Targeting the tumor-draining area

Marieke Herbert-Fransen 2012

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Local immunotherapy and its effect on the systemic T cell response

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Ramsey Nasr

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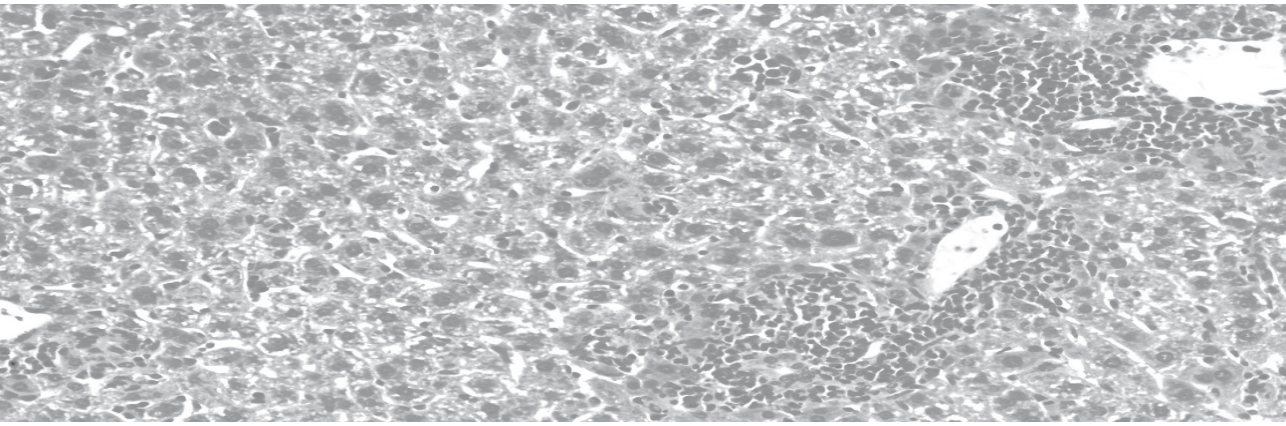
General introduction



General Introduction

Our immune system has evolved to protect us from disease and death caused by pathogens including bacteria and viruses. It comprises two separate but interacting compartments, the innate and the adaptive immune system. The innate immune response constitutes the first line of defense against pathogens that have broken through physical barriers such as the skin and mucosal layers. Innate immune cells, including macrophages, dendritic cells (DCs), as well as neutrophils, basophils, eosinophils and granulocytes harbor receptors (e.g. Toll like receptors) that recognize specific conserved patterns on pathogens known as PAMPs (pathogen associated molecular patterns). After recognition of PAMPs, the innate immune cells become activated which leads to enhanced phagocytosis of the pathogens by these cells and production of substances that can destroy the pathogens. In addition, immune signaling agents, such as cytokines and chemokines are secreted that cause inflammation and attraction of other immune cells. NK cells are rapidly responding innate immune cells, which cause destruction of virus-infected cells and tumor-cells by secreting granzymes and perforin that induce apoptosis. NK cells are regulated by an array of activating and inhibiting receptors on their surface (1). Compared to adaptive immune cells (i.e. B and T cells), innate immune cells have a limited repertoire of recognition and do not posses the ability to generate memory against pathogens, an exclusive characteristic of the adaptive immune system.

The adaptive immune system consists of B cells and T cells. B cells are responsible for the humoral response of the adaptive immune repertoire. Upon encountering cognate antigen, they divide from low numbers of precursor cells into large numbers. Subsequently, B cells either mature into memory B cells or into plasma cells, which secrete antibodies that can neutralize pathogens or tag them for destruction by the innate immune cells. The cellular component of the adaptive response consists of T cells. T cells are divided in $CD8^+$ T cells, cytotoxic T cells, and $CD4^+$ T cells, T helper cells and γδ T cells. T cell precursors originate in the bone-marrow and develop in the thymus from immature to mature T cells by T cell receptor (TCR) rearrangement and selection for specificity. In the thymus many epitopes (derived from self proteins) are presented by the individual's major histocompatibility (MHC) molecules, and T cells are selected based on affinity towards these MHC-presented epitopes. T cells with no affinity to the MHC/epitope complex die of neglect, because they are unable to recognize the basic structure of the MHC in which peptides are presented (lack of positive selection). T cells carrying T cell receptors with strong affinity for self-epitopes are eliminated, because they risk causing auto-immunity (negative selection). T cells with low affinity for self MHC-presented epitopes are allowed to expand

(positive selection) (2). After these selection processes the T cells leave the thymus and circulate in the periphery, residing mostly in secondary lymphoid tissue such as lymph nodes and spleen. This is where the naïve T cells will first encounter high affinity foreign epitopes.

Effective priming of T cells is dependent on 1. High affinity TCR engagement with MHC molecules presenting the cognate foreign epitope, 2. Interaction with costimulatory molecules such as CD80, CD86, and various TNF-(receptor) family molecules like CD40, OX40-L, 41BB-L and CD70, present on the cell surface of antigen presenting cells (APCs) such as DCs. TCR signals and costimulatory signals operate together in the immunological synapse to provide long lasting stimulation 3. Cytokines secreted by the APCs, mainly IL-2, IL-12 and IFN-alpha. The development of naive T cells into effector cells with different functions and kinetics, is controlled by the presence and strength of all three types of signals (TCR-, Costimulation-, Cytokine-mediated).

T lymphocytes

T cells are divided roughly in $CD8^+$ T cells, which recognize epitopes presented in MHC Class I molecules, and $CD4^+$ T cells, which recognize epitopes presented in MHC Class II molecules. $CD4^+$ T cell differentiation displays great plasticity, incorporating many subsets, some of which are definite, others retain the capacity to switch from one subset to another, according to present knowledge. There are at least 5 different categories known: T helper 1(Th_1), T helper 2 (Th_2), T helper 17 (Th_{17}), Regulatory T cells (T_{reg}), and Follicular helper T cells (T_{fh}). The subset distinction is based on the cytokine expression profile: IFN-gamma for Th_1, IL-4 and IL-5 for Th_2 and IL-17 for Th_{17}, or the transcription factor responsible for subset differentiation, T-bet for Th_1, Gata-3 for Th_2, Ror-γt for Th_{17}, FoxP3 for T_{reg} and Bcl6 for T_{fh} cells (3, 4). The subsets have different roles in the immune system. Th_1 cells activate DCs via CD40-CD40L interaction, effectively licensing the DC to prime $CD8^+$ T cells into CTLs (5, 6) and provide help to $CD8^+$ T cells via cytokine production. T_{reg} cells inhibit T cell responses against self-antigens, thereby keeping auto-immunity at bay. Both Th_2 and T_{fh} cells are involved in the activation of B cells, and Th_2 cells also are important in regulating the innate immune response against parasites.

$CD8^+$ T cells appear to be less heterogeneous but are also found to secrete various cytokines and various stages of activation and development, from naïve to full effector CTLs and memory cells, are found (7). Several reports describe the existence of regulatory $CD8^+$ T cells, but further studies are required in order to elucidate their precise physiological role.

Antigen presentation/DC

Antigens are generally proteins and sometimes carbohydrates nucleic acids or lipopeptides and can be derived from self molecules, pathogens and/or other non-self materials. Antigen is taken up by professional antigen-presenting cells (APCs); B-cells, DCs and macrophages, and processed for presentation. Material that is taken up can be either processed in endosomal compartments and loaded directly onto MHC II molecules, or proteins can be further cleaved by the proteasome into fragments which are then transported into the ER by the TAP transporter, where they can be loaded onto the MHC I molecule. Furthermore, material taken up can be stored in compartments that facilitate antigen supply to MHC Class I for several days (8). Peptides presented in MHC I molecules are either derived from the biosynthetic pathway, or in the case of DCs also from exogenously ingested proteins, a process referred to as cross-presentation (9). Cross-presentation is important in the priming of tumor-specific $CD8^+$ T cells, by APCs that have taken up necrotic tumor cell material and present it in MHC I molecules, either in the tumor or the tumor-draining LN (10). Peptides presented in MHC II molecules, present mainly on APCs and B cells, are generally derived from antigen that has been taken up by the APCs and becomes processed via the socalled endosomal route (11).

Lymph nodes

The lymph node (LN) plays a pivotal role in the immune system. Located at strategic places in the body, lymph nodes are the meeting point for different immune cells and create the architectural conditions for a productive primary or secondary immune response following first, respectively additional encounter with antigen. APCs arrive in the lymph node with the afferent lymph, while the majority of lymphocytes enter the LN from the bloodstream via high endothelial venules (12). Once in the T cell area or the B cell follicle DCs display, based on information received in the periphery, antigens in the context that instructs the lymphocytes to differentiate into adequate effector cells that are required for the situation at hand. Not only APCs appear in the LN through the afferent lymph, the interstitial fluid also drains to the LN into the subcapsular sinus. From here, the highly specialized conduit system distributes the fluid containing only molecules smaller then 70 kD to the B cell follicles and the T cell zone in the paracortex, where they can be taken up and presented by resident APCs (13, 14). An abundant influx of activated DCs, and danger signals, such as pro-inflammatory cytokines, heat shock proteins or uric acid can cause the lymph node to become reactive, swelling up to several times its original size, due to additional influx from both DCs via the afferent lymph and lymphocytes via the bloodstream, and reduced egress of lymphocytes from the LN (15-17). This

process accelerates the normal kinetics of APCs and lymphocyte interactions and provides a pro-inflammatory environment.

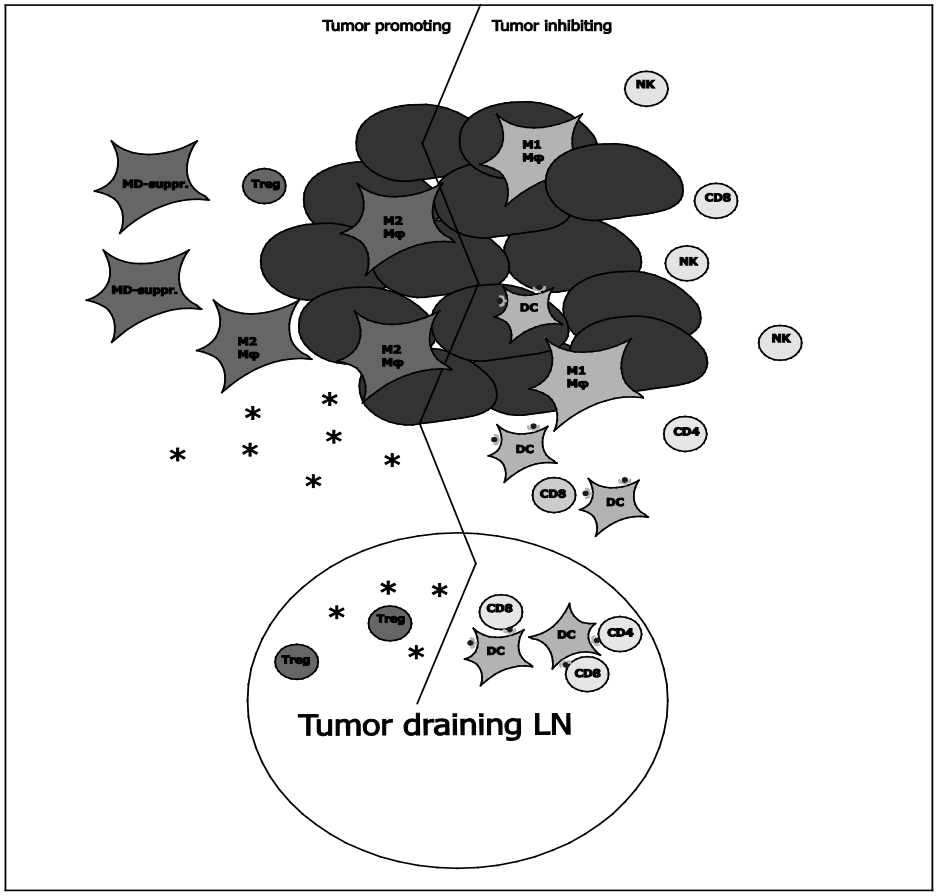


Figure 1: Schematic overview of immunological processes within the tumor microenvironment and tumor-draining LN. Within the tumor microenvironment and tumor-draining lymph nodes immune cells and processes are active, with opposing effects. Tumor promoting inflammation consists of cells like M2-macrophages (M2-Mφ), myeloid-derived suppressive cells (MD-suppr.) and T_{reg} cells and soluble factors like TGF-ß, IL-6 and VEGF. Tumor inhibiting immunity consists of cells like NK cells, M1-macrophages (M1- Mφ), $CD8^+$ T cells, $CD4^+$ T cells, and dendritic cells (DC).

Cancer immune surveillance

In 1891 the concept of cancer immune surveillance was first postulated, by William Coley, who described the ability of the immune system to recognize and possibly kill tumor cells (18). This notion was generally overlooked until Burnet revived it in the 1960's (19). He elaborated on the idea that the immune system is keeping emerging tumor cells in check, and is capable of preventing malignant cells from growing out. Not until decades later data from experimental animal models and descriptive studies in patients were published that started to

elucidate the molecular and cellular basis behind this theory, as reviewed by Swann and Smyth (20). In recent years significant numbers of T cells specific to tumor associated antigens have been identified in cancer patients, and several studies have correlated pro-inflammatory immune infiltration with improved prognosis (21, 22). The concept of immune surveillance was extended by Dunn et al. who described the phenomenon called immuno-editing; immunological pressure put upon malignant cells by lymphocytes ultimately causing the development of tumor cells capable of evading the immune system (23, 24). This evasion can be shaped by several different mechanisms, including MHC Class I downregulation, antigenic drift, secretion of immune suppressive agents, such as TGF-beta, IDO or IL-10, and attraction and expansion of regulatory T cells. Many immunotherapy strategies have been studied to intervene with these processes, with varying success rates.

Tumor immunotherapy

Modulating the immune system in order to clear tumors and metastases has been the goal of extensive studies in the past few decades. Strategies are generally based on either enhancing the tumor specific T cell repertoire, or blocking tumor induced immune suppression. Cancer immunotherapies generally employ one of four different methods; direct targeting of tumor-associated antigens by monoclonal antibodies, vaccination to drive effector T cell responses to tumor-associated antigens, adoptive transfer of tumor-specific T or NK cells or immune-modulating monoclonal antibodies.

Vaccination has long been used to strengthen the immune response against pathogens, and is now being used to boost anti-tumor T cell or B cell responses in various different forms. Among the most promising for tumor immune therapy are vaccinations with synthetic long peptides, and DCs loaded with tumor antigen (25-32). Each approach is aimed at expanding the tumor specific T cell repertoire and redirecting existing tumor specific T cells into pro-inflammatory effector cells.

Adoptive transfer strategies have employed tumor specific T cells present in tumor tissue or peripheral blood by expanding them in vitro and infuse them back into the patients. This process is laborious and costly; however, some promising results have been obtained, proving the potential of tumor-specific T-cells (33-36). New techniques, designed to increase the number of T cells recognizing tumor antigens by introducing T cell receptors through gene transfer are hopeful, and are currently being investigated for their potential to be used in the clinic (37, 38).

Monoclonal antibodies are now established as targeted therapies for several diseases including malignancies. Some antibodies target specifically tumor

cells, such as Herceptin and Rituximab, and tag thereby the tumor cells for destruction, leading to enhanced antigen presentation, which indirectly also enhances the anti-tumor immune response (39-41). Additionally, a plethora of immune-modulating antibodies is available, which can all be used to stimulate the anti-tumor immune response. Certain antibodies activate the $CD8^+$ T cell, such as 4-1BB agonists, and others activate DCs presenting tumor antigen, such as CD40 agonists, or block inhibitory signals for T cells, like PD-1 and CTLA-4 blocking antibodies (40-46). The use of monoclonal antibodies is expected to grow in the next few decades, with more molecules being investigated for their role in therapeutic settings.

Outline of this thesis

This dissertation deals with the role of local immune stimulation in the lymph node and tumor microenvironment and its effect on systemic $CD8^+$ T cell responses, in particular the anti-tumor $CD8^+$ T cell responses.

In **chapter 2** the use of a slow-release system is described to deliver the immune-activating agonistic CD40 antibody to the tumor-draining area, and the advantages of this method over systemic administration of the antibody. The local, slow-release administration was very effective in activating a systemic anti-tumor effector $CD8^+$ T cell response, to such an extent that a tenfold lower dose of antibody could be used without loss of efficacy. Adverse side-effects, analyzed by organ histology and liver enzymes in the blood, were much lower upon local anti-CD40 antibody delivery compared to systemic administration. The local delivery of anti-CD40 antibody resulted in a systemic anti-tumor $CD8^+$ T cell response, capable of clearing distant tumors expressing identical tumor antigens.

Chapter 3 shows that slow-release local administration of CTLA-4 blocking antibody can also activate a tumor-specific $CD8^+$ T cell response and cause tumor regression, while lowering systemic adverse side-effect as compared to systemic administration. CTLA-4 blocking antibody is being widely used in clinical trials, and its use has been complicated by induction of auto-immune disease. Here we show that using a local low dose injection of CTLA-4 blocking antibody in a slow-release formulation is equally effective in activating a tumor-specific $CD8^+$ T cell response, capable of eradicating tumor cells as systemic high dose treatment.

The influence of local lymph node activation on systemic T cell responses is further analyzed in **chapter 4**. $CD8^+$ T cell priming generally occurs in a locally inflamed lymph node, called a reactive LN, due to the presence of pathogens. The role of the inflammatory milieu on the priming and fate of $CD8^+$ T cells was studied by separating the TCR-MHC interaction from the inflammatory cues, by priming briefly in vitro followed by transfer to mice with or without a CpG-induced

reactive lymph node. The primary $CD8^+$ T cell response was not influenced by the presence of a reactive lymph node, however, after a boost vaccination in the memory phase, $CD8^+$ T cells primed in the presence of a reactive LN displayed a strong quantitative advantage over control $CD8^+$ T cells. The reactive LN, which remained swollen with enhanced cellularity for a pronounced period of time, was envisaged to act as a shelter for CD8+ T cells while undergoing contraction after the primary response.

