumor immunity. *Cancer Res* **64**, 4024-9 (2004).
7. Weigel, B.J., Rodeberg, D.A., Krieg, A.M. & Blazar, B.R. CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma. *Clin Cancer Res* **9**, 3105-14 (2003).
8. Teitz-Tennenbaum, S. et al. Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration. *Cancer Res* **63**, 8466-75 (2003).
9. den Brok, M.H., Nierkens, S., Figdor, C.G., Ruers, T.J. & Adema, G.J. Dendritic cells: tools and targets for antitumor vaccination. *Expert Rev Vaccines* **4**, 699-710 (2005).
10. Novellino, L., Castelli, C. & Parmiani, G. A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol Immunother* **54**, 187-207 (2005).
11. Moudgil, K.D. & Sercarz, E.E. Understanding crypticity is the key to revealing the pathogenesis of autoimmunity. *Trends Immunol* **26**, 355-9 (2005).
12. Banchereau, J. & Palucka, A.K. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* **5**, 296-306 (2005).
13. Albert, M.L., Sauter, B. & Bhardwaj, N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86-9 (1998).
14. Edinger, A.L. & Thompson, C.B. Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* **16**, 663-9 (2004).
15. Skoberne, M., Beignon, A.S. & Bhardwaj, N. Danger signals: a time and space continuum. *Trends Mol Med* **10**, 251-7 (2004).
16. Tong, Y., Song, W. & Crystal, R.G. Combined intratumoral injection of bone marrow-derived dendritic cells and systemic chemotherapy to treat pre-existing murine tumors. *Cancer Res* **61**, 7530-5 (2001).
17. Tanaka, F. et al. Intratumoral injection of dendritic cells after treatment of anticancer drugs induces tumor-specific antitumor effect in vivo. *Int J Cancer* **101**, 265-9 (2002).

18. Emens, L.A., Machiels, J.P., Reilly, R.T. & Jaffee, E.M. Chemotherapy: friend or foe to cancer vaccines? *Curr Opin Mol Ther* **3**, 77-84 (2001).
19. Lake, R.A. & Robinson, B.W. Immunotherapy and chemotherapy--a practical partnership. *Nat Rev Cancer* **5**, 397-405 (2005).
20. Goldberg, S.N. et al. Image-guided tumor ablation: standardization of terminology and reporting criteria. *J Vasc Interv Radiol* **16**, 765-78 (2005).
21. Ruers, T. & Bleichrodt, R.P. Treatment of liver metastases, an update on the possibilities and results. *Eur J Cancer* **38**, 1023-33 (2002).
22. Garcea, G., Lloyd, T.D., Aylott, C., Maddern, G. & Berry, D.P. The emergent role of focal liver ablation techniques in the treatment of primary and secondary liver tumours. *Eur J Cancer* **39**, 2150-64 (2003).
23. Livraghi, T. et al. Hepatocellular carcinoma and cirrhosis in 746 patients: long-term results of percutaneous ethanol injection. *Radiology* **197**, 101-8 (1995).
24. Curley, S.A. Radiofrequency ablation of malignant liver tumors. *Ann Surg Oncol* **10**, 338-47 (2003).
25. Shibata, T. et al. Small hepatocellular carcinoma: comparison of radio-frequency ablation and percutaneous microwave coagulation therapy. *Radiology* **223**, 331-7 (2002).
26. Bown, S.G. et al. Photodynamic therapy for cancer of the pancreas. *Gut* **50**, 549-57 (2002).
27. Nikfarjam, M. & Christophi, C. Interstitial laser thermotherapy for liver tumours. *Br J Surg* **90**, 1033-47 (2003).
28. Neeleman, N., Wobbes, T., Jager, G.J. & Ruers, T.J. Cryosurgery as treatment modality for colorectal liver metastases. *Hepatogastroenterology* **48**, 325-9 (2001).
29. Jolesz, F.A. & Hynynen, K. Magnetic resonance image-guided focused ultrasound surgery. *Cancer J* **8 Suppl 1**, S100-12 (2002).
30. Deardorff, D.L., Diederich, C.J. & Nau, W.H. Control of interstitial thermal coagulation: comparative evaluation of microwave and ultrasound applicators. *Med Phys* **28**, 104-17 (2001).
31. Andry, G., Hamoir, M. & Leemans, C.R. The evolving role of surgery in the management of head and neck tumors. *Curr Opin Oncol* **17**, 241-8 (2005).
32. Itano, A.A. et al. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* **19**, 47-57 (2003).
33. Delamarre, L., Pack, M., Chang, H., Mellman, I. & Trombetta, E.S. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* **307**, 1630-4 (2005).
34. Bevan, M.J. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**, 1283-8 (1976).
35. Wolkers, M.C., Brouwenstijn, N., Bakker, A.H., Toebes, M. & Schumacher, T.N. Antigen bias in T cell cross-priming. *Science* **304**, 1314-7 (2004).
36. Datta, S.K. et al. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J Immunol* **170**, 4102-10 (2003).

37. Delamarre, L., Holcombe, H. & Mellman, I. Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *J Exp Med* **198**, 111-22 (2003).
38. Steinman, R.M., Hawiger, D. & Nussenzweig, M.C. Tolerogenic dendritic cells. *Annu Rev Immunol* **21**, 685-711 (2003).
39. Jonuleit, H. et al. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. *Int J Cancer* **93**, 243-51 (2001).
40. De Vries, I.J. et al. Maturation of Dendritic Cells Is a Prerequisite for Inducing Immune Responses in Advanced Melanoma Patients. *Clin Cancer Res* **9**, 5091-5100 (2003).
41. Matzinger, P. Tolerance, danger, and the extended family. *Annu Rev Immunol* **12**, 991-1045 (1994).
42. Sauter, B. et al. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* **191**, 423-34 (2000).
43. Gallucci, S., Lolkema, M. & Matzinger, P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* **5**, 1249-55 (1999).
44. Restifo, N.P. Building better vaccines: how apoptotic cell death can induce inflammation and activate innate and adaptive immunity. *Curr Opin Immunol* **12**, 597-603 (2000).
45. Rock, K.L., Hearn, A., Chen, C.J. & Shi, Y. Natural endogenous adjuvants. *Springer Semin Immunopathol* **26**, 231-46 (2005).
46. Somersan, S. et al. Primary tumor tissue lysates are enriched in heat shock proteins and induce the maturation of human dendritic cells. *J Immunol* **167**, 4844-52 (2001).
47. Shi, Y., Evans, J.E. & Rock, K.L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-21 (2003).
48. Barrat, F.J. et al. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* **202**, 1131-9 (2005).
49. Ali, M.Y. et al. Activation of dendritic cells by local ablation of hepatocellular carcinoma. *J Hepatol* (2005).
50. Nikfarjam, M., Muralidharan, V., Su, K., Malcontenti-Wilson, C. & Christophi, C. Patterns of heat shock protein (HSP70) expression and Kupffer cell activity following thermal ablation of liver and colorectal liver metastases. *Int J Hyperthermia* **21**, 319-32 (2005).
51. Mailliard, R.B. et al. alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res* **64**, 5934-7 (2004).
52. Sporri, R. & Reis e Sousa, C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol* **6**, 163-70 (2005).

53. Pulendran, B. Variegation of the immune response with dendritic cells and pathogen recognition receptors. *J Immunol* **174**, 2457-65 (2005).
54. Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F. & Lanzavecchia, A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* **6**, 769-76 (2005).
55. Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* **5**, 987-95 (2004).
56. Krieg, A.M. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* **20**, 709-60 (2002).
57. Furumoto, K., Soares, L., Engleman, E.G. & Merad, M. Induction of potent antitumor immunity by in situ targeting of intratumoral DCs. *J Clin Invest* **113**, 774-83 (2004).
58. Krieg, A.M. Antitumor applications of stimulating toll-like receptor 9 with CpG oligodeoxynucleotides. *Curr Oncol Rep* **6**, 88-95 (2004).
59. van Mierlo, G.J. et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. *J Immunol* **173**, 6753-9 (2004).
60. Meng, Y. et al. Successful combination of local CpG-ODN and radiotherapy in malignant glioma. *Int J Cancer* (2005).
61. Pratesi, G. et al. Therapeutic synergism of gemcitabine and CpG-oligodeoxynucleotides in an orthotopic human pancreatic carcinoma xenograft. *Cancer Res* **65**, 6388-93 (2005).
62. Stumbles, P.A. et al. Cutting edge: tumor-specific CTL are constitutively cross-armed in draining lymph nodes and transiently disseminate to mediate tumor regression following systemic CD40 activation. *J Immunol* **173**, 5923-8 (2004).
63. Nowak, A.K., Robinson, B.W. & Lake, R.A. Synergy between chemotherapy and immunotherapy in the treatment of established murine solid tumors. *Cancer Res* **63**, 4490-6 (2003).
64. Croft, M. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol* **3**, 609-20 (2003).
65. Egen, J.G., Kuhns, M.S. & Allison, J.P. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat Immunol* **3**, 611-8 (2002).
66. Demaria, S. et al. Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. *Clin Cancer Res* **11**, 728-34 (2005).
67. Zou, W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* **5**, 263-74 (2005).
68. Sakaguchi, S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* **6**, 345-52 (2005).
69. Ercolini, A.M. et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J Exp Med* **201**, 1591-602 (2005).
70. Dudley, M.E. et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J Clin Oncol* **23**, 2346-57 (2005).