In **chapter 5**, the advantages and disadvantages of the use of dextran-based microparticles as slow-release system for the delivery of immune-activating antibodies such as agonistic CD40 in the tumor-draining area are described. Dextran-based microparticles can be tailored to release antibodies in desired pharmacokinetics, leading to an even further decrease of adverse side-effects, as compared to previously described Montanide-ISA 51. However, dextran-based particles were unexpectedly found to have a stimulating effect on tumor-outgrowth. This effect coincided with the appearance of large, ulcerated swellings at the site of injection.

In **chapter 6**, the issues presented in this thesis are discussed. The knowledge gained in the work shown here, compared with and strengthened by related published work, is used to state the opinion that targeting the tumor-draining lymph node and/or tumor microenvironment for immune-activating therapy against tumors must be seriously considered.

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Chapter 2

2

Local activation of CD8 T cells and systemic tumor eradication without toxicity via slow release and local delivery of agonistic CD40 antibody.

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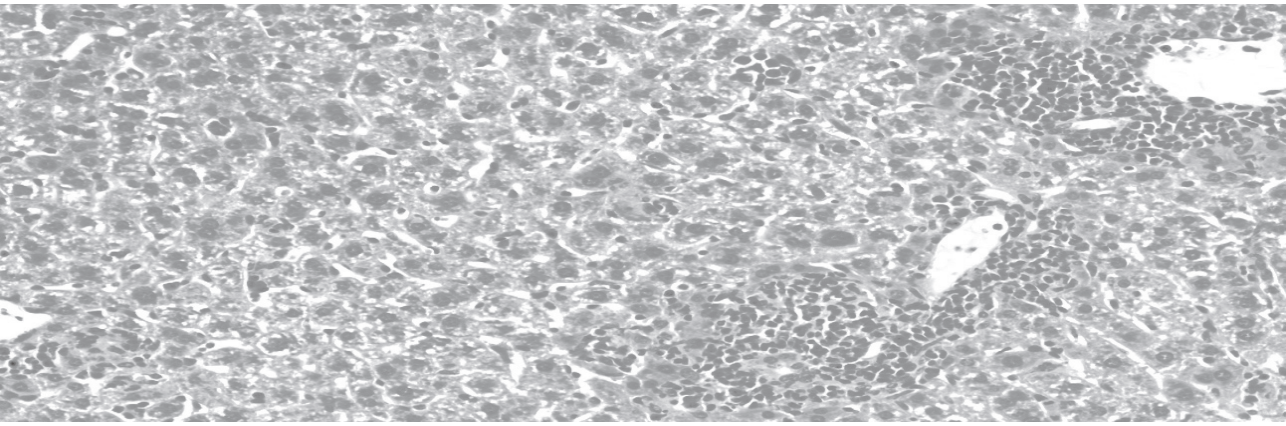

Abstract:
Immune-therapy against tumors using anti-CD40 agonistic antibodies has been extensively studied in pre-clinical animal models and recently also in clinical trials. Although promising results have been obtained, antibody-related toxicity has been a limiting factor. We reasoned that strict local activation of tumor-specific CD8 T cells through stimulation of CD40 on the dendritic cells in the tumor area while excluding systemic stimulation might be sufficient for effective tumor eradication and can limit systemic toxicity.
Experimental design:
Pre-clinical in vivo models for immunogenic tumors were used to investigate the potential of delivering a non-toxic dose of agonistic anti-CD40 antibody to the tumor region, including draining lymph node, in a slow-release formulation (Montanide).
Results:
The delivery of anti-CD40 monoclonal antibody, formulated in slow release Montanide ISA-51, reprograms CTLs by inducing local but not systemic dendritic cell activation, resulting in effective tumor-specific CTL responses, which eradicate local and distant tumors. Adverse side-effects, assayed by organ histology and liver enzymes in the blood, were much lower upon local anti-CD40 antibody delivery as compared to systemic administration. The local delivery of anti-CD40 antibody activates only CTLs against antigens presented in the tumor-draining area, because unrelated distant tumors expressing different tumor antigens were not eradicated.
Conclusions:
These results establish a novel therapeutic principle that local delivery and slow release of agonistic anti-CD40 antibody to the tumor-draining area effectively activates local tumor-specific CD8 T cells to become systemic effectors without causing systemic toxicity or non-specific CTL activation. These findings have important implications for the use of anti-CD40 therapies in patients.

Statement of translational relevance:
Systemic delivery of agonistic anti-CD40 antibodies induces good anti-tumor immune responses in pre-clinical models but dose-limiting toxicity hampers clinical success. We have used a novel delivery system based on the slow-release agent Montanide ISA-51 to distribute agonistic CD40 antibody in the lymphoid drainage area of the tumor, which stimulates local but not systemic dendritic cells. Local dendritic cell activation results in a robust systemic anti-tumor CD8 T-cell response and both local and distant tumor eradication without the side effects associated with the standard systemic administration of anti-CD40

antibody. These results indicate an important novel delivery platform for the use of anti-CD40 antibody and conceivably other immune stimulatory therapies in cancer patients.

2

Introduction

CD8+ T cells (cytotoxic T lymphocytes (CTLs)) recognize and kill specific target cells based on their T cell receptors (TCRs) that are selected to recognize antigens presented by MHC class I molecules [1]. Since many tumors express aberrant antigens, CD8+ T cells have the potential to eradicate these tumors [2-4]. The induction of anti-tumor T-cell priming, however, is often ineffective. One of the reasons for this phenomenon is that dendritic cells (DCs), which cross-present the tumor antigens in tumor-draining lymph nodes are poorly activated due to a lack of danger signals and insufficient CD4+ T-cell help [5-8]. Methods to effectively activate those DCs and thereby enhance the anti-tumor CTL response may thus provide significant improvement for the treatment of cancer.

The tumor necrosis factor receptor (TNFR) family member CD40 is a stimulatory molecule and constitutively expressed on a large variety of cells, including DCs, B cells, macrophages, and endothelial cells [9]. Engagement of CD40 on DCs provides potent maturation signals leading to improved T-cell mediated tumor rejection [10, 11]. Several in vivo studies have shown that CD40 ligation by systemically delivered agonistic antibodies can induce robust anti-tumor immune responses, either as monotherapy or in combination with TLR ligands, cytokines and chemotherapy, indicating clinical potential. [12-17]. However, serious side effects of treatment with agonistic CD40 antibodies in the clinic have been reported, which include cytokine release syndrome and liver function abnormalities [18]. Adverse effects have also been reported for several other therapies that are based on antibodies that mediate systemic immune activation, such as CTLA-4 blocking antibodies and agonistic anti-CD28 antibodies [18-21]. Previously, we have shown that tumor antigens are predominantly presented by DCs in the tumor-draining lymph node, which results in detectable amounts of tumor-specific CD8+ T cells trapped within that lymph node [22, 23]. We hypothesize that local tumor antigen presenting DCs are the main target for agonistic anti-CD40 antibodies. To investigate the possibility to more specifically target the antibody treatment to the tumor-draining area and prevent systemic immune activation and toxicity, we investigated in the present study the utilization of local antibody injection in a slow release formulation. We found that local injection of agonistic CD40 antibody is more effective in enhancing the anti-tumor response than a similar dose given systemically and that a slow-release formulation is attractive to use in clinical settings since it resulted in substantial decrease in toxicity compared to systemic delivery. Importantly, although the treatment is strictly local, the tumor-specific T cells spread systemically and eradicate both local and distant related tumors.

Material and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. C57BL/6 Kh (B6, H-2^b) were bred at the LUMC animal facility. The experiments were approved by the animal experimental committee of the University of Leiden.

Tumor experiments

Mouse embryonic cells transformed by the early region 1A of human adenovirus type 5 (Ad5E1A) plus EJ-ras (AR6) [24] were cultured in Iscove's modified Dulbecco's medium (IMDM): (BioWhittaker, Verviers, Belgium) supplemented with 4% FCS, 50 µM 2-mercaptoethanol and 100 IU/ml penicillin/streptavidin. EG7 tumor cells expressing the full-length OVA antigen were cultured in IMDM (Invitrogen Life Technologies, Rockville, MD) supplemented with 8% v/v FBS (Greiner), 50 µM 2-ME, 2 mM glutamine, 100 IU/mL penicillin (complete medium) supplemented with 400 µg/mL G418 (Gibco) [25]. AR6 tumor cells do not express CD40 and EG7 tumor cells express low levels of CD40 on their cell surface.

The AR6 (E1A expressing) tumor cells (7.5 x 10^6) were injected s.c. into 7-13 week-old male mice in 200 µl of PBS. Treatment was started 8-14 days after tumor inoculation, when palpable tumors were present. EG7 tumor cells (1 x 10^6) were injected s.c. into 7-13 week old male mice in 200 µl of PBS. Treatment was started 3 days later. Secondary tumors were injected one day before start of treatment. Tumor size was measured twice weekly in three dimensions and mice were killed when tumor size exceeded 1 cm^3.

Flow cytometry

Single-cell suspension of blood and spleens, after erythrocyte lysis, and lymph nodes were stained with anti-CD8a (clone 53-6.7), anti-CD62L (clone MEL14), anti-CD11c (clone HL3), anti-CD70 (clone FR70), CD90.1 (Thy1.1; clone OX-7), CD19 (clone 1D3) and $CD3_\varepsilon$ (clone 145-2C11) all from BD Bioscience, anti-KLRG1 (clone 2F1) from Southern Biotech, and $E1A_{234\text{-}243}$-loaded H-$2D^b$ tetramers and $OVA_{257\text{-}264}$–loaded H-$2K^b$ tetramers.

Agonistic CD40 antibody treatment

The FGK-45 hybridoma cells producing an agonistic anti-CD40 Ab were provided by A. Rolink (Basel Institute for Immunology, Basel, Switzerland) [26]. Hybridomas were cultured in Protein Free Hybridoma Medium (Gibco), and mAbs were purified using a Protein G column. FGK-45 antibody contained <2 IU/mg endotoxin. Mice treated with high dose, systemic treatment were injected with 100 micrograms of antibody in PBS intravenously, on three consecutive

days. Montanide/antibody emulsions were made by mixing different dosages of antibody in PBS 1:1 with montanide (Montanide ISA-51, Seppic), and vortexing for 30 minutes.

Serum analyses

Serum samples were taken from mice at several time points after treatment. ALAT and ASAT analyses were performed by the department of Clinical Chemistry of the LUMC hospital according to standard protocols. Anti-CD40 antibody concentrations were analyzed by ELISA with anti-rat antibodies (BD bioscience).

Histology

Liver, lung and kidney were isolated from mice at several time points after treatment. Tissues were fixed with formalin and embedded in paraffin, 4 μm sections were made and sections were stained with H&E staining. Images were captured using a Zeiss Axioskop 40 microscope and processed using axiovision AC software.

In vivo cytotoxicity assay

In vivo cytotoxicity was determined using as target cells Thy1.1^{+} splenocytes. Target cells were labeled with 5 μM CFSE and pulsed with $E1A_{234\text{-}243}$, SGPSNTPPEI peptide (0.5 μg/mL for 90 min at 37°C) or labeled with 0.5 μM CFSE and pulsed with control peptide. Target cells were mixed in a 1:1 ratio of E1A-pulsed to control cells and injected i.v. (5×10^6 cells of each population) into naïve and tumor-bearing mice, 9 days after being treated with low dose anti-CD40 in montanide, high dose systemic anti-CD40 or no treatment. Three days after injection of the target cells, spleens were isolated and the number of CFSE^{hi} and control CFSE^{lo} Thy1.1^{+} target cells was determined by flow cytometry. The percentage of specific killing is calculated as follows: [1 - (ratio tumorbearing/ ratio naive)] × 100%. Ratio is defined as the number of E1A CFSE^{hi} target cells/ number of control CFSE^{lo} target cells.

Results:

Local treatment with a low dose of agonistic anti-CD40 antibody in a slow-release formulation combines effective treatment and decreased toxicity. In order to determine the most optimal anti-tumor treatment with agonistic anti-CD40 antibodies, we compared the effects of different administration methods and antibody dosage on both tumor eradication efficacy and toxicity in organs such as liver. Mice were inoculated subcutaneously with adenovirus protein E1A expressing tumor cells (AR6), previously described to be eradicated by $CD8^+$ T-cells [24], which grew into palpable tumors over 10 days. Subsequently, these tumor-bearing mice were treated with 1) a standard dose (100 ug) of agonistic anti-CD40 antibody delivered systemically during three consecutive days, 2) a low dose (30 ug) delivered systemically (i.v.), 3) a low dose delivered locally (s.c.) in saline, or 4) a low dose delivered locally in a slow-release formulation (Montanide-ISA-51) [27]. The subcutaneous treatments were injected in the area between the tumor and the tumor-draining inguinal lymph node. As is shown in figure 1A, mice receiving local treatment either in Montanide or in saline displayed an equal percentage of tumor eradication and survival and both of these treatments are equally effective in clearing the tumor as the standard systemic i.v. treatment. The low dose i.v. injected group, however, showed no evidence of tumor growth reduction, and as a consequence most mice in this group did not survive. These results indicate that a low dose of anti-CD40 can be equally effective as a high dose to eradicate tumors provided that it is injected closely to the tumor and/or tumor-draining lymph node.

We assessed the toxicity caused by the aforementioned treatments by analyzing the liver enzymes ALAT and ASAT, known to be indicative for tissue damage [28], in serum one day after antibody treatment. The levels of these liver enzymes were substantially higher in the group treated with systemically administered CD40 antibody as compared to local treatments. Remarkably, toxic effects of local treatment were further decreased to base-line levels when CD40 antibody was administered in the slow release (Montanide) formulation (figure 1B). This correlated with concentration of anti-CD40 antibody found in the serum of these mice one day after treatment (Supplemental Figure 1).

Comparison of the kinetics of the ALAT and ASAT levels between the low-dose plus slow-release treatment and the standard systemic high-dose treatment, showed that these liver enzymes were elevated especially during the first days after systemic treatment and suggest that tissue damage follows (Figure 1C). The administration of a high dose (150 μg) of anti-CD40 antibody in a slow release formulation causes also liver inflammation as determined by liver enzyme elevation (data not shown). Histological analysis performed at several time-points after treatment, showed that indeed damage to the liver was evident

(Figure 1D). At day 3 post-treatment, the livers from mice that received systemic high-dose anti-CD40 treatment were severely affected. Livers displayed lobular foci with a minor component of portal inflammation. The lobular foci of inflammation consisted of lymphocytes (predominantly $CD3^+$), plasma cells and histiocytes (including $F4/80^+$ macrophages) (data not shown). At day 7 post-treatment, lobular inflammations had mostly subsided but portal inflammation (with lymphocytes) was enhanced.

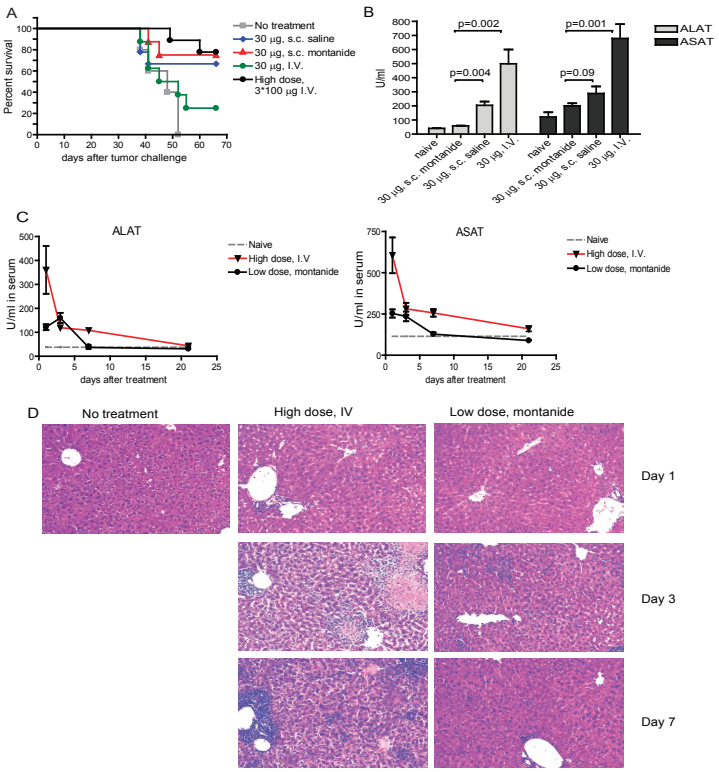

Figure 1: **Low dose local treatment with anti-CD40 gives lower toxicity then high dose systemic treatment.**

Tumor-bearing mice were treated with different dosages and administration methods of anti-CD40 agonist antibody, 8 days after tumor inoculation. Tumor-survival after administration of 30 µg of antibody either intravenously, subcutaneously in saline or in montanide was compared to the standard protocol of 3 subsequent high dose systemic injection of antibody. 8 mice per group, representative of 3 experiments (A). Liver enzyme concentrations in serum, 24 hours after treatment with different administration methods of 30 µg of antibody. Student T-test revealed significant differences between groups. (IV vs montanide p= 0.002 and 0.001 for ALAT and ASAT respectively, montanide vs saline p=0.004 and 0.09 for ALAT and ASAT respectively) 4 mice per group, representative of 2 experiments (B). Liver enzymes in serum in time after treatment with 30 µg of antibody subcutaneously in montanide, compared to standard protocol. 7 mice per group, representative of two experiments (C). Histological sections of liver at day 1, 3 and 7 after treatment with 30 µg of antibody subcutaneously in montanide, compared to standard protocol. Representative sections of groups of 3 mice (D).