71. O'Mahony, C. Genital warts: current and future management options. *Am J Clin Dermatol* **6**, 239-43 (2005).
72. Peng, G. et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* **309**, 1380-4 (2005).
73. Romagnani, C. et al. Activation of human NK cells by plasmacytoid dendritic cells and its modulation by CD4+ T helper cells and CD4+ CD25hi T regulatory cells. *Eur J Immunol* **35**, 2452-8 (2005).
74. Munz, C., Steinman, R.M. & Fujii, S. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J Exp Med* **202**, 203-7 (2005).
75. Sivori, S. et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A* **101**, 10116-21 (2004).

Summary

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Nederlandse Samenvatting

Summary

Our immune system is dedicated to protect the body from invading microorganisms, like bacteria and viruses. This complex defence system comprises many different cells, mainly leukocytes, which orchestrate the resistance to pathogens. In this, **dendritic cells** (DCs) are important players that function as the system's sentinels. They reside in peripheral tissues as immature DCs where they continuously sample their environment in search for antigens. They take up and degrade these antigens into small pieces (peptides), which are presented on the MHC molecules on the surface of the DCs. The context the DC perceives when taking up the antigens has important immunological consequences. If the antigens are, for instance, derived from bacteria, the DC will decide to initiate an immune response. On the other hand, if the antigens are derived of structures from the own body (self-antigens), then the DC will help to create a quiescent state and initiate tolerance for the antigen.

In the past decades, it has become clear that DCs can sense the difference between these two situations. The signals that a DC receives when it encounters an antigenic structure appear to be responsible for this. Microbes often carry common structures essential for their existence, and these Pathogen-Associated Molecular Patterns (PAMPs) can be recognized by specialized receptors in the immune system. **Toll-like receptors** (TLR) are well known examples of this. When a DC recognizes 'danger', it will undergo a phenotypical change called 'maturation'. Upon maturation, DCs migrate from the site of antigen encounter to the draining lymph nodes (LN). Here they interact and present the MHC-peptide complexes to naïve T cells. Only mature DCs have up-regulated expression levels of so called costimulatory molecules, which enable them to translate their 'sense of danger' into a full activation of the T cells. In the following fight against the infection, activated T cells play a very important role.

There are, however, situations in which (by mistake) immunity to self-antigens is created. If these responses are not dampened they will result in autoimmune disease. **Regulatory T cells** play an important role in the regulation of T cell responses. Regulatory T cells (Treg, CD4+CD25+ cells) inhibit auto-reactive immune responses and suppress T cells. It is generally believed that these Treg cells are actively induced in the thymus and suppress other cells via cell-contact-dependent mechanisms, although the exact mechanism is still not elucidated.

Immunotherapy applying *ex vivo* generated DCs has been successfully introduced in a variety of clinical vaccination protocols. They can be artificially loaded outside the body with relevant peptides to initiate immune responses inside the body. Also peptides from tumor antigens can be used for loading onto DCs. This kind of vaccination has been extensively studied the past years and has proven to be feasible and effective in some cancer patients. Importantly, because DC maturation near

tumors is often hampered and Treg activity is suppressing anti-tumor immunity, vigorous immune responses are not readily observed in all patients.