In contrast, livers from mice receiving a low dose anti-CD40 antibody in Montanide displayed much less extensive signs of liver damage on day 3 post-

treatment and on day 7 only remnant signs of inflammation were visible. The lungs and kidneys in mice that were treated with a high dose systemic anti-CD40 also showed more severe damage, as evidenced by lymphocytic infiltration, compared to these organs in mice that had received low dose local treatment (data not shown). Together these data show that a lower dose and local subcutaneous injection of CD40 agonistic antibody is sufficient for tumor eradication and that this treatment, if applied in a slow-release formulation, caused the least immune-mediated toxicity.

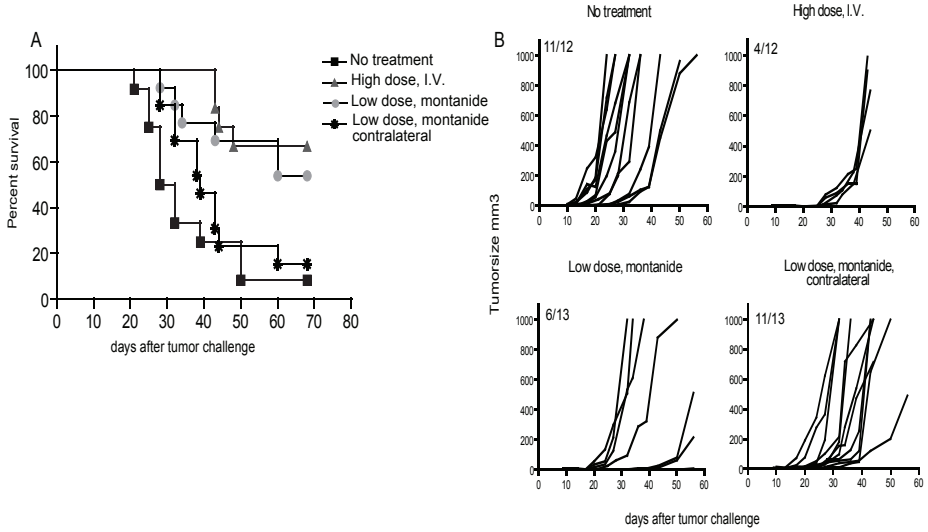

Figure 2 **Low dose local treatment gives similar anti-tumor response as standard treatment, and is a strictly local treatment.**
Survival and tumor-growth of tumor-bearing mice after treatment with 30 µg of anti-CD40 agonist antibody in montanide, subcutaneously compared to standard protocol, and compared to injection into the contralateral flank, 8 days after tumor inoculation. Data presented as cumulative survival from two independent experiments, 12 or 13 mice per group. Kaplan-Meier test revealed a significant difference between local treatment and no treatment, and local treatment and contralateral treatment (p=0.002 and p=0.03 respectively) (A). Data presented as tumor-growth in each mouse, 6 or 7 mice per group, number of mice that died of tumor-burden indicated in upper left-hand corner. Representative of three experiments (B).

Local delivery of low-dose anti-CD40 in Montanide is essential for therapeutic efficacy.

To examine whether a low dose anti-CD40 therapy in a slow-release formulation (Montanide) is only effective to induce an anti-tumor response when delivered close to the tumor, we tested whether contralateral injection could also induce tumor eradication. As is shown in Figure 2, tumor growth and survival of mice treated with low dose anti-CD40 in Montanide close to the tumor was comparable to those of mice treated with high dose systemic anti-CD40. Mice treated with low dose Montanide- formulated antibody in the contralateral flank, however, did not eradicate the tumor (Figure 2 A, B). When tumor-bearing mice were treated

with a high dose of anti-CD40 in Montanide (150 microgram), either close to the tumor, or in the opposite flank (contralateral flank) the anti-tumor effects were similar, indicating that only a low dose is confined in its therapeutic action to the tumor-draining area (Supplemental Figure 2A). Mice locally injected with a Montanide depot lacking anti-CD40 antibody, did not show any anti-tumor effect (Supplemental Figure 2B). These data demonstrate that the therapeutic effect of low dose agonistic CD40 antibody treatment in Montanide was strictly dependent on local delivery in the vicinity of the tumor and/or tumor draining lymph node

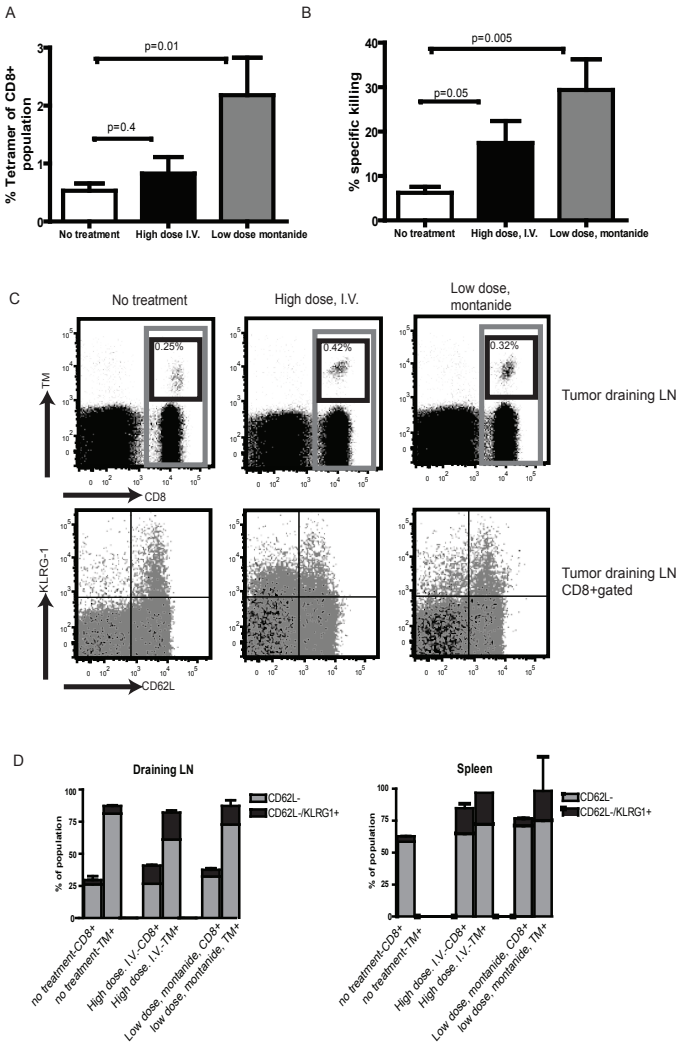

Figure 3 **Systemic CTL response after local, low dose, montanide treatment with anti-CD40 antibody.** Quantity and functionality of the anti-tumor CTL response in blood after local, low dose, montanide treatment with anti-CD40 agonist antibody, compared to standard treatment. 8 days after start of treatment, CD8+, tetramer+ T-cells were analyzed in blood. Student T-test revealed significant differences between no treatment and low dose, montanide treatment groups (p= 0.04). (A) Nine days after start of treatment specific lysis was determined. Mann-whitney test revealed significant differences between no treatment and low dose, montanide treatment groups (p<0.0001). Two experiments pooled, 9 mice per group. (B) Phenotypic analyses of tumor-specific CTLs in tumor-draining lymph nodes and spleen, 10 days after start of treatment. Mice were sacrificed and CD8+, tetramer+ cells were analyzed in tumor-draining LN and spleen for CD62L and KLRG-1 expression. Upper panels display representative samples of CD8 and tetramer staining of tumor-draining lymph nodes. Lower panels show CD62L and KLRG-1 expression of CD8+ cells (in grey) and CD8+, tetramer+ cells (in black) in tumor-draining lymph nodes (C). Bar graphs indicate mean and SEM of CD62L low (grey) and CD62L low/KLRG-1 high cells (black) of CD8+ and tetramer+ cells in tumor-draining lymph node (left panel) and spleen (right panel) of 4 mice per group (D). One representative experiment of two.

Local treatment with low dose CD40 agonist causes systemic CTL responses equal to high dose systemic treatment

The induction of anti-tumor eradication by local treatment with anti-CD40 in the vicinity of a tumor could be explained by the activation of local CD8 T cells to become systemic effector CTL. In order to examine this possibility, we analyzed tumor-specific $CD8^+$ T cells in secondary lymphoid organs of treated and untreated mice. The anti-tumor CTL response was clearly detectable systemically in blood at day 8 post-treatment with both the standard high dose i.v. protocol and the locally administered low dose treatment in Montanide, in comparison to non-treated mice, indicating that the local treatment is capable to induce a potent systemic CD8 T cell response (Figure 3A). In order to determine the functionality of these tetramer-positive CTLs, we performed an in vivo cytotoxicity assay. Specific lysis of target cells in vivo correlated with the presence of tumor-specific (tetramer+) $CD8^+$ T cells in blood. Both treated groups of mice specifically killed target cells loaded with the tumor antigen to a similar extent, in contrast to the non-treated group (Figure 3B).

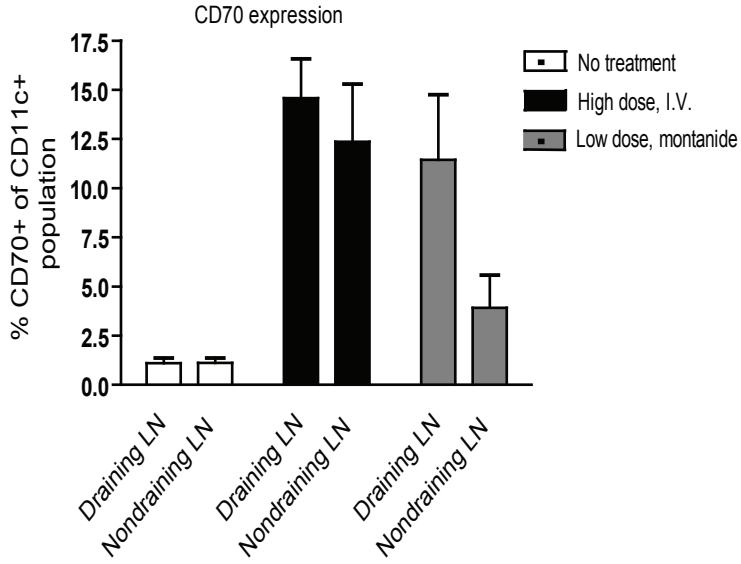

Figure 4 **DC activation in tumor-draining lymph node after local treatment**
CD70 expression on CD11c high cells in tumor-draining and non-draining lymph nodes of tumor-bearing mice, non-treated, high systemic dose treated of low dose, montanide treated. 4 mice per group, one representative experiment of two.

We next investigated phenotypic effects of these treatments on the cell-surface of tumor-specific CTLs in spleen and lymph node. By examining effector T cell markers CD62L and KLRG-1 on tetramer+ T cells, we could determine whether the different administration routes activated tumor-specific T-cells in a qualitatively different manner. The down-modulation of the homing receptor CD62L and the up-regulation of the Killer cell lectin-like receptor G1 (KLRG-1, marker for effector cells) were similar in tumor-draining lymph nodes and

spleens after either high dose systemic or low dose local treatments (Figure 3C, D). In non-treated mice, however, the KLRG1 expression on tetramer-positive $CD8^+$ T cells was lower in the draining lymph nodes. The tumor-specific CTL response in this group could not be detected systemically in blood and spleen. To determine the effects of CD40-mediated DC maturation in tumor draining versus non-draining lymph nodes of tumor-bearing mice, we measured the expression of the TNF ligand family member CD70 on the cell surface of DCs [29-31]. In mice that were treated intravenously, DCs in both tumor-draining and non-draining lymph nodes showed high expression of cell surface CD70 which indicates systemic activation of DCs (Figure 4). In contrast, the CD70 expression on the cell-surface of DCs in mice that received local treatment in Montanide formulation was strongly up-regulated in tumor-draining but not non-draining lymph nodes. Similar results were obtained with staining of the costimulatory molecule CD80 (B7.1). Treating mice with Montanide alone or Montanide containing control antibody did not mature DC in the LN (data not shown). Thus, predominantly the DCs in the draining lymph node (LN) of mice treated with the low dose of anti-CD40 in Montanide are activated, whereas the DCs in lymphoid organs distant from the tumor-draining area remain immature.

Local treatment can eradicate a distant tumor.

We hypothesized that even though an antibody treatment is delivered locally, it could still be effective in eradicating metastasized (secondary) related (presenting the same tumor antigens) tumors due to the induction of a systemic CTL response. To test this hypothesis, we used a model for metastasis in which we inoculated groups of mice subcutaneously with tumor cells in the right flank (first tumor), except for one group which was not inoculated. Eight days later, when tumors in the right flank were palpable, we inoculated all groups subcutaneously with tumor cells in the left flank (second tumor). Mice were treated the next day, either with high dose anti-CD40 antibodies i.v., or with low-dose anti-CD40 antibody in Montanide either injected locally close to the palpable primary tumor in the right flank or injected in the right flank where no tumor cells were inoculated. Tumor growth of tumors on both flanks was measured regularly and the anti-tumor CTL response was analyzed in blood. As depicted in Figure 5, tumor-specific $CD8^+$ T-cells were observed in the blood of the systemically treated group as well as in the blood of the group that was treated locally near the right flank tumor. Tumor-specific CTLs were not demonstrable in the blood of mice that were treated subcutaneously in the right flank and were not inoculated with tumor cells at this site. Consistent with these findings, the growth of the secondary tumor on the left flank is strongly inhibited in the groups treated with systemic high-dose anti-CD40 antibody and in the group treated with the low-dose of anti-CD40 antibody in Montanide, which was administered

close to the tumor in the right flank (Figure 5C,D; groups II and III). Mice that received no primary tumor and were injected with the low-dose anti-CD40 in the right flank succumbed to rapid outgrowth of the secondary tumor (Figure 5B, C; group IV). Taken together, these studies show that local treatment with anti-CD40 antibody in a slow-release formulation is effective in prompting systemic tumor-specific CTL expansion and eradication of distant related tumors.

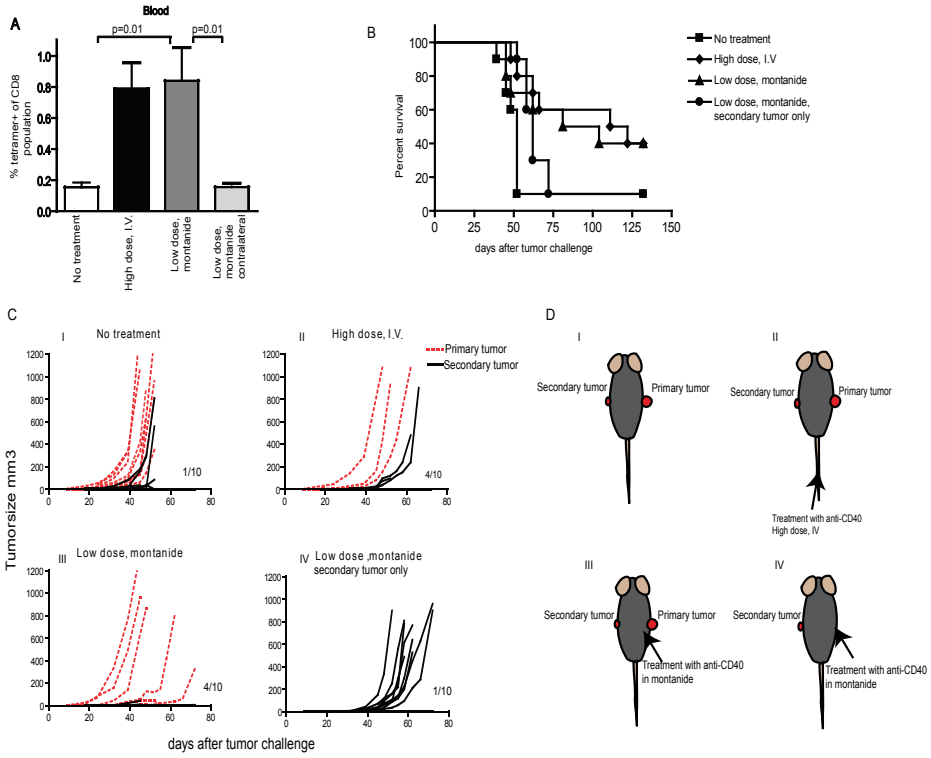

Figure 5 **Local treatment can eradicate a distant tumor.**
Eradication of secondary tumor by tumor-specific CTLs activated by local treatment with low dose anti-CD40 in montanide, compared to standard treatment. Data are presented as cumulative survival (10 mice per group), representative experiment of two. Kaplan-Meier test reveals differences between no treatment group and low dose, montanide group (p= 0.05) and between no treatment and high dose IV (p= 0.05) and between low dose, montanide and low dose, montanide contralateral (p= 0.1). Tumor growth was measured and data are presented for each independent mouse, primary (red dotted line) and secondary tumor (black line). Between brackets is noted how many mice were tumor free at the end of the experiment. (C). Tumor inoculation and treatment scheme (D)

Local treatment of primary tumors is capable of eradicating distant secondary tumors, but not unrelated tumors.

In order to substantiate that local treatment activates exclusively specific anti-tumor CTL responses that eradicate related (but not unrelated) distant tumors, thereby avoiding systemic T cell activation which may cause unwanted side effects such as

immune pathology and auto-immunity, we decided to employ a combination of two unrelated tumors (AR6, mouse embryonic cell line expressing adenovirus protein E1A and EG7 lymphoma expressing OVA protein) in one experimental model. By utilizing these tumor cells as first and/or second tumors, we were able to investigate the tumor-clearing potential of CTLs activated by local anti-CD40 treatment and the role of tumor-antigens presented at the local injection site (see scheme in Figure 6).

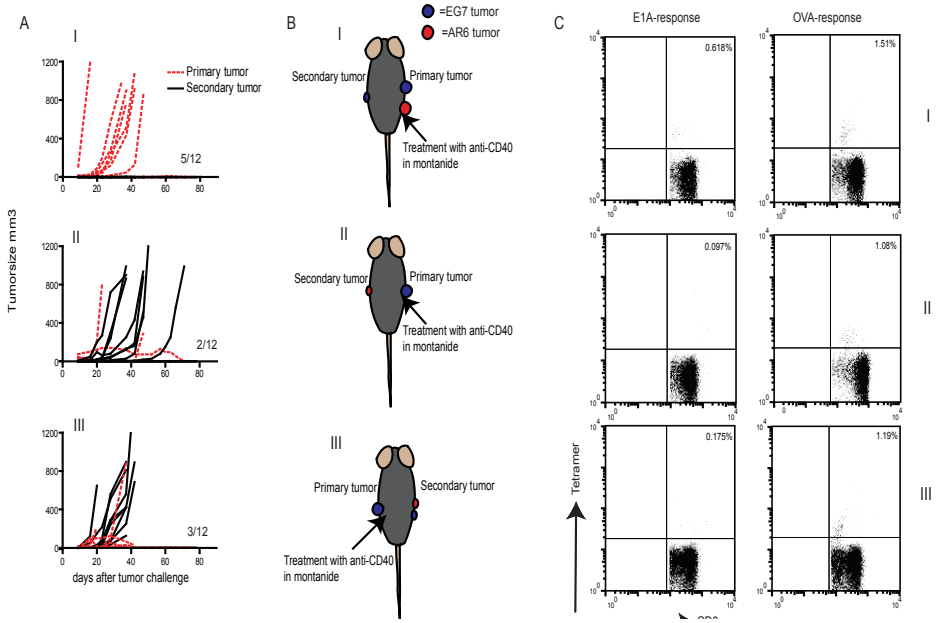

Figure 6 **Tumor-eradicating response is activated by locally available tumor-antigen.**
Eradication of secondary tumor by tumor-specific CTL activated by local treatment with low dose anti-CD40 in montanide. Dependence of tumor-eradicating capacity of CTL on locally available tumor-antigen. Tumor growth was measured and data are presented for each independent mouse, primary (red dotted line) and secondary tumor (black line). Between brackets is noted how many mice were tumor free at the end of the experiment. (A). Tumor inoculation and treatment scheme (B). Specific CTL response in blood at day 11 after treatment (C). 12 mice per group, one representative experiment of two.

First, we established that local anti-CD40 treatment was successful in generating tumor-specific CTL responses and tumor eradication following EG7 tumor challenge (data not shown). Next, we challenged mice with two unrelated tumors according to scheme (fig 6B). We found that the related secondary tumor was completely eradicated when a specific CTL response was activated by the primary tumor (Figure 6, group I), but grew out when unrelated tumors (expressing different tumor antigens than the second tumor) were employed as a first tumor (Figure 6, group II). In mice that received EG7 as a primary tumor, and were injected with both AR6 and EG7 tumor cells as distant tumors (Figure 6, group III), the outgrowing tumor was a solitary AR6 tumor as determined

by histology (suppl. Fig. 3). In all cases tumor eradication was accompanied by a systemic CTL response specific for the antigen present at the treatment site (Fig 6C). These results indicate that local treatment with anti-CD40 activates CTLs specific for tumor antigens that are presented in the vicinity of the local treatment area and in case of metastasis of related tumors these CTLs possess potent systemic anti-tumor cytotoxicity.

Discussion

We here report a novel administration method for anti-CD40 agonistic antibody as a monotherapy against immunogenic tumors. By using a slow-release agent (Montanide) to deliver the antibody close to the tumor-draining lymph node, we were able to induce activation of tumor-specific CTLs as defined by expansion and differentiation into potent effectors as well as effusion to the systemic circulation. Importantly, we were also able to reduce the adverse side effects, including liver toxicity as observed with I.V. injections. This treatment is only effective when injected close to the tumor, but is capable of activating a systemic CTL response that can eradicate tumors at distant locations. The systemic CTL response is specific for tumor antigens present at the site of treatment, which avoids unwanted systemic T cell activation and its associated detrimental side effects such as immune pathology and auto-immunity.

Previously, we reported that both NK cells and $CD4^+$ T cells are not critically involved in the anti-tumor effect of anti-CD40 antibody treatment against our tumor-model but that cytotoxic $CD8^+$ T cells play a crucial role [10, 22]. Together with the knowledge that the tumor antigen was presented mainly by $CD11c^+$ cells in the tumor-draining lymph node [22], this lead us to focus on targeting the CD40 activating antibody to these organs in order to activate the tumor-antigen presenting APC, which, in their turn, activate the tumor-specific CTL. We hypothesize that activation of DCs in the lymph node is instrumental in the systemic anti-tumor response caused by our local treatment. Our finding that DCs in the tumor draining LN after local treatment express CD70 whereas the DCs in non-draining LN do not, underlines the local induction of immune responses, which nevertheless induce a systemic anti-tumor CTL response that can eradicate distant related tumors.

Considering the presence of CD40 molecules on numerous cell types distributed through the body and their role in various processes, it is not unexpected that toxicity caused by systemic treatment with an agonist antibody against CD40 can occur but thus far this has been a largely ignored phenomenon in preclinical animal models. In addition, some groups reported that very high dosages of anti-CD40 induce deleterious effects on CD4 and CD8 T cell responses [32, 33].

Several studies have been published on the effects of targeting the tumor or the

tumor-draining lymph node, using tumor-specific antibodies or cellular vaccines producing cytokines and antibodies [34-37]. Although these studies have successful outcomes, they are all technically challenging and require knowledge regarding tumor-specificity. The method of administration as reported here is relatively straightforward and applicable to different types of immunogenic tumors. Jackaman et al. and we have previously shown that intratumoral injections of anti-CD40 led to eradication of local and distal tumors. However, these studies have not addressed the toxicity as described here. [38] By using a slow-release delivery method to target anti-CD40 agonistic antibody to the tumor-draining area, we were able to drastically decrease the dose needed for effective anti-tumor CTL activation and significantly reduce antibody-mediated side-effects. We found that toxicity, as measured by serum liver enzyme levels, in mice after systemic injection reaches plateau levels already at an injected dose of 10 mg, whereas anti-tumor CTL activation is not effective at this dose, or even at 30 mg. We therefore conclude that systemic injection of agonistic CD40 antibody has no proper therapeutic window (unpublished observations). Our findings have important implications for the use of anti-CD40 in the clinic, as CD40 agonist antibody therapy in a clinical trial was found to be associated with biological and anti-tumor activity but was hampered by dose-limiting toxicity [18]. Therefore, we propose the use of our treatment method in a clinical trial in order to reduce toxicity and achieve full anti-tumor efficacy.

Some studies which describe the use of anti-CD40 as an immunotherapy against tumors conclude that monotherapy is not sufficient for tumor-eradication but that this treatment has to be combined with cytokines, TLR ligand signaling or chemotherapy [13-15, 39]. We agree that anti-CD40 monotherapy as described in this study is not always capable of tumor eradication and that the use of combinatorial treatments will be beneficial and we propose that our superior administration method (i.e. delivery of a low dose agonistic CD40 antibody in a slow-release formulation in the tumor-draining area) is also valuable when combining anti-CD40 treatment with other agents.

In conclusion, this study shows that precision guiding of tumor-specific CTL from tumor-draining lymph nodes by local delivery of immunostimulants to DCs cross-presenting tumor antigen constitutes a novel way to elicit systemic therapeutic CTL responses. This approach lends itself without difficulty to clinical exploratory trials, because Montanide–ISA-51 delivery is safe in human individuals [40] and because appropriate agonistic antibodies against human CD40 are available [18].

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Chapter 3

Slow Release and Local Delivery of CTLA-4 blocking Antibody induces Tumor Eradication without Toxicity and is Dependent on CD8+ T cells

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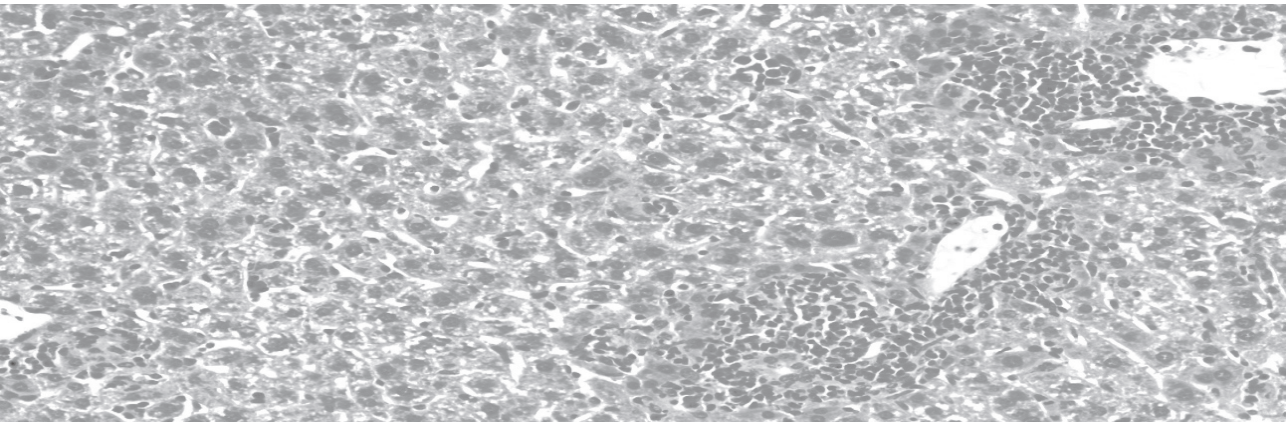

Abstract

Blockade of CTLA-4 by antibodies has potentiated anti-tumor T cell responses in both pre-clinical models and clinical trials. However, treatment with CTLA-4 blocking antibodies is associated with auto-immune and inflammatory side-effects. In this study, we propose a novel administration method for CTLA-4 blocking antibodies. By injecting the antibodies in a subcutaneous slow-release delivery formulation close to a tumor-draining lymph node, we show that an eightfold lower dose of antibody is as effective in activating a tumor-eradicating T cell response as systemic delivery. The significantly decreased levels of antibody in the serum cause less adverse events and reduce the risk of auto-immunity. The main target and effector cells in the tumor-model described here are $CD8^+$ T cells, whereas $CD4^+$ T cells do not play a prominent role in the antibody-mediated tumor eradicating effect. These results call for investigation of a similar delivery system of CTLA-4 blocking antibody in the clinic to reduce toxic side effects.

Introduction

T cell mediated immunotherapy holds great potential for the treatment of human malignancies. A crucial element of this therapy is the ability of $CD8^+$ T cells (cytotoxic T lymphocytes (CTLs)) to recognize and kill tumor cells that express tumor associated antigens [1;2]. Different types of tumor-associated antigens can be targeted such as those arising through mutations (e.g. p53, BCR-ABL and RAS), differentiation antigens (Tyrosinase, gp100, MART-1, Mucin), viral antigens, (HPV E6/E7, EBNA-1) and overexpressed antigens (WT, MDM2, HER-2/neu). Therapeutic interventions aimed at enhancing the efficacy of anti-tumor $CD8^+$ T cell responses are necessary to achieve clinical efficacy.

Effective priming of T cells requires antigenic stimulation of the T cell receptor in conjunction with costimulatory signals. B7.1 (CD80) and B7.2 (CD86) are costimulatory molecules expressed on antigen presenting cells (APCs), which bind to CD28 and CTLA-4 on T cells [3;4]. CD28 is constitutively expressed on T cells and provides essential costimulatory signals, whereas CTLA-4 is inducibly upregulated on convential T cells and inhibits the T cells activation. Several mechanisms of CTLA-4 inhibition have been proposed. CTLA-4 has been shown to outcompete CD28 for B7 ligation, inhibiting the positive activation effect of CD28. This was established in cells with CTLA-4 molecules containing non-functional cytoplasmic tails. These cells were still able to inhibit T cell responses [5-7]. Recent studies described that this result could also be due to back-signaling to B7, described to induce IDO, a metabolic enzyme expressed on APC that catabolises tryptophan, leading to starvation of T cells [8]. This latter finding has been implicated to be one of the mechanism through which T regulatory cells suppress T cell responses via APCs, as T regulatory cells constitutively express CTLA-4, which is important for their suppressive phenotype [9]. And lastly, CTLA-4 signaling has been shown to be responsible for reversing the TCR-stop; effectively ending the process of activation by detachment of the immunological synapse and increased T cell motility [10;11].

Blocking the interaction of CTLA-4 with B7.1 and B7.2 has been demonstrated to improve antitumor T cell responses in pre-clinical tumor models and in cancer patients [12-16]. Recently, promising clinical results have been obtained with CTLA-4 blockade in melanoma patients that have led to approval by the FDA for treatment of advanced melanoma [17]. However, CTLA-4 treatment is accompanied by auto-immune and inflammatory side effects such as colitis, dermatitis, uveïtis and hypophysitis.

Previously, we have shown that local delivery of agonistic antibody against CD40 in the tumor-draining area was equally effective in activating tumor-specific $CD8^+$ T cell responses leading to tumor eradication, with strongly decreased treatment-induced toxicity in comparison with systemic administration [18].

In this study we show that local injection of a CTLA-4 blocking antibody in the slow-release formulation Montanide ISA-51 in tumor bearing mice leads to an effective anti-tumor $CD8^+$ T cell response and tumor eradication, while levels of systemic antibody in serum remain low. The treatment was dependent on $CD8^+$ T cells whereas $CD4^+$ T cells do not play a major role. Thus, local CTLA-4 treatment induces tumor eradication by directly enhancing tumor-specific $CD8^+$ T cell responses.

Material and Methods:

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. The experiments were approved by the Animal Experimental Committee of the University of Leiden.

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Tumor experiments

MC-38 cells expressing ovalbumin (MC38-ova) [19] were cultured in Iscove's modified Dulbecco's medium (IMDM): (BioWhittaker, Verviers, Belgium) supplemented with 4% FCS, 50 µM 2-mercaptoethanol and 100 IU/ml penicillin/ streptomycin. The tumor cells (0.5×10^6) were injected s.c. into 8-12 week-old female mice in 200 µl of PBS. Treatment was started 7-10 days after tumor inoculation, when palpable tumors were present. Mice were sacrificed when tumors reached a size of 1000 mm^3 to avoid unnecessary suffering.

Flow cytometry

Single-cell suspensions of spleens underwent erythrocyte lysis, and were subsequently stained with CD8a (clone 53-6.7), CD4 (clone RM4-5), and $CD3_\varepsilon$ (clone 145-2C11) mAbs (BD Bioscience), and $OVA_{257-264}$–loaded H-$2K^b$ tetramers. All stained cells were analyzed on a FACScalibur (Becton Dickinson) and data analysis was performed with Flowjo (treestar).

Antibody treatment

Hybridoma cells producing a CTLA-4 blocking Ab clone 9H10 [5], a depleting CD8 mAb (clone 2.43) or a depleting CD4 mAb (clone GK1.5) were cultured in Protein Free Hybridoma Medium (Gibco), and mAbs were purified using a Protein G column. Mice treated systemically with CTLA-4 blocking mAb received 200 micrograms mAb (high dose) in PBS intraperitoneally on day 0 and day 3 or received 50 micrograms mAb (low dose) at day 0. Mice treated locally with a low dose CTLA-4 blocking mAb received 50 micrograms mAb in montanide, subcutaneously on day 0. Montanide/ 9H10 antibody emulsions were made by mixing antibody in PBS 1:1 with montanide (Montanide ISA-51, Seppic), and

vortexing for 30 minutes. To deplete $CD8^+$ or $CD4^+$ T cells mice received an i.p. administration of 100 microgram anti-CD4 or anti-CD8 on day -1, 2, 7, 14, 21 after tumor inoculation. The efficiency of T cell subset depletion was measured by staining of blood lymphocytes for cell surface CD4 and CD8 (using non-competitive mAbs) and indicated a consistent depletion of >98% of the total T cell populations. All control mice received in parallel similar amounts of isotype control rat IgG.

Serum analyses

Serum samples were taken from mice at several time points after CTLA-4 treatment. ALT and AST analyses were performed by the department of Clinical Chemistry of the LUMC hospital according to standard protocols. Auto antibodies were analyzed in serum with the Anti-Nuclear Antibodies-ELISA kit (US Biological, Swampscott, MA, USA) according to manufacturer's instructions. CTLA-4 blocking antibodies levels in serum were detected in an ELISA using purified and biotin-labeled mouse anti-hamster antibodies (clone 192-1) from BD bioscience.

Results

Tumor eradication by local treatment with a low dose of CTLA-4 blocking antibody is equally effective as high dose systemic treatment.