In **Chapter 1 and 2**, a general introduction is given about the regulation of immune responses, the characteristics and properties of DCs, and their role in current anti-cancer vaccines. Although promising data have been obtained with DC vaccination, a better understanding of how DCs induce and modulate immunity is necessary to optimally exploit the DC's capacities. The first part of chapter 2 therefore describes current insights in antigen loading, activation and migration of DCs and their impact on the application of *ex vivo* generated DC vaccines. Current vaccines mostly use tailor-made *ex vivo* generated DCs to induce immunity, but it has become evident that this is a costly and labour intensive procedure. Efforts to load and activate DCs directly *in vivo* will greatly facilitate the application of DC-based vaccines. The second part of chapter 2 discusses this subject and describes novel approaches of loading and activation of DCs directly *in situ*. However, DC vaccines will only then become fully effective when we learn more about *in vivo* inhibitory processes that prevent the immune system to become fully activated. So finally, the possible obstacles that should be overcome to induce long-lasting immunity in therapeutic settings are considered.

Chapter 3 describes a newly developed mouse model for *in situ* tumor destruction. This model was used to illustrate our view on *in situ* loading of dendritic cells. Tumor-destructing techniques, like radiofrequency ablation, allow eradication of large tumors, but at the same time may provide the immune system with an antigen source for the induction of anti-tumor immunity. We use the murine B16-OVA melanoma cell line to explore: 1) the immunological consequences of *in situ* tumor destruction and 2) the efficacy of a combination approach of tumor destruction and immunostimulation. Applying this model system we demonstrate that following radiofrequency ablation, a weak but detectable immune response develops, directed against OVA, but also against a broader range of B16 antigens. Adoptive transfer experiments further indicate that anti-tumor reactivity could be transferred to naïve mice by splenocytes. Administration of a blocking antibody against the co-inhibitory molecule cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) at the time of tumor destruction strongly enhances anti-tumor immunity resulting in long-lasting tumor protection. These results were our first proof that *in situ* tumor destruction could provide a useful antigen source for the induction of anti-tumor immunity, provided that additional immune modulation is administered.

Chapter 4 describes the first data on involvement of DCs in the initiation of immune responses following ablation. It also provides a comparison between the immune effects induced by cryo ablation and radiofrequency ablation. In this chapter we demonstrate that upon tumor destruction by radiofrequency ablation, up to 10% of

the total draining lymph node DCs acquire antigens. Interestingly, after tumor destruction by cryo ablation this amount is doubled. Compared to conventional DC vaccination, far more antigen positive DCs could be discerned in the lymph node after ablation. Analysis of DC maturation revealed that both destruction methods are able to enhance maturation. Finally, we show that *in situ* tumor ablation can again be efficiently combined with immune modulation by anti-CTLA-4 antibodies, but also with regulatory T cell depletion by anti-CD25 antibodies. These combinations lead to *in vivo* enhancement of tumor-specific T cell numbers, protecting them from lethal tumor challenges. The data in chapter 4 were our first evidence that *in situ* tumor destruction in combination with immune modulation creates a unique, '*in situ* DC-vaccine'. Interestingly, this strategy could form a promising, readily applicable treatment in the clinic, for which no prior knowledge of tumor antigens is necessary.

In **Chapter 5** this view is further explored. Using cryo ablation of B16-OVA tumors that were injected with marker proteins we could see that tumor antigen positive DCs were more mature compared to antigen negative DCs, while maturation was further enhanced by ablation. But most importantly, we showed that *in situ* tumor ablation synergizes with the TLR9 ligand CpG-ODN in inducing DC maturation. Only the combination treatment enabled *in vivo* cross-presentation of the antigens taken up, leading to superior DC function *in vivo*. The combination treatment was far more effective in the eradication of local and systemic tumors than either treatment modality alone. The data suggest that the complex interplay between antigen uptake, mediators released by the destruction treatment, and immunological skewing by carefully timed exogenous stimuli are of key importance for *in situ* DC-vaccination. This implicates that a combination of an optimal kind of *in situ* tumor destruction with an optimal kind of immune stimulation exists.