We previously described that a low dose of agonistic CD40 antibody delivered locally in a slow-release formulation (Montanide-ISA-51) was very effective in inducing systemic anti-tumor immunity without strong systemic side-effects. We hypothesized that this administration technique would also be applicable to other immune modulating antibodies, such as CTLA-4 blocking antibody. To verify this, mice were inoculated subcutaneously with MC-38-ova tumor cells (murine coloncarcinoma cells expressing ovalbumin in the cytoplasm). Seven days after tumor inoculation, when palpable tumors were present, treatment was started. Mice underwent either the standard systemic treatment of CTLA-4 blocking antibody (2 injections of 200 microgram intraperitoneally) or were treated locally by receiving one injection of 50 microgram in montanide close to the tumor. Both the high dose systemic and low dose local treatment with CTLA-4 blocking antibody was able to induce tumor eradication as compared to non-treated mice (Figure 1a and b). Mice treated with a systemic administration of the low dose, 50 microgram, were not able to clear the tumor, indicating that this dose is only effective when delivered into the tumor-draining area (Figure 1c). To determine whether tumor eradication correlated with enhanced tumor-specific T cell responses we analyzed the magnitude of the $CD8^+$ T cell response in tumor-bearing mice treated with CTLA-4 blocking antibody. The tumor-specific $CD8^+$ T cell response in the spleen and blood, as analyzed by tetramer

staining, was enhanced in mice that underwent either the high systemic dose or the low dose local treatment as compared to untreated mice (Figure 1d and data not shown). Thus, local low dose of blocking CTLA-4 treatment is similar in tumor-eradicating capabilities and induction of tumor specific CTL responses as high dose systemic treatment.

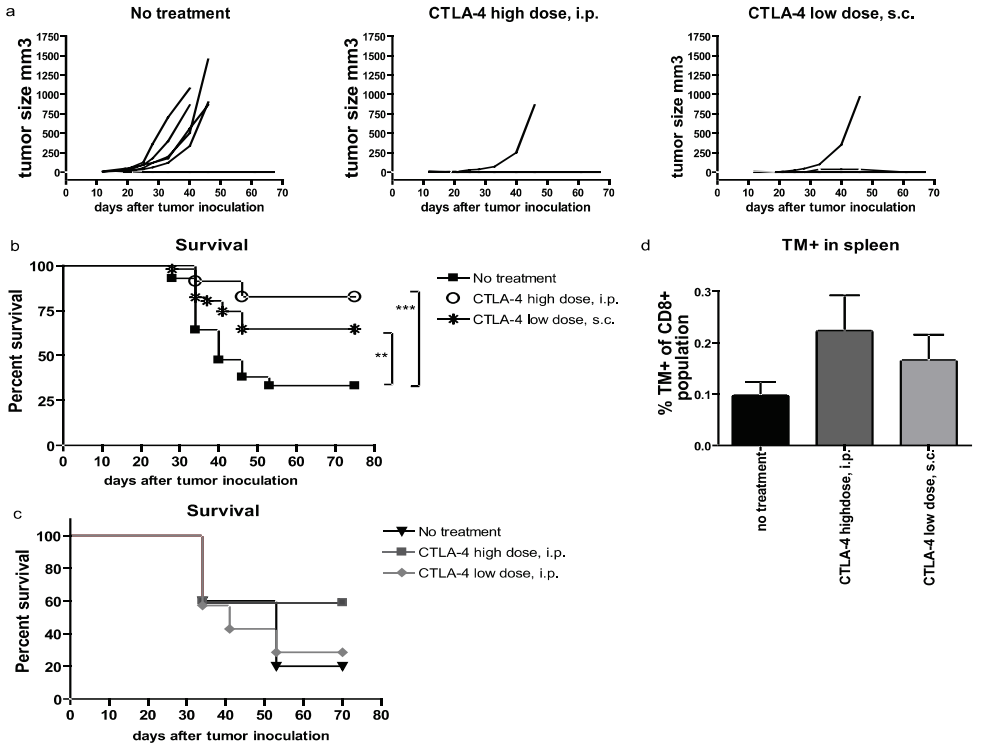

Fig. 1 Local treatment with a low dose of CTLA-4 blocking antibody induces effective tumor eradication. Mice bearing palpable MC-38-ova tumors were treated with two intraperitoneal injections with high dose (2 x 200 µg) of CTLA-4 blocking antibody (standard treatment), or one subcutaneous, local, injection with low dose (50 µg) CTLA-4 blocking antibody in slow-release agent Montanide ISA51. Tumor growth was measured at regular intervals. **a** Data presented as tumor growth in each mouse, 10 mice per group. **b** Survival curve. Shown are pooled data of 4 independent experiments, 32 mice per group. Kaplan-Meier test revealed significant differences between non-treated group and local treated group or intraperitoneal treated group, 0.002 (**) and 0.0002 (***) respectively. **c** Survival curve of mice treated with either high dose (2 x 200 µg) of CTLA-4 blocking antibody, intraperitoneally (standard treatment) or low dose (50 µg) intraperitoneally. n=7 mice per group. **d** CTL response after low dose, local, treatment with CTLA-4 blocking antibody.: Nine days after start of treatment, tetramer$^+$ CD8$^+$ T cells were analyzed in spleen (mean ± SE, n=10 mice per group), data pooled of two independent experiments. Student T-test revealed a significant difference between treated groups and non-treated group. ($p < 0.05$ for both treated groups).

Local slow-release administration of CTLA-4 blocking antibody decreases adverse events.

In order to determine the CTLA-4 blocking antibody levels in the serum, we performed an anti-hamster ELISA on serum samples, taken at different intervals after start of treatment. As depicted in figure 2a, antibody concentrations in

the high dose systemically treated mice were more than 1000 fold increased compared to local treatment with a low dose. The CTLA-4 antibody levels in the latter group were only slightly elevated compared to background due to the combined effects of lower dose and slow local delivery. This difference in antibody concentrations between the systemically and locally treated groups persisted for at least 14 days.

Considering the strongly decreased concentration of antibody in the serum in locally treated mice, we hypothesized that this treatment would induce lower adverse side-effects than systemic administration. In order to determine this, we analyzed the liver enzymes ALT and AST, known to be indicative for tissue damage [20], in serum samples of treated mice at several time-points after administration of the antibodies. As indicated in Figure 2b and c, liver enzyme levels were decreased in mice treated with a low dose of CTLA-4 blocking antibody in Montanide, compared to mice treated with the high intraperitoneal dose of CTLA-4 blocking antibody.

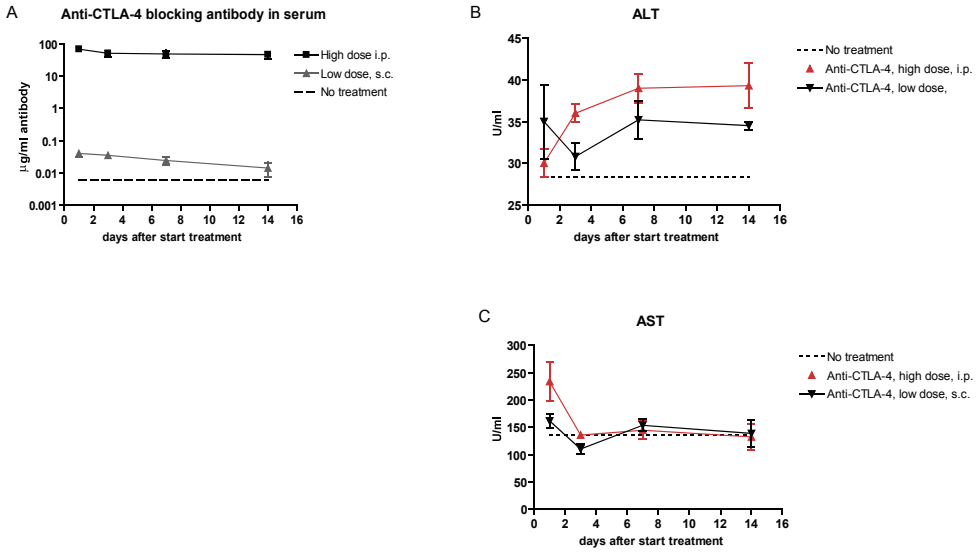

Fig. 2 Local treatment with a low dose of CTLA-4 blocking antibody results in decreased treatment induced toxicity as compared to high dose, systemic treatment. Shown are CTLA-4 antibody concentrations and liver enzyme levels in serum in time after treatment with low dose (50 µg) local treatment or high dose (2 x 200 µg), intraperitoneal treatment. **a** Antibody concentrations in serum. **b** ALT levels in serum. **c** AST levels in serum. (mean ± SE, n=5 mice per group)

Since CTLA-4 blocking treatment in patients can induce serious autoimmune and inflammatory side-effects, we analyzed the serum-levels of anti-nuclear antibodies (ANA) in the mice after treatment, at several time-points between start of treatment and day 14, as ANAs are a strong indication of autoimmunity

[21]. However, we could not detect a rise in serum ANA levels in either high dose, intraperitoneal treatment or low dose antibody-treated mice, at any of the time-points (data not shown).

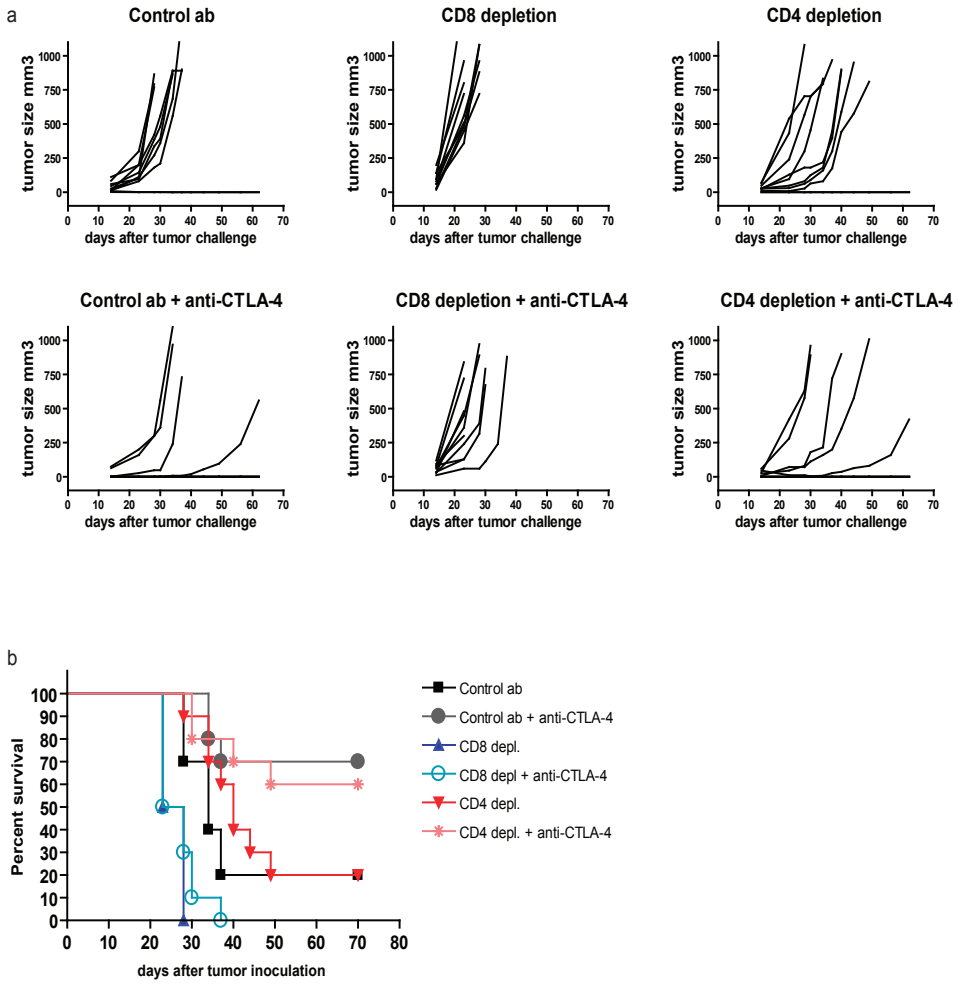

Fig. 3 CD8⁺ T cells are the main effector cells involved in tumor eradication and the main target of local treatment with CTLA-4 blocking antibodies. Mice were depleted of CD8⁺ or CD4⁺ T cell populations, starting one day before tumor inoculation, for three weeks. 8 days after tumor inoculations, when palpable tumors had formed, treatment was started. Mice were treated with a locally provided low dose (50 μg), CTLA-4 blocking antibody, or left untreated. Tumor growth was measured at regular intervals. **a** Data represents tumor growth in each mouse, 8 mice per group. **b** Survival curve. Data is representative of two independent experiments

Local treatment depends strictly on induction of tumor specific CD8+ T cell responses.

To assess whether CD8⁺ and/or CD4⁺ T cells populations were important for the efficacy of local CTLA-4 treatment, we injected tumor bearing mice for three

weeks with $CD8^+$ or $CD4^+$ T cell depleting antibodies, starting one day before tumor inoculation. Seven days after tumor inoculation, when palpable tumors had formed, half of the mice in each group were treated with a low dose of CTLA-4 blocking antibody that was administered locally in Montanide. Tumors in mice depleted of $CD8^+$ T cells grew out at a faster rate than in control mice, regardless of CTLA-4 treatment. In contrast, mice depleted of $CD4^+$ T cells, responded identical to CTLA-4 treatment as the control group, indicating that the presence of $CD4^+$ T cell populations were not involved in tumor eradication in this model (Figure 3a and b). Together these data emphasize that in our tumor model $CD8^+$ T cells are primarily responsible for tumor eradication and mainly targeted by CTLA-4 blockade.

Discussion

In this study we show that local treatment of tumor bearing mice with CTLA-4 blocking antibody in a slow-release formulation is very effective in activating a tumor-specific $CD8^+$ T cell response, capable of tumor eradication. We further show that the $CD8^+$ T cell itself is the main effector cell responsible for clearing the tumor, and most likely also the main target for the CTLA-4 treatment. Treatment-induced side effects were reduced by this local administration strategy compared to systemic administration, and the lower concentration of antibody in the serum should reduce the risk of auto-immune and inflammatory problems connected to clinical treatment with CTLA-4 blocking antibody.

Local treatment with CTLA-4 blocking antibody to induce tumor eradication has been described before [22;23]. In these studies, the CTLA-4 blocking antibody treatment was given in combination with either CpG or GM-CSF secreting vaccines. Here, we show that the local administration is also applicable for CTLA-4 blocking antibody as monotherapy, and that using a slow-release delivery system further decreases systemic levels of antibody, and thereby adverse side-effects.

Contrary to previous studies in mice using CTLA-4 blocking antibody [14;22;24], $CD4^+$ T cells do not play an essential role in our tumor model, as evidenced by the fact that CTLA-4 blocking in CD4 depleted mice showed similar anti-tumor activity as in non-depleted control mice. This might be related to the presence of a tumor antigen (in our model ova) that induces a strong $CD8^+$ T cell response. Also, it is conceivable that regulatory T cells play a minor role in this tumor model, causing the $CD8^+$ T cell to be the major target cell for the CTLA-4 blocking antibody. However, we can not exclude that opposing effects might occur due to depletion of both effector/helper $CD4^+$ T cells and suppressive $CD4^+$ Tregs, creating a net neutral effect of CD4 T cell depletion.

CTLA-4 blocking antibody treatment did not lead to increase in autoantibody levels in this study, whereas clinical data shows that patients treated with

CTLA-4 blocking antibodies suffered from autoimmune and inflammatory side effects. This could be explained by the fact that patients are treated over a long period of time, whereas in animal models such as this study, treatment is limited to a few weeks. Additionally, the antibody used in mice studies, 9H10, has a shorter half-life than the antibodies used in patients, which can also contribute to the stronger adverse side-effects seen in clinical trials.

In conclusion, this study shows that local delivery of CTLA-4 blocking antibody elicits tumor eradication with a relatively low dose needed, which leads to a decrease in treatment-induced toxicity. The main target cells of CTLA-4 treatment in this model are tumor-specific $CD8^+$ T cells, which are enhanced in number after treatment and found to be essential for tumor eradication. This approach lends itself without difficulty to clinical trials, because Montanide–ISA-51 delivery is safe in human individuals [25], and because appropriate human CTLA-4 blocking antibodies are available [17].

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Chapter 4

Separate roles for antigen recognition and lymph node inflammation in CD8+ memory T-cell formation.

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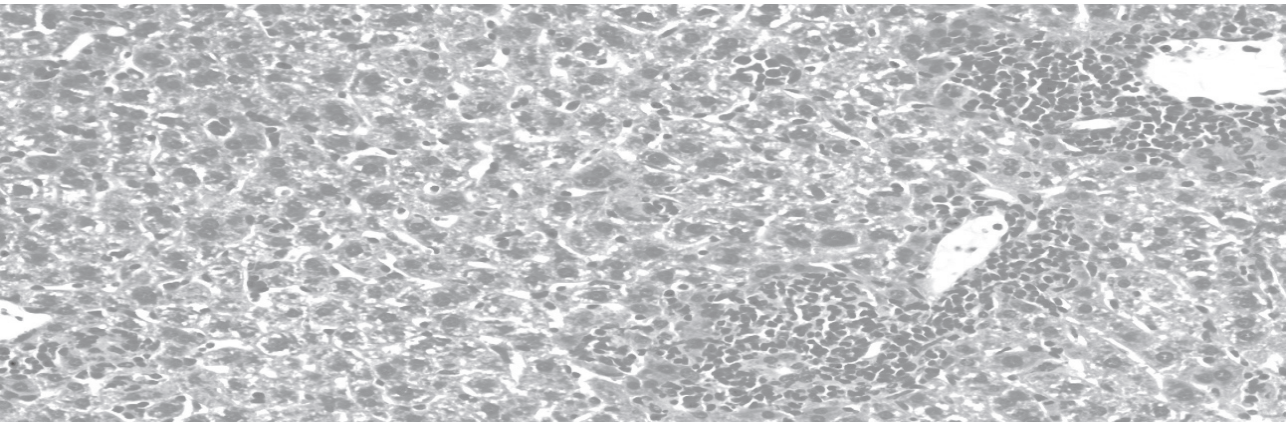

Abstract

Priming of naive CD8+ T-cells by pathogens or vaccines generally involves their interaction with antigen-loaded dendritic cells (DCs) in the context of an inflamed lymph node. Lymph node activation fosters DC and T-cell encounter, and subsequently provides newly primed T-cells with nurturing conditions. We dissected these two aspects by infusing in vitro primed CD8+ T-cells into naïve recipient mice harboring a single activated lymph node and comparing the fate of these T-cells with those infused into control recipients. Brief (20 hr) in vitro priming empowered the T-cells to expand in vivo without further antigen stimulation. This primary response was not affected by the presence or absence of a non-specifically activated lymph node. In contrast, in vivo antigenic challenge after contraction of the primary response resulted in significantly stronger secondary T-cell responses in mice harboring activated lymph nodes, demonstrating that the availability of an activated lymph node supported the generation of T-cell memory in an antigen unrelated manner. The presence of an activated lymph node during the expansion and contraction phase of the primary response did not endow T-cells with an instructional program for increased survival or secondary expansion, but primarily served to conserve increased numbers of T-cells.

Introduction

The initiation of T-cell responses upon primary encounter of pathogens involves the delivery of antigen and pathogen-associated molecular patterns (PAMPs) to secondary lymphoid organs draining the infected areas of the body. As a result, immunogenic peptide antigens are presented in MHC molecules at the surface of dendritic cells (DCs). Furthermore, the PAMPs trigger a sequence of events that orchestrate effective interaction between antigen-loaded DCs and naïve T-cells, including the expression of costimulatory molecules at the DC surface and the production of pro-inflammatory cytokines and chemokines that recruit and arouse a variety of immune cells. (1) The latter process causes lymph nodes to develop a state of inflammation and swelling, also referred to as lymph node congestion or activation(2). Chemokines like CCL9 and CCL21, cytokines including TNF-alpha and IFN-α/ß, and the chemo-attractant receptor S1P all play an important role in enhancing the cellularity of the lymph node during an immune response, thereby creating an inflammatory micro-environment supportive of T-cell priming.(3-8) The significance of PAMPs such as Toll-like receptor ligands in the induction of T-cell immunity has been demonstrated in numerous experimental models, showing that delivery of antigen without PAMPs as immune-adjuvants results in T-cell tolerance. Even though the importance of PAMPs for inducing costimulatory signals through DC activation as well as for triggering lymph node activation is broadly recognized, and the nature of the DC costimulatory signals has been studied in great detail (9,10), much less is known about the impact of lymph node activation on the effector and memory phases of the T-cell response. In the present study, we have focused on the latter aspect by separating DC-T cell engagement and lymph node activation in place and time. Our experimental data reveal that the availability of an inflamed lymph node during the primary response increases the magnitude of the secondary response through the conservation of larger numbers of T-cells.

Material and Methods:

Mice:

C57BL/6 jico (B6) mice were obtained from Jackson laboratories. OT-I TCR Tg RAG-1$^{-/-}$ mice were from W. R. Heath (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) and were bred in the animal facility of LUMC.v The experiments were approved by the animal experimental committee of Leiden University Medical Center.

Cell culture:

All in vitro cell culture and assays were performed in IMDM (Invitrogen Life

Technologies, Rockville, MD) supplemented with 8% v/v FBS (Greiner), 50 μM 2-ME, 2 mM glutamine, 100 IU/ml penicillin (complete medium). D1 cell line, a long-term growth factor-dependent immature splenic DC line derived from B6 mice, was kindly provided by P. Ricciardi-Castagnoli (University of Milano-Bicocca, Milan, Italy) and cultured as described (11). BM-derived DC were isolated from C57BL/6 jico femurs, cultured for 10 days in the same manner as D1 cells, before activation. The engineered APC cell line MEC.B7.SigOVA (SAMBOK) was generated as described (12), briefly, the mouse embryonic fibroblast cell line MEC-1 was transfected with CD80 and the minigene SigOVA, which encodes the $OVA_{257-264}$ (SIINFEKL) peptide directed to the ER, leading to efficient loading of the peptide onto MHC Class I molecules K^b. For in vitro priming, engineered APC were cultured at 70000 cells/well in 24 well plates overnight. The next day, nonadherent cells and cell debris were removed by washing the wells twice. OT-I cells were then added to the wells (0.5×10^6/well) in 2 ml of medium and plates were centrifuged for 1 min at 1000 rpm to initiate cell contacts. After 20h of co-culture the nonadherent OT-I cells were gently harvested, washed and adoptively transferred into recipient mice.

Lymph node activation:
D1 cells were cultured in D1 medium, maturation was induced by adding 6 μg/ml LPS (E-coli derived, serotype 026, B6, Sigma) 24 hours before injection. Cells were harvested with EDTA, washed three times with PBS, and injected, 2×10^6 in 30 μl PBS into the right hind leg footpad of mice.

In vivo challenge:
50 μg of synthetic short OVA peptide (SIINFEKL) was injected with 15 μg CpG 1826 (synthesized in the Leiden Institute of Chemistry) in 30 μl PBS into the right hind leg footpad of mice.

Flowcytometry:
All antibodies were purchased at BD and eBioscience. Intracellular cytokine staining was performed with the Cytofix/Cytoperm kit (Becton Dickinson) according to manufacturer's instructions.

In vitro proliferation assay;
Lymph nodes from mice were isolated from recipient mice and single cells suspensions were generated by mincing through cell strainers. Subsequently, cells were stained with CD8 and CD45.1 mAbs, and OT-I cells were purified by flowcytometric sorting. Equal amounts of cells were co-cultured with 100.000 irradiated spleen cells, and 1 μg/ml SIINFEKL, or medium control. 36 hours

later, cells were pulsed with 1 μC $_3$H per well, and analyzed 24 hours later.

Virus-infection:
Mice were infected with 5× 10^4 pfu influenza A/WSN/33 (WSN)-OVA_I (13) through intra-tracheal inoculation. After 5 days mice were sacrificed, and lungs were isolated in TRizol (Gibco) and homogenized. RNA was extracted and purified using the Qiagen RNeasy kit, cDNA was synthesized using 7.5 microgram RNA of each sample, and 12.5% cDNA was used in the RT-PCR reaction, in triplicate, and correlated to actin expression (primer/probe Applied Biosystems). cDNA synthesis and RT-PCR were performed using primer-sequences as described before (14)

Results
Activated lymph nodes provide increased storage for antigen-experienced T-cells
Lymph node activation is an intrinsic aspect of T-cell activation by infectious pathogens and vaccines. We separated the T-cell priming event from lymph node activation in space and time by exploiting an experimental model consisting of TCR-transgenic OT-I CD8+ T-cells and engineered APCs expressing high levels of the cognate ovalbumin (OVA)-derived peptide epitope and the costimulatory ligand CD80. Prior work had demonstrated that a 20-hr in vitro encounter with these APCs empowered naïve OT-I cells to vigorously proliferate in vivo upon transfer to naïve syngeneic recipient (15) Furthermore, these T-cells were shown to develop into long lived memory cells, capable of clonal expansion and protective effector function in response to secondary antigen encounter (16). Importantly, progression of the 20-hr primed OT-I T-cells through primary expansion, contraction and memory phases required neither in vivo exposure to antigen, nor the context of an inflamed, congested lymph node. This model therefore offered a unique opportunity to compare the in vivo behavior of primed CD8+ T-cells in the absence versus presence of a non-specifically activated lymph node. Activation of a single, popliteal lymph node in recipient mice was induced by footpad injection of activated syngeneic DCs (D1 cells or isolated BM-DCs) that were not loaded with antigen. Work by others has shown that this results in inflammation of the targeted lymph node within 1 day (8). The use of DCs not loaded with OVA antigen enabled us to separate the impact of antigen exposure and lymph node congestion in time and space. Comparison of the behavior of 20-hr ex vivo primed OT-I cells upon injection into DC-injected versus control mice revealed very few differences in the kinetics and magnitude of the primary response.

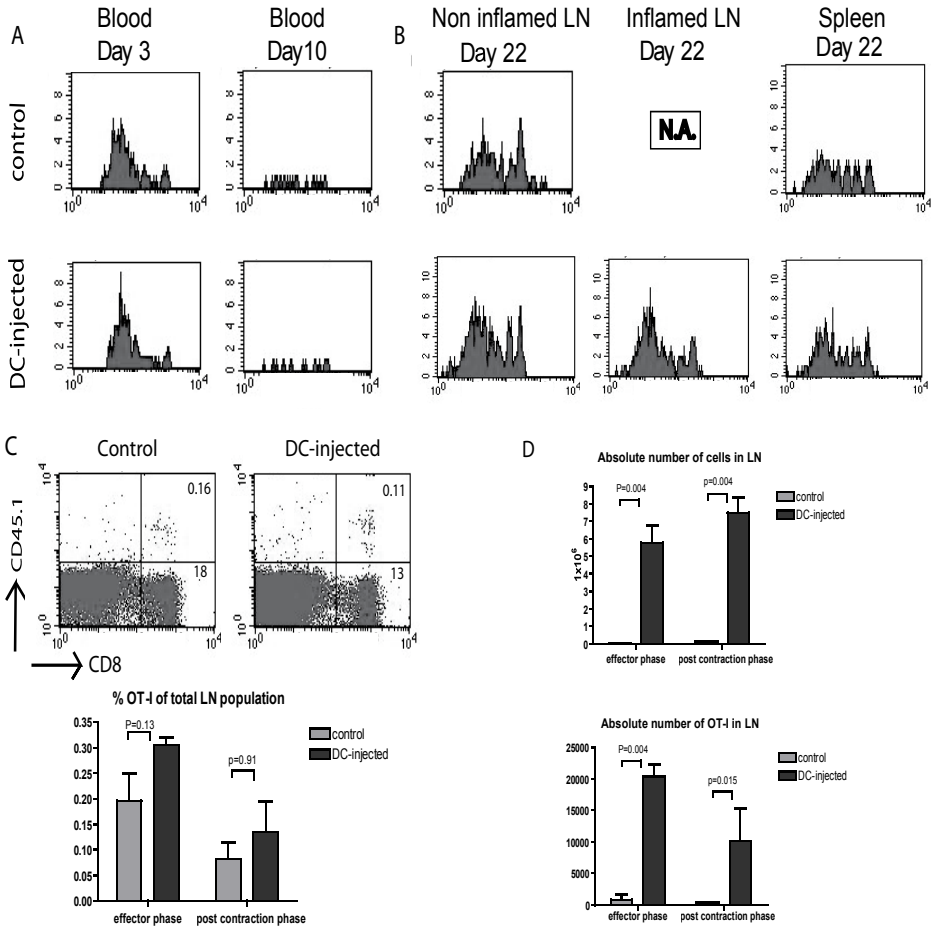

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Figure 1: Kinetics and magnitude of the primary in vivo response by 20 h in vitro primed OT-I cells.
CFSE-labeled naïve CD45.1 OT-I cells were cultured during 20 hrs with engineered APCs expressing OVA antigen and CD80, after which T-cells were separated from the APCs and injected into antigen-naïve B6 mice (1 ×106 per mice) as described previously (15). Recipient CD45.2 mice either did not receive prior treatment, or were injected into the footpad of the right hind leg with 2 × 106 in vitro, LPS-activated DCs (D1 cells), not loaded with antigen, 24 hrs prior to T-cell infusion . Blood samples were isolated at days 3 and 10 after start of T-cell activation. At day 22, mice were sacrificed and spleen and lymph nodes isolated. Panels A/B and C/D were from subsequent experiments with similar outcome. OT-I cells were gated by CD8 and CD45.1 A. Representative examples of groups of 4 mice (A, B) of proliferative response (CFSE) and relative OT-I cell counts in blood of DC-injected and control mice. B. Accumulation of memory OT-I cells during the post contraction phase in popliteal lymph nodes and spleen of DC-injected and control mice. Right-hand lymph nodes of DC-injected mice were inflamed. Contra-lateral, left-hand lymph nodes from DC-injected mice served as internal control. N.A. = not applicable. Three independent experiments were performed (A and B) C. Percentage of OT-I cells, in relation to total cell counts, in congested, right-hand lymph nodes of DC injected mice and the same lymph nodes from control mice in post contraction phase. Comparison revealed no difference in both effector and post contraction phase. D. Absolute total cell count and OT-I T cell count in congested, right-hand lymph nodes of DC injected mice and the same lymph nodes from control mice. Mann-Whitney test revealed significant differences in total and OT-I numbers in effector phase as well as in memory phase, 5 mice per group, two experiments pooled. Four independent experiments performed in total (C and D)

In both cases, the percentage of circulating OT-I cells peaked at day 3 after transfer, followed by a rapid contraction due to the absence of cognate antigen (Fig 1A). Three weeks after transfer, at a time when OT-I numbers in the blood were below detection, memory T-cells could still be detected in the spleen and lymph nodes (Fig 1B). As observed in the primary phase, percentages of memory OT-I cells differed neither between DC-injected and control mice (Fig 1C). However, the absolute total cell count was increased in the inflamed lymph nodes compared to contra-lateral lymph nodes in DC–injected mice and lymph nodes in control mice. Consequently, also the absolute numbers of OT-I cells were amplified in these lymph nodes, both at the peak of the effector phase and during the post-contraction phase of the primary response (Fig 1D). For inflamed lymph nodes, the total cell count was even larger in the post-contraction phase (day 22) than during the effector phase, in line with the notion that the size of the inflamed lymph nodes gradually increases and remains fully enlarged over at least 3 weeks. (data not shown). Taken together, our data suggest that the primary role of an inflamed, enlarged lymph node is to provide an expandable storage reservoir for memory T-cells.

Memory T-cell storage is important for the magnitude of the secondary T-cell response

Our initial observations prompted us to test how accumulation of greater numbers of memory T-cells in a single inflamed lymph node would impact on the systemic CD8+ T-cell response upon secondary antigen encounter. We delivered a secondary antigenic challenge to the in vitro primed OT-I cells through injection of ovalbumin peptide and CpG ODN into the footpad of the right hind leg, the same footpad into which mice with inflamed lymph nodes received their initial DC injection. In line with the greater number of memory T-cells available in these LNs in DC-injected mice, secondary responses in these mice were stronger than in control mice (Fig 2A, B). Moreover, the secondary responses in DC-injected mice were stronger than as compared to the primary responses recorded in these same mice, reminiscent of a textbook example of the relative magnitude of primary and secondary T-cell responses. Differences between DC-injected and control mice were most prominent in the blood and lung (Fig 2B), illustrating that secondary challenge of the DC-injected mice resulted in increased frequencies of effector T-cells capable of leaving the lymphoid organs and migrating to potential target tissues. Differences in OT-I T-cell frequencies were the least prominent in the draining lymph nodes, in line with this migratory behavior of effector T-cells and the increased overall cellularity of these lymph nodes (Fig 1D). Similar results were obtained in mice with an activated lymph node induced by injecting

bone-marrow derived DC. (data not shown).
Interestingly, the secondary responses in control mice did not exceed the magnitude of the primary responses (Fig 2A). Our experiments therefore reveal that the availability of an inflamed lymph node during the primary response is important for the generation of an enhanced secondary T-cell response. Because secondary antigen challenge in our experiments involved co-delivery of CpG ODN, causing efficient activation of the draining lymph nodes in both DC-injected and control mice, we deem it unlikely that the already inflamed status of these lymph nodes in DC-injected mice is a key determinant in facilitating the secondary response of the T-cells. The most likely explanation for the stronger secondary response in DC-injected mice is therefore the greater number of CD8+ memory T-cells conserved after contraction of the primary response elicited from the inflamed lymph nodes.

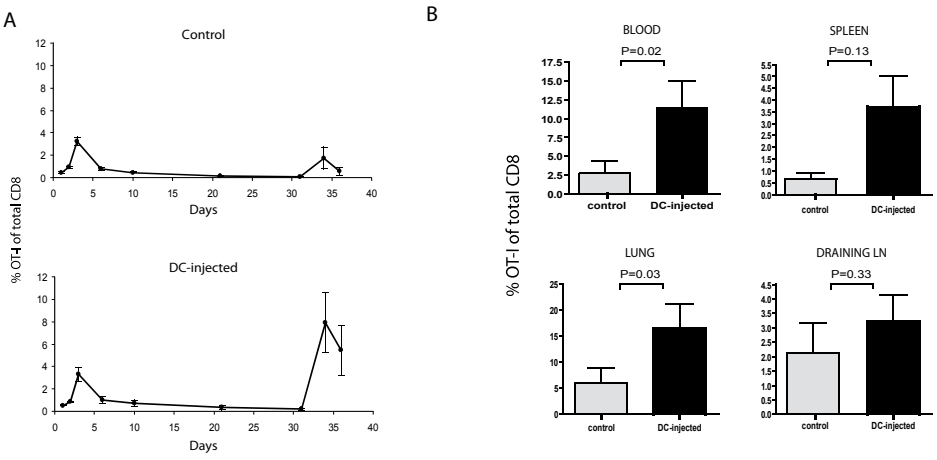

Figure 2: Presence of inflamed LN during primary response increases magnitude secondary T-cell response
Primary responses of ex vivo primed OT-I cells were followed over time in the blood in mice that had received a footpad-injection of LPS-activated DCs, not loaded with antigen, and in control mice. At day 28, after contraction of the primary response, mice were challenged with 50 µg synthetic OVA peptide (SIINFEKL) comprising the OT-I T-cell epitope in combination with 15 µg CpG. This antigen challenge was delivered into the footpad of the right hind leg, at the same site of DC injection. **A.** Kinetics of OT-I in blood in control and DC-injected mice, mean of three mice per group with standard error of the mean. Arrow indicates time of antigen challenge. Representative of three experiments **B.** Mean values for percentages of OT-I cells in different compartments around peak of secondary response (day 8 after secondary challenge) for 14 mice/group. Mann-Whitney test revealed statistically significant differences between groups in blood and lung (p=0.02 and p=0.03 respectively), but not in spleens and draining LNs (p=0.13 and p=0.33 respectively). Two independent experiments performed.