Chapter 6 describes that immunological stimulation by TLR-ligands not only affects DCs, but also Tregs. Although mature DCs are more competent inducers of immunity, T cell responses will not be optimal when negative regulation by Tregs is still present. However, in this chapter we demonstrate that this is not always the case. The data showed that direct triggering of Tregs by a TLR2 ligand temporarily ceased their suppressive capacities. Consistent with this, we demonstrate in an *in vivo* infection model that the TLR-ligand can modulate outgrowth of microbes. In the context of our ablation model, we therefore suggest that modulation in the effector (T cell) phase of immune responses can be an effective tool in conjunction to modulation in the initiation (antigen/DC) phase.

Finally, in **Chapter 7**, a general discussion and future prospective on the topics presented in this thesis are given.

Nederlandse Samenvatting

Het immuunsysteem beschermt ons lichaam tegen binnengedrongen micro-organismen, zoals bacteriën en virussen. Dit complexe verdedigingssysteem bestaat uit vele verschillende cellen, voornamelijk leukocyten, die de weerstand tegen pathogenen coördineren. **Dendritische cellen** (DCs) spelen hierbij een belangrijke rol. Zij functioneren als het ware als de poortwachters van het immuunsysteem. DCs bevinden zich in allerlei perifere weefsels in het lichaam, waar ze veelal als onrijpe DCs de omgeving verkennen. Ze nemen daarbij steeds een sample van hun omgeving op zoek naar antigenen. Deze antigenen worden opgenomen en eenmaal binnen gedegradeerd tot kleinere fragmenten (peptides). De peptides worden vervolgens aan de buitenkant van de cel gepresenteerd op zogenaamde MHC moleculen. De context die een DC waarneemt op het moment dat hij antigenen opneemt heeft belangrijke immunologische consequenties. Wanneer een DC bijvoorbeeld antigenen opneemt van bacteriën, zal hij hoogst waarschijnlijk een immuunrespons initiëren. Echter, wanneer de antigenen afkomstig zijn van structuren van het eigen lichaam (zelfantigenen), dan zal de DC proberen een immuunrespons te onderdrukken en tolerantie te ontwikkelen voor het antigeen.

In de afgelopen jaren is het duidelijk geworden dat DCs het verschil tussen deze twee situaties kennen. De signalen die een DC krijgt op het moment van antigen opname blijken hiervoor verantwoordelijk. Micro-organismen bezitten vaak gemeenschappelijke structuren die onmisbaar zijn voor hun voortbestaan. Deze Pathogeen Geassocieerde Moleculaire Patronen (*engels:* PAMPs) kunnen worden herkend door gespecialiseerde receptoren binnen het immuunsysteem. De zogenaamde **'Toll-like' receptoren** (TLRs) zijn bekende voorbeelden hiervan. Wanneer een DC (via zijn TLRs) 'gevaar' waarneemt, ondergaat hij een uiterlijke verandering, maturatie geheten. Nadat maturatie heeft plaatsgevonden, migreren DCs van de plek waar ze het antigen opnamen naar de drainerende lymfeknopen. In de lymfeknopen gaan ze een interactie aan met naïeve T cellen waaraan ze de MHC-peptide complexen presenteren. Alleen de gematureerde DCs hebben grote hoeveelheden zogenaamde co-stimulatoire moleculen op hun oppervlak, welke hen in staat stellen hun perceptie van het 'gevaar' te vertalen in een volledige activatie van de T cel. In het daarop volgende gevecht tegen de infectie spelen geactiveerde T cellen een zeer grote rol.

Er komen echter situaties voor waarin (per ongeluk) een immuunrespons tegen zelfantigenen is gecreëerd. Indien deze responsen niet onder controle worden gehouden zullen ze resulteren in auto-immuunziekten. **Regulatoire T cellen** (Treg, CD4+CD25+ cellen) spelen een belangrijke rol in de regulatie van T cel responsen. Treg cellen remmen zelfgerichte immuunresponsen en onderdrukken gewone T cellen.

Ook weten we dat deze Treg cellen actief worden geïnduceerd in de thymus en dat ze andere cellen remmen via een celcontact afhankelijk mechanisme en cytokines als IL-10 en TGF-β.