To further analyze the conditions in inflamed lymph nodes in DC-injected mice, we performed footpad injection of CFSE-labeled DCs. As shown in Fig 3A, this results in a rapid increase, within 1 day, of the absolute numbers of various types of cells, including T-cells, B-cells and CD11c+ DCs. The increase in CD11c+ cell numbers cannot be attributed by the injected DCs, because CFSE-high cells were found to constitute less than 0.5% of the CD11c+ population

(data not shown). The host-derived cells recruited to the inflamed lymph node displayed an activated state, as illustrated by the increased frequencies of IL-12 producing CD11c+ cells and TNF-alpha producing CD3+ cells (Fig 3B). There findings strengthened the notion, based on experiments shown in Fig.1C/D, that the injection of activated DCs triggers an overall accumulation of APCs and lymphocytes rather than a selective accumulation and/or activation of antigen-specific OT-I T-cells. Accordingly, we did not find significant differences in the activation status, as determined by IFN-gamma production, of ex vivo primed OT-1 cells between inflamed and resting lymph nodes of DC-injected and control mice respectively, neither in the effector phase, nor in the post contraction phase of the primary response (Fig 3C). Evaluation of additional markers that were found to be associated with T-cell activation and memory cell formation, in particular CD62L, CD127, CD122, CCR7 and TRAIL (17-19), also failed to reveal a qualitative difference between the OT-I T-cells in resting versus inflamed lymph nodes (supplementary figure 1 and data not shown).

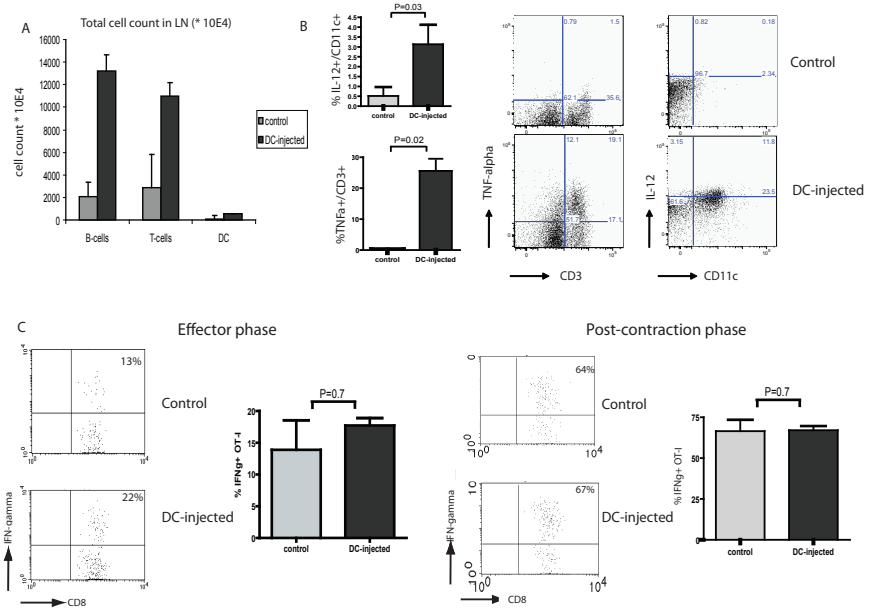

Figure 3: Conditions in the inflamed LN

Cellularity and cytokine production in inflamed and control LN was analyzed 24h after footpad-injection of LPS-activated DC's. Single cell suspensions of LN were stained for CD19, CD3 and CD11c **A**:. Absolute numbers of cells in inflamed and control LN. **B**: Percentage cytokine producing cells in inflamed and control LN 24h after footpad-injection of DC. Cytokine production was analyzed by intracellular cytokine staining following PMA/ionomycin activation. In each experiment 4 mice per group were used. Mann-Whitney test revealed statistically significant differences in IL-12p40 and TNF-alpha production between groups (p=0.03 and p=0.02 respectively). Two independent experiments performed. **C**: IFN-gamma production by OT-I CD8+ T-cells at day 3 and 26 after start of T-cell activation. Single cell suspensions of LN were analyzed by intracellular cytokine staining following 3h stimulation with SIINFEKL peptide in vitro. Mann-Whitney test revealed no significant difference between groups in effector and post-contraction phase (p=0.7 in both phases). Groups of three mice, three independent experiments performed.

The possibility of a qualitative rather than a mere quantitative difference between OT-I cells in DC-injected and control recipient mice, was further investigated by harvesting OT-I memory T-cells from mice with inflamed lymph nodes in the early post-contraction phase (12 days after infusion), and transferring these – in identical numbers - to either DC-injected or control recipients (Fig 4A). T-cells were allowed to rest for another 15 days in new recipients, after which they encountered a secondary antigenic challenge through footpad injection of OVA-peptide/CpG. Analysis of the secondary in vivo OT-I responses showed that the availability of an inflamed lymph node in the recipient mice was of key importance for a strong, systemic memory T-cell response (Fig 4B). Thus, post-contraction OT-I T-cells harvested from inflamed lymph nodes only persisted in greater quantities when transferred into DC-injected recipients, and therefore did not exhibit an intrinsic capacity for survival independent of the availability of an inflamed lymph node. In reciprocal experiments, we found that post-contraction OT-I cells harvested from control mice led to stronger secondary responses in magnitude when infused into DC-injected mice than when infused into control mice (Fig 4C) supporting the notion that inflamed LN supports memory T cell maintenance and CD8+ memory T-cells 'educated' in DC-injected and control mice did not differ intrinsically. In order to determine if the OT-I T cells from DC-injected mice reacted differently to antigenic stimuli, OT-I T-cells cells were recovered, at day 7 and day 24 after adoptive transfer, from lymph nodes of DC-injected and control mice, and co-cultured in vitro with feeders and specific peptides. Proliferation to the specific peptide was analyzed by tritium incorporation. (Fig 4D) Although a trend towards more proliferation at day 7 in the DC-injected group was observed, this difference was not significant. At day 24, no difference between OT-I cells from DC-injected versus control mice to the antigenic challenge was observed. . This supports our hypothesis that the amplified response in vivo in DC-injected mice is due to quantitative difference caused by the larger absolute numbers of antigen-experienced T-cells in the inflamed lymph node.

Importance of memory T-cell conservation by inflamed lymph nodes in anti-viral immunity

So far, our experiments demonstrated that secondary challenge at the site of prior lymph node activation can result in a superior memory T-cell response. In additional experiments, we delivered the secondary antigenic challenge, consisting of ovalbumin peptide and CpG ODN, in the ipsilateral footpad. Antigen delivery at this site resulted in a secondary T-cell response comparable to that elicited by antigen delivery in the foot pad near the inflamed lymph

node, showing that the locally conserved memory T-cells can be recruited into secondary responses triggered by antigenic delivery at distal sites (data not shown). To further assess the physiological significance of memory CD8+ T-cell conservation in inflamed, local lymph nodes, we infected OT-I recipient, DC-injected and control mice intra-tracheally with recombinant influenza virus encoding the ovalbumin antigen (13)

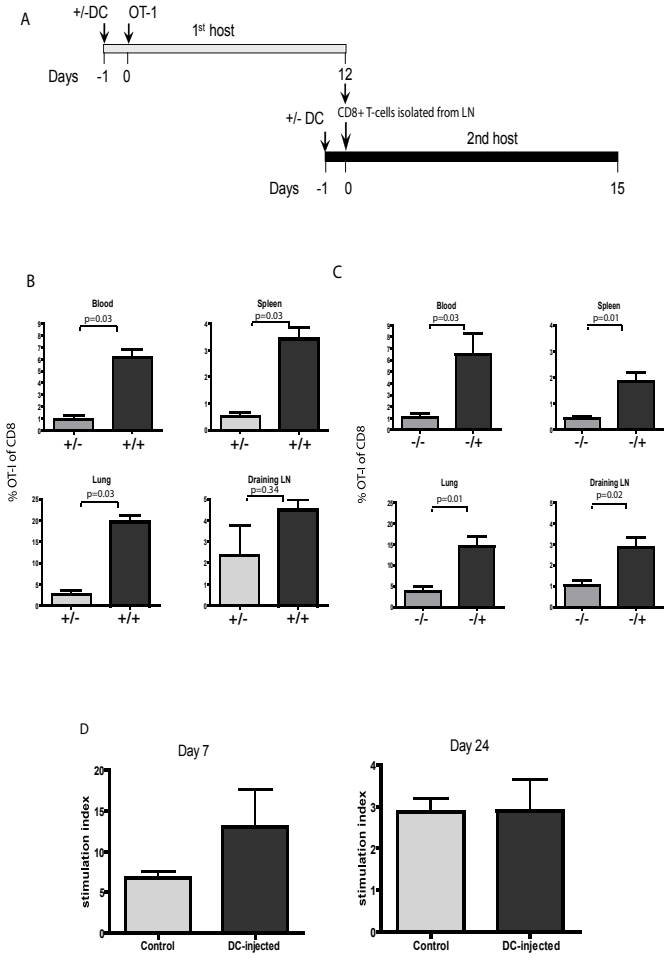

Figure 4: T-cells educated in inflamed LN are not intrinsically changed

T-cells recovered from inflamed or control LN were counted and adoptively transferred in identical numbers (10^3 OT-I cells/recipient) into new recipients with or without inflamed LN. Response to a boost vaccination was analyzed in blood, spleen, lung and draining LN. **A**: Schematic cartoon of experimental design. **B**: Mean values for percentages of OT-I cells educated in mice with inflamed LN in different compartments around peak of secondary response (day 8 after secondary challenge) 4 mice/ group. Mann-Whitney test revealed significant differences for blood, spleen and lung (p=0.03 for all compartments) but not draining LN (p=0.34) between groups. **C**: Mean values for percentages of OT-I cells educated in control mice in different compartments around peak of secondary response (day 8 after secondary challenge) 4 mice/group. Mann-Whitney test revealed significant differences for blood, spleen, lung and draining LN (p=0.03, p+0.01, p=0.01 and p=0.02 respectively) between groups. Two independent experiments performed (A and B)

T-cells recovered from inflamed or control LN at day 7 and day 24 were isolated by flow-cytometric sorting and co-cultured with irradiated spleen cells and SIINFEKL peptide in equal amounts (1250 cells/well for day 7, 500 cells/well for day 24). After 36 hours, cells were pulsed with tritium and incorporation was measured 24 hours later. Stimulation index was calculated as tritium count with peptide divided by tritium count of medium control. **C:** Stimulation index of OT-I cells recovered on day7 and day 24 respectively. Groups of 5 (day 7) and 10 (day 24) mice. Mann-Whitney test revealed no significant differences between groups. Mann-Whitney test revealed no significant differences on day 7 and day 24 (p=0.25 and 1.0 respectively).

As shown in Fig 5, DC-injected OT-I recipients have a significantly lower amount of virus particles in their lungs. This can be explained by the fact that DC-injected recipients develop a memory T-cell response superior to that of control OT-I

recipients, which is capable of more efficiently controlling the viral infection.

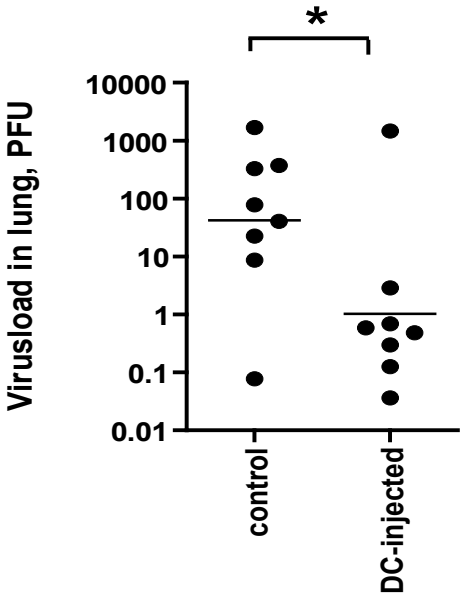

Figure 5: Presence of inflamed LN during primary response enhances anti-viral immunity

A secondary response to an influenza virus containing the SIINFEKL epitope is more vigorous in mice with an inflamed LN, induced by injecting LPS-activated DC not loaded with antigen into the footpad of mice, during the primary response. Mice with an inflamed LN or control mice were adoptively transferred with in vitro activated OT-I T-cells. After 35 days mice were infected with 5×10^4 PFU influenza virus through intra-tracheal injection. 5 days later viral load in the lungs was determined by quantitative PCR. Mann-Whitney test revealed significant reduction of virus RNA in DC-injected mice compared with control mice. (P=0.05) 8 mice per group.

4 Discussion

By separating DC-T-cell engagement and lymph node activation in space and time, we demonstrated that the presence or absence of an activated lymph node has no impact on the magnitude and kinetics of the primary response, at least in the absence of cognate antigen, but clearly influences the magnitude of the memory T-cell response upon secondary antigen encounter. Furthermore, our data show that presence or absence of an activated lymph node does not imprint intrinsic differences into the T-cells that would result in alternate survival or memory programs. Instead, the activated lymph nodes appear to primarily offer increased storage space for antigen-experienced T-cells during the contraction and memory phases of the primary response. This offers opportunities for using lymph node inflammation in conditioning patients for adoptive T-cell transfer strategies. Aswell as the current method of lympho depletion, lymph node inflammation

can create a nurturing environment for newly injected T-cells. Our findings differ from those emerging from studies that have looked at the impact of signals at the DC-T-cell interface on the development of T-cell memory. For instance, the presence of costimulatory signals involving the 4-1BB and OX40 pathways during T-cell priming imprints a survival program that promotes development of T-cell

memory and enhanced secondary T-cell responses(20,21) (22). Furthermore, the presence of CD4+ T-cells during CD8+ T-cell priming was shown to render CD8+ T-cells insensitive to TRAIL-mediated death during the secondary response, thereby endowing them with the capacity to mount an enhanced memory T-cell response (17,23). These prior studies and our present work imply that T-cell programming during DC-T-cell engagement and the availability of enlarged lymph nodes for T-cell storage are expected to synergize in the generation of potent T-cell memory.

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Chapter 5

Effectiveness and Side-Effects of Slow-Release Systems in CD40 agonistic Antibody Immunotherapy of Cancer.

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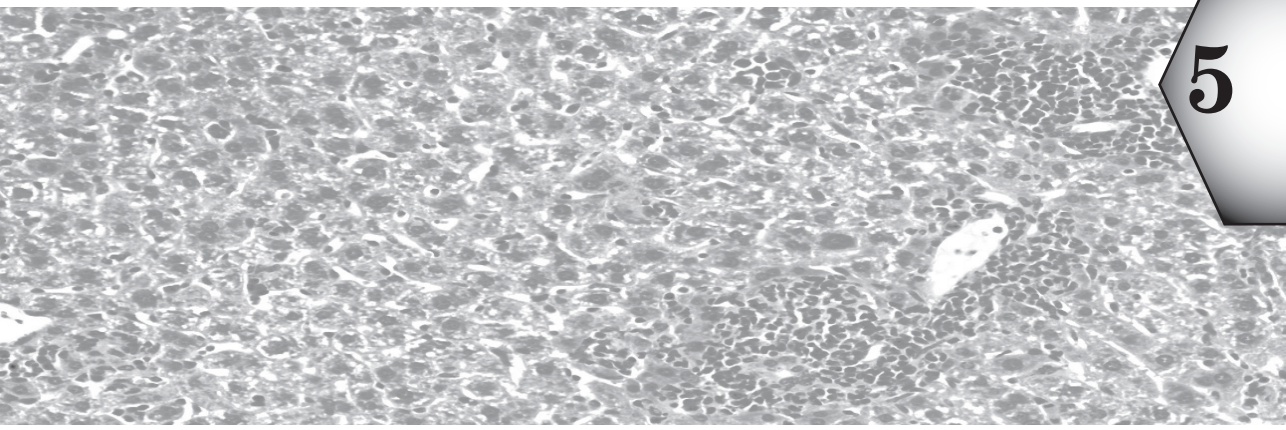

Abstract
Slow-release delivery has great potential for specifically targeting immune-modulating agents into the tumor-draining area. In prior work we showed that local treatment of slowly delivered anti-CD40 antibody induced robust anti-tumor CD8+ T cell responses without systemic toxicity. We now report on the comparison of two slow-release delivery systems for their use in antibody-based immunotherapy of cancer. Anti-CD40 antibody delivered locally in either Montanide ISA 51 or dextran-based microparticles activated tumor-specific T cell activation. Both slow-release methods decreased systemic side-effects significantly compared to systemic administration of anti-CD40 agonistic antibody. However, dextran-based microparticles caused serious local inflammation associated with enhanced outgrowth of tumors instead of the tumor clearance observed with delivery in Montanide. We therefore conclude that Montanide ISA 51 is to be preferred as a slow-release agent for immunotherapy of cancer

Introduction:

CD8+ T cells have the potential to eradicate tumors since many tumors express aberrant antigens [1, 2]. The induction of anti-tumor T-cell priming, however, is often ineffective [3]. The inactive state of dendritic cells (DCs) cross-presenting tumor antigen in the draining lymph node (LN) is one of the main reasons. Inadequate CD4+ T cell help and lack of danger signals are responsible for the inactive state of the DC[4].

Strategies aimed at activating anti-tumor CD8+ T cells directly or indirectly via tumor antigen presenting DCs have now been shown to be therapeutically useful in the treatment of cancer [5-7]. Monoclonal antibodies that modulate the immune system, such as 4-1BB, OX40 and CD40 agonists and CTLA-4 and PD-1 blocking antibodies, are available, and many are currently being investigated in pre-clinical and clinical studies [5-7]. Systemically activating the immune system, however, is perilous since this can cause adverse side-effects and risk initiating auto-immunity [8-10].