Immuuntherapie met buiten het lichaam (*ex vivo*) gemaakte DCs heeft een succesvolle plek weten te bemachtigen binnen de klinische vaccinatieprotocollen. Deze DCs kunnen in kweek beladen worden met de relevante peptiden om vervolgens, na teruggegeven te zijn aan het lichaam, immuunresponsen te genereren. Ook tumorantigenen kunnen worden gebruikt voor belading van DCs. In recente jaren is deze vorm van vaccinatie intensief bestudeerd en het is gebleken dat de methodiek praktisch mogelijk is en zelfs effectief in sommige patiënten. Het is daarbij belangrijk te vermelden dat in veel patiënten geen voldoende sterke immuunresponsen waarneembaar zijn. Tegenwerking van DC maturatie in de buurt van tumoren en T cel remming door regulatoire T cellen (tumoren zijn immers afkomstig van eigen weefsels) zijn hiervoor aannemelijke verklaringen.

In **Hoofdstuk 1 en 2** wordt een algemene introductie gegeven over de regulatie van immuunresponsen, de eigenschappen van DCs en hun rol binnen de huidige antikanker vaccins. Alhoewel veelbelovende resultaten zijn geboekt met DC vaccinatie, is een grotere kennis over hoe DCs immuniteit induceren en reguleren nodig om de capaciteiten van de DC volledig te kunnen benutten. Het eerste gedeelte van hoofdstuk 2 beschrijft daarom de huidige inzichten in antigen belading, activatie en migratie van DCs en hun invloed op het gebruik van *ex vivo* geproduceerde DC vaccins. De huidige vaccins gebruiken veelal *ex vivo* op maat gemaakte DCs om immuniteit te induceren, maar het wordt steeds duidelijker dat dit een uiterst kostbare en arbeidsintensieve methode is. Belading en activatie van DCs in het lichaam zelf zouden een grote sprong voorwaarts betekenen voor de toepassing van DC vaccinatie. Het tweede gedeelte van hoofdstuk 2 behandelt dit onderwerp en beschrijft de recente benaderingen om DCs direct *in vivo* te beladen. Elke vorm van DC vaccinatie zal echter pas effectief zijn wanneer negatieve regulatie door het immuunsysteem kan worden verminderd. Aan het eind van hoofdstuk 2 wordt daarom bediscussieerd welke obstakels nog moeten worden genomen om het mogelijk te maken langdurige immuniteit in therapeutische settings te creëren.

Hoofdstuk 3 beschrijft een nieuw ontwikkeld muismodel voor *in situ* (lokaal in het lichaam) tumor destructie. Dit model gebruiken we om onze visie op *in vivo* belading van DCs te illustreren. Tumor destructie technieken als radiofrequentie ablatie zijn in staat grote tumoren te elimineren, maar op hetzelfde moment kunnen ze een bron van tumorantigenen scheppen die gebruikt kan worden voor de inductie van immuunresponsen. We gebruiken de muizen-melanoom cellijn B16-OVA om te onderzoeken wat A) de immunologische consequenties zijn van *in situ* tumor destructie

en B) de gevolgen zijn van een benadering waarin we tumor destructie combineren met immuunstimulatie. Met dit model tonen we aan dat volgend op radiofrequentie ablatie zich een zwakke maar detecteerbare immuunrespons ontwikkelt. Deze is gericht tegen het eiwit OVA, maar ook tegen andere B16 antigenen. Experimenten waarbij we T cellen (splenocyten) transplanteren, laten verder zien dat de antitumor reactiviteit over kan worden gedragen op naïeve muizen. Wanneer nu een blokkerend antilichaam tegen het immuun remmende molecuul 'cytotoxic T lymphocyte-associated antigen 4' (CTLA-4) gegeven word op het moment van tumordestructie, word de antitumor respons sterk verhoogd. Dit resulteert dan ook in langdurige bescherming tegen tumor uitgroei. Deze resultaten zijn ons eerste bewijs dat *in situ* tumor destructie een effectieve bron van antigenen kan bieden voor de inductie van antitumor immuniteit, gegeven dat additionele immuunmodulatie wordt toegediend.

Hoofdstuk 4 beschrijft onze eerste data over de betrokkenheid van DCs in de initiatie van immuunresponsen volgend op ablatie. Ook biedt het een vergelijking tussen de geïnduceerde effecten door radiofrequentie ablatie en cryo ablatie. In dit hoofdstuk tonen we aan dat, na *in situ* tumor destructie door radiofrequentie ablatie, bijna 10% van alle drainerende lymfeknoop DCs antigenen op hebben genomen. Interessant is echter dat na tumordestructie via cryo ablatie deze hoeveelheid is verdubbeld. Wanneer we dit vergelijken met een conventioneel DC vaccin, zien we dat met beide ablatie methoden enorm veel meer antigeen beladen DCs in de drainerende lymfeknoop komen. Uiteindelijk laten we zien dat beide vormen van *in situ* tumor ablatie wederom uitstekend kunnen worden gecombineerd met anti-CTLA-4 antilichamen. Deze keer laten we echter ook zien dat *in vivo* depletie van regulatoire T cellen een even effectieve strategie is. Deze combinaties leiden tot verhoogde aantallen tumor-specifieke T cellen in het lichaam, die de muizen bescherming bieden tegen opnieuw geïnjecteerde en normaal dodelijke tumor-challenges. De resultaten van hoofdstuk 4 waren ons eerste bewijs dat *in situ* tumordestructie in combinatie met immuunmodulatie een uniek '*in situ* DC vaccin' kan vormen. Dit is uiterst interessant, omdat deze strategie een veelbelovende, direct toepasbare, behandeling in de kliniek zou kunnen worden. Er is immers geen voorafgaande kennis van de juiste tumorantigenen nodig om dit vaccin toe te passen.

In **Hoofdstuk 5** wordt deze visie verder uitgewerkt. Met behulp van marker eiwitten tonen we aan dat na cryo ablatie van B16OVA tumoren de tumorantigen positieve DCs meer gematureerd zijn vergeleken met antigen negatieve DCs. Maturatie wordt daarop nog eens verder verhoogd door ablatie. Het belangrijkste resultaat is echter dat *in situ* tumor ablatie in synergie blijkt te werken met de effecten die het TLR9 ligand CpG-ODN teweeg brengt. Alleen de combinatie behandeling leidt tot zogenaamde cross-presentatie van de opgenomen antigenen, wat een superieure functie van deze DCs weergeeft. De combinatie is vervolgens veruit het meest effectief

in het elimineren van locale of systemische tumoren, vergeleken met elke behandeling apart. De resultaten suggereren dat het complexe samenspel van antigen opname, endogene mediatoren die vrijkomen tijdens de destructie van de tumor en zorgvuldig getimede immuunmodulatie door exogene TLR-liganden een zeer belangrijk aspect is in deze vorm van *in situ* DC vaccinatie. Dit samenspel kan mogelijk een voordeel bieden op andere recent voorgestelde vormen van *in vivo* belading van DCs.

Hoofdstuk 6 beschrijft dat immunologische stimulatie via TLR-liganden niet alleen effecten heeft op DCs, maar ook op Tregs. Alhoewel gematureerde DCs zeer goed immuunresponsen kunnen induceren, zal een T cel respons nooit optimaal zijn wanneer negatieve regulatie door Tregs aanwezig blijft. Zoals in hoofdstuk 4 al beschreven was, leidt Treg depletie immers tot verhoging van de immuunrespons. In dit hoofdstuk tonen we aan dat er situaties bestaan waarin Tregs hun remmende capaciteit op een natuurlijke manier tijdelijk verliezen. De resultaten laten zien dat directe stimulatie van Tregs via het TLR2 ligand Pam_3Cys (ook aanwezig op bepaalde bacteriën) tijdelijk hun suppressie kan stilleggen. In samenhang hiermee tonen we aan dat het TLR2 ligand uitgroei van micro-organismen kan moduleren in een *in vivo* infectie model. In de context van ons ablatie model kunnen we dus bedenken dat modulatie in de effector fase van de immuunrespons (T cellen) een effectieve behandeling kan zijn in samenspel met modulatie in de initiatie fase (DCs en antigeen).

Uiteindelijk wordt in **Hoofdstuk 7** een algemene beschouwing en toekomstvisie op de in dit proefschrift gepresenteerde onderwerpen gegeven.

Throughout this thesis the following abbreviations are used

^{111}In	111-Indium (isotope)
AICD	Activation-induced cell death
APC	Antigen presenting cell *or* allophycocyanin (fluorophore)
CD8	Cluster of differentiation (number 8)
CFSE	Carboxyfluorescein diacetate succinimidyl ester (fluorophore)
CLSM	Confocal laser scanning microscopy
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen (number 4)
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate (fluorophore)
FCS	Foetal calf serum
GM-CSF	Granulocyte/macrophage colony stimulating factor
HSP	Heat shock protein
IgG	Immunoglobulin (type G)
IFN-γ	Interferon (type gamma)
IL-2	Interleukin (number 2)
KLH	Keyhole limpet hemocyanin
LTC-4	Leukotriene (type C4)
LPS	Lipopolysaccharid
LN	Lymph node
MIP	Macrophage inflammatory protein
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
mAb	Monoclonal antibody
mDC	Myeloid dendritic cell
NK cell	Natural killer cell
CpG-ODN	Oligodeoxynucleotide containing CG motif
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
pDC	Plasmacytoid dendritic cell
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
RFA	Radiofrequency ablation
Treg	Regulatory T cell
PE	R-phycoerythrin (fluorophore)
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TGF-β	Transforming growth factor (type beta)
TNF-α	Tumor necrosis factor (type alpha)

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Curriculum Vitae

Martijn den Brok werd geboren op 26 januari 1978 te Veghel. Na het eindexamen Gymnasium β aan het Gymnasium Bernrode te Heeswijk-Dinther in 1996, begon hij aan de opleiding Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen (heden Radboud Universiteit Nijmegen). Tijdens deze studie liep hij stages bij de afdelingen Toxicologie (Dr. Ir. PTJ Scheepers en Prof. Dr. RP Bos) en Gastro-enterologie (Dr. A Naber). Tevens behaalde hij de aantekening Proefdierdeskundige (ex. Art. 9 van de Wet op de Dierproeven). In augustus 2000 slaagde hij voor het doctoraal examen en in datzelfde jaar werd hij aangesteld als Junior Onderzoeker bij de afdeling Tumor immunologie van het UMC St. Radboud (Prof. Dr. CG Figdor). Onder leiding van Prof. Dr. GJ Adema en Dr. TJM Ruers van de afdeling Chirurgie begon hij aan het promotie onderzoek waarvan de resultaten nu voor u liggen. Tijdens zijn promotie nam hij deel aan diverse nationale en internationale congressen, waaronder het Keystone congres 'Basic aspects of tumorimmunology' in Amerika, waar hij zijn onderzoeksresultaten presenteerde.

Publications

Martijn H.M.G.M. den Brok, Roger P.M. Sutmuller, Robbert van der Voort, Erik J. Bennink, Carl G. Figdor, Theo J.M. Ruers, Gosse J. Adema. *In situ* tumor ablation creates an antigen source for the generation of anti-tumor immunity. *Cancer Research, 2004 Jun; 64(11):4024-4029*

Martijn H.M.G.M. den Brok, Stefan Nierkens, Carl G. Figdor, Theo J.M. Ruers, Gosse J. Adema. Dendritic cells: tools and targets for anti-tumor vaccination. *Expert Reviews In Vaccines, 2005 Oct; 4(5):699-710*

Roger P.M. Sutmuller, **Martijn H.M.G.M. den Brok**, Matthijs Kramer, Erik J. Bennink, Liza W.J Toonen, Bart-Jan Kullberg, Leo A. Joosten, Shizuo Akira, Mihai Netea, Gosse J. Adema. Toll-like receptor 2 controls expansion and function of regulatory T cells. *Journal of Clinical Investigation, 2006 Feb;116(2):485-494*

Martijn H.M.G.M. den Brok, Roger P.M. Sutmuller, Stefan Nierkens, Erik J. Bennink, Cathelijne Frielink, Liza W.J. Toonen, Otto C. Boerman, Carl G. Figdor, Theo J.M. Ruers, Gosse J. Adema. Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumor immunity. *Submitted for publication*

Martijn H.M.G.M. den Brok, Roger P.M. Sutmuller, Stefan Nierkens, Erik J. Bennink, Liza W.J. Toonen, Carl G. Figdor, Theo J.M. Ruers, Gosse J. Adema. Synergy between *in situ* tumor destruction and TLR9 stimulation results in a highly effective *in vivo* dendritic cell vaccine. *Submitted for publication*

Martijn H.M.G.M. den Brok, Theo J.M. Ruers, Gosse J. Adema. *In situ* tumor destruction: a perfect opportunity for the induction of anti-tumor immunity via dendritic cells. *In preparation*