Previously we have shown that local administration of an agonist antibody against the TNF receptor family member CD40 can activate the local tumor antigen presenting DC, thereby indirectly activating the anti-tumor CD8+ T cell response. By using a slow-release system, Montanide-ISA51, this treatment was local, with significantly decreased adverse side-effects compared to the standard systemic treatment. This local, non-toxic, treatment, however, was capable of activating a systemic CD8+ T cell response, associated with eradication of both local and distant tumors [10]. We hypothesize that this strategy could be successful for many monoclonal antibodies aimed at activating the anti-tumor CD8+ T cells, tumor-antigen presenting DCs or blocking tumor induced immune suppression.

We now report on the exploration of two different slow-release systems, in order to specifically target the antibodies to the tumor-draining area at a release rate suitable for an antibody. In particular we compared the potential of dextran-based microparticles as a slow-release system for targeting immune-modulating antibodies in the tumor-draining area, with delivery in Montanide ISA 51.

Dextran-based microparticles are reportedly a well-regulated slow-release agent, which can be tailored to display the exact pharmaco-dynamic kinetics required for the encapsulated substance, with possibilities to mix particles encapsulating different substances with different release rates [11-13]. Dextran is a water-soluble polysaccharide that has good biocompatability, relative stability and a large number of hydroxyl groups, making it widely used as a polymeric building block for the design of hydrogels. Dextran-based injectable microparticles loaded with proteins can be prepared in an aqueous two-phase system avoiding the use of organic solvents. We have previously shown that the release of proteins from

these particles depends on the equilibrium water content and their degradation rate [14].

To investigate the use of dextran-based microparticles for delivering immune-modulating antibodies in the tumor-draining area, we analyzed the release kinetics and biological activity of antibodies encapsulated in different dextran-based microparticles. Agonistic CD40 antibody was used as our model immune-modulating antibody. We show that dextran-based microparticles can deliver agonistic CD40 antibodies with slow-release kinetics that can lower adverse side-effects by decreasing the serum levels of the antibodies. Our studies show that both dextran and Montanide release systems induce robust CD8+ T cell responses. However, dextran-based particles caused serious local inflammation, creating subcutaneous ulcerating lumps at the site of injection associated with enhanced outgrowth of tumors. We therefore conclude that Montanide ISA51 should be preferred as slow-release delivery system for anti-CD40 agonistic antibody treatment for cancer immunotherapy.

Material and methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. C57BL/6 Kh (B6, H- 2^b) were bred at the LUMC animal facility. The experiments were approved by the Animal Experimental Committee of the University of Leiden.

Tumor experiments

Mouse embryonic cells transformed by the early region 1A of human adenovirus type 5 (Ad5E1A) plus EJ-ras were cultured in Iscove's modified Dulbecco's medium (IMDM): (BioWhittaker, Verviers, Belgium) supplemented with 4% FCS, 50 µM 2- mercaptoethanol and 100 IU/ml penicillin/streptavidin. The E1A expressing tumor cells (7.5×10^6) were injected s.c. into 7-13 week-old male mice in 200 µl of PBS. Treatment was started 8-14 days after tumor inoculation, when palpable tumors were present. Tumor size was measured twice weekly in three dimensions and mice were killed when tumor size exceeded 1 cm^3 .

Flow cytometry

Single-cell suspension of blood and spleens, after erythrocyte lysis, and lymph nodes were stained with anti-CD8a (clone 53-6.7), CD90.1 (clone OX-7),) CD3ε (clone 145-2C11), CD19 (clone 1D3), and CD3e (clone 145-2C11), anti-CD11c (clone HL3) and CD86 (clone GL1) all from BD Bioscience and $E1A_{234\text{-}243}$-loaded H-$2D^b$ tetramers

Agonistic CD40 antibody treatment

The FGK-45 hybridoma cells producing an agonistic anti-CD40 Ab were

provided by A. Rolink (Basel Institute for Immunology, Basel, Switzerland) [17]. Hybridomas were cultured in Protein Free Hybridoma Medium (Gibco), and mAbs were purified using a Protein G column. Mice treated with high dose, systemic treatment were injected with 100 micrograms of antibody in PBS intravenously, on three consecutive days. Montanide/antibody emulsions were made by mixing different dosages of antibody in PBS 1:1 with Montanide (Montanide ISA-51, Seppic), and vortexing for 30 minutes.

Dextran-based microparticles encapsulating anti-CD40 antibody

Dextran-based microparticles were prepared as described elsewhere [18]. In brief; for dextran microspheres with a protein load of 1% w/w (weight antibody/ weight dex-hema) dextran-HEMA (hydroxyethyl methacrylate derivatized dextran) with a DS of 9.7 (degree of HEMA substitution; the number of HEMA groups per 100 glucose units, synthesis described in van Dijk-Wolthuis et al. [19] was dissolved in an aqueous solution containing the antibody. All solutions had been deoxygenized by flushing with nitrogen for 5 minutes. The ratio of dex-HEMA solution to the PEG aqueous solution was varied, in order to obtain particles with different release kinetics. The PEG/dex-Hema/water system was vortexed for 1 minute and subsequently the dextran-bound HEMA groups were polymerized using TEMED and KPS to yield crosslinked dextran microparticles. After 1 hour of incubation, the microparticles were washed three times with PBS, to remove traces of PEG, and freeze-dried. Average particle size diameters and size distributions of the microspheres were measured with an AccuSizer 780 with water as diluent.

In vitro release assay

Microparticles were weighed, dispersed in PBS and incubated at 37˚C under rotation. Samples were taken at regular intervals, and protein content was determined by Bradford analysis[20]. Percentage of protein release was calculated as amount of protein analyzed divided by total protein encapsulated. Total protein encapsulated was determined by Bradford analysis of the burst release of microspheres at pH 11.

Serum analyses

Serum samples were taken from mice at several time points after treatment. ALAT and ASAT analyses were performed by the department of Clinical Chemistry of the LUMC according to standard protocols [21]. Anti-CD40 antibody concentrations were analyzed by ELISA with anti-rat antibodies (BD bioscience).

In vivo analysis of strain 42 proliferation

Spleen and lymph nodes were isolated from Strain 42 mice, and CD8+ T cells were enriched using the BD Imag CD8+ enrichment kit, according to manufacturer's instructions. 1 million CD8+ T cells were injected intravenously into recipient mice, bearing palpable AR6 tumors. 4 days later, mice were treated with anti-CD40 in Montanide, encapsulated in different dextran-particles subcutaneously, close to the tumor, or left untreated. Blood samples were taken at regular intervals, and the percentage of Strain 42 cells of the CD8+ population was determined by flowcytometry, using the CD90.1 congenic marker, and serum levels of anti-CD40 antibody was determined by ELISA.

Results

In vitro and in vivo release from dextran-based microparticles

In order to determine whether dextran-based microparticles could be a suitable slow-release system for the use in tumor immunotherapy, we prepared dextran-based microparticles containing CD40 agonistic antibody, with different water content. By adjusting the water content, the antibody release characteristics of the particles can be modulated. The size of the particles ranged from 5 to 30 µm. The protein encapsulation was determined to be 95%. The in vitro release kinetics is depicted in figure 1A. Approximately 80% of the antibody was released from the microparticles over 30 day. Increase in water in the particle correlated with increase in rate of release. The mix of equal amounts of particles with 70%, 60% and 50% of water showed a constant rate of release, whereas the 50% water particles showed an initial delay of several days. We next established the in vivo release of antibody from particles, and correlated this to the biological activity of the antibody in the well-defined adenovirus induced AR6 tumor model[15]. Tumor-bearing mice were injected with enriched CD8+ T cells from strain 42 mice, a TCR transgenic mouse strain whose CD8+ T cells recognize the E1A epitope of the AR6 tumor. Subsequently these mice were injected sc with various dextran-particles containing anti-CD40 antibody. We used a dose of 30 µg of antibody, as was previously described to be very effective in Montanide [16]. The level of serum antibody was determined at various intervals, and correlated to the proliferation of the strain 42 CD8+ T cells. Figure 1B shows that the in vivo release of the antibody did not correlate with release kinetics in vitro, as neither the 70% nor 50% water containing particles nor the mix of 70, 60 and 50% particles displayed any delay in release in vivo. Additionally, the release of antibody from the dextran-particles was detectable in vivo for a maximum of 12 days, whereas the in vitro release continues for more than 20 days. However, the level of antibody in the serum was lowest in the 50% water particles in line with the expected slowest release rate. This obviously shows that other factors play a

role in degrading anti-CD40 particles in vivo.

Importantly, each of the delivery conditions was biologically active, since in every group a proliferative response of the Strain 42 T cells was activated as depicted in figure 1C. Each of the dextran-treated groups activated a stronger response than the Montanide treated mice, and the 50% water- containing dextran-particle group showed the highest proliferative capacity. We therefore concluded that the particles with 50% water were the most suitable formulation, since this had advanced biological activity locally, but showed the least amount of antibody systemically.

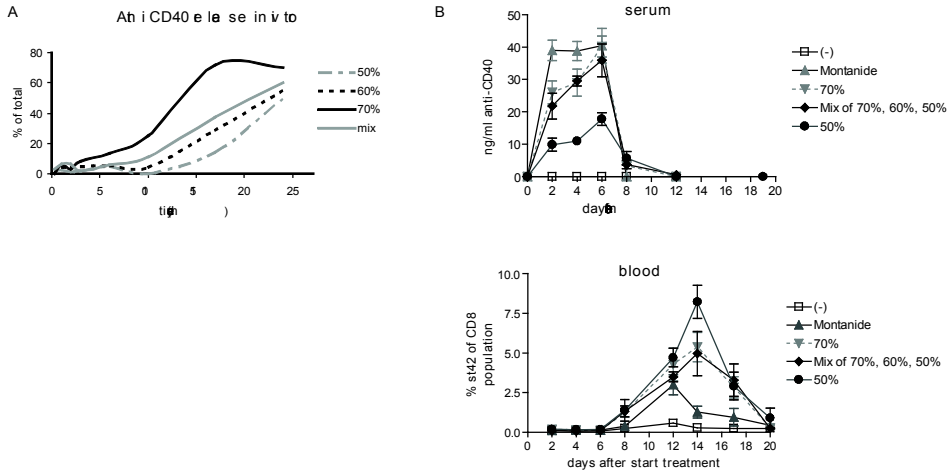

Figure 1: In vitro and in vivo release of dextran-based encapsulated antibody. Dextran-based microparticles were dispersed in PBS, and incubated at 37 ˚C. Samples were taken at regular intervals, and antibody levels were determined by Bradford analysis (A). In vivo release of antibody, correlated with biological activity of released antibody. Tumor bearing mice were injected with 1x 10^6 enriched strain42 CD8+ T cells, and treated with anti-CD40 antibody in dextran-based microparticles or anti-CD40 in Montanide. Bloodsamples were taken at regular intervals, and antibody levels were analyzed in serum by ELISA, and strain42 CD8+ T cell proliferation by flowcytometry (B) (mean ± SE, n= 4 mice per group). Data are representative of two independent experiments.

CD40-induced toxicity

As we previously published, systemic treatment with CD40 antibody causes strong adverse side-effects, as analyzed by serum levels of liver enzymes and tissue damage assessed histologically [16]. In order to determine the adverse side-effects caused by treatment with 50% water containing microparticles, compared to high dose systemic treatment or low dose Montanide treatment. We assessed serum levels of liver enzymes ALAT and ASAT, as they are indicative for liver and tissue damage respectively. Compared to the high dose systemic treatment of anti-CD40 antibody, both low dose treatments showed clear decrease in ALAT and ASAT levels, as was expected from our previous study with Montanide (Fig 2A). When comparing only Montanide with the dextran formulations, however, the ALAT levels were even lower in the dextran-group,

as predicted from serum levels of antibody, whereas for ASAT levels there is no difference between dextran-treated and Montanide-treated mice (Fig 2B). We therefore conclude that the low level systemically released antibody in 50% water-containing dextran-particles induces no detectable systemic toxicity.

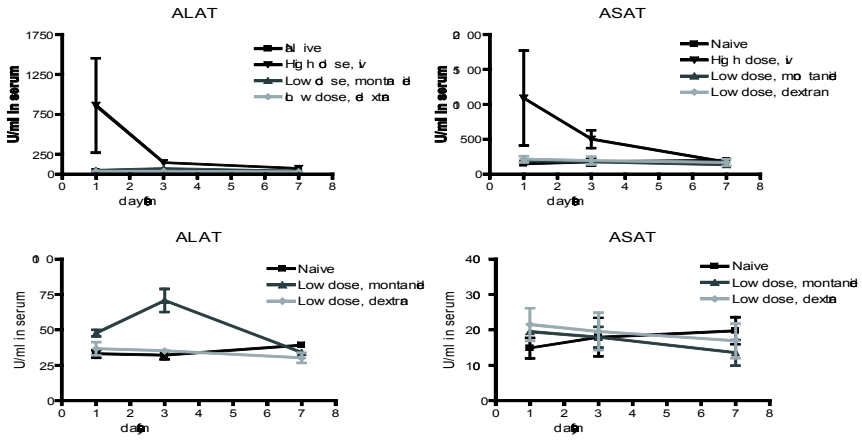

Figure 2: Systemic toxicity caused by antibody treatment. Mice were treated with different dosages and administration methods of anti-CD40 agonist Ab. Serum samples were taken at regular intervals. (A); ALAT and ASAT levels in serum, mice treated with high dose anti-CD40 antibody (2 x 100 µg) intravenously compared to mice treated with low dose (30 µg), either in Montanide, or encapsulated in dextran-based microparticles. (B); ALAT and ASAT levels in serum, low dose (30 µg) anti-CD40 antibody in Montanide treated mice compared to low dose (30 µg) dextran-based microparticles treated mice. (mean ± SE, n=5 mice per group).

Activation of the endogenous anti-tumor response

To examine whether a low dose (30 ug) of anti-CD40 antibody delivered in 50% water-containing dextran-based microparticles could activate an endogenous anti-tumor CD8+ T cell response, we treated tumor-bearing animals with different formulations of slow-release vehicles with agonistic anti-CD40 antibody.Ten days later we sacrificed the mice, and analyzed the presence of tumor-specific CD8+ T cells with tetramer staining and specific interferon-gamma expression in spleen. As shown in figure 3A and B, both high dose treatment and the low dose Montanide treatment activate a strong tumor-specific CD8+ T cell response, both detectable with tetramer and cytokine expression. In contrast, the dextran-based treatment activated a much lower tumor-specific CD8+ T cell response, similar to levels of untreated mice in the tetramer-staining, but slightly elevated in interferon-gamma expression as compared to untreated controls. Unexpectedly, dextran-based treatment seemed less effective in activating the endogenous response than in activating the TCR transgenic CD8+ T cells. The anti-CD40 was capable of activating the dendritic cells in the injection area, as the CD86 (B7.2) expression on CD11c+ cells in the LN draining the injection site was increased (fig 3C). The B7.2 expression on DC was highest in the Montanide treated mice,

and mice treated with dextran particle-packaged antibody displayed an elevation of B7.2 expression compared to non-treated mice, but lower than the Montanide treated group.

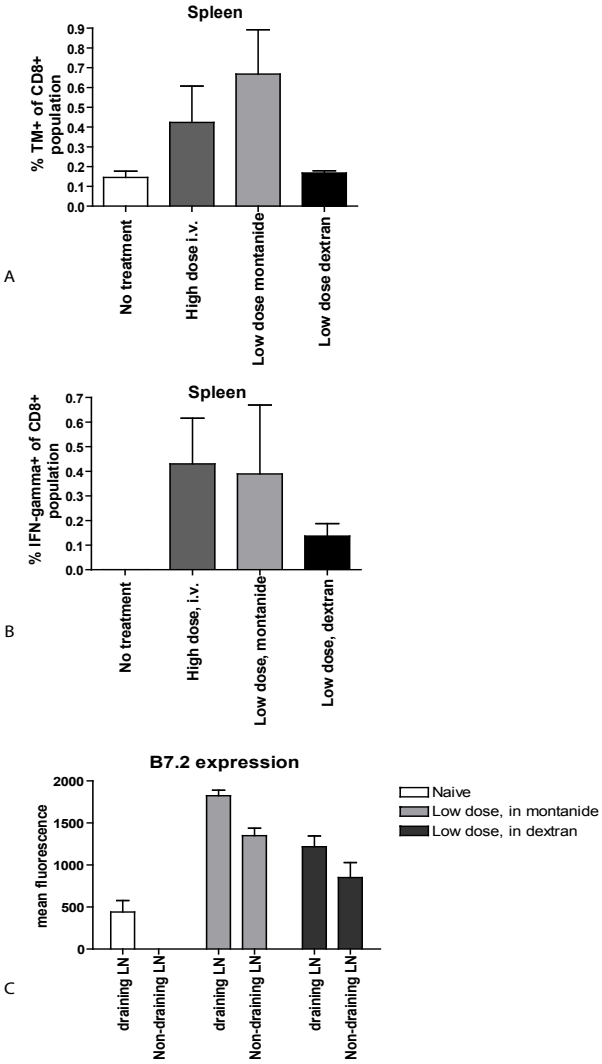

Figure 3: Endogenous immune response after dextran-based treatment. Tumor bearing mice were treated with high dose (3*100 µg) intravenously compared to mice treated with low dose (30 µg) anti-CD40 antibody, either in Montanide, or encapsulated in dextran-based microparticles. Spleen were analyzed for tetramer+ CD8+ T cells and IFN-gamma expression 9 days after start of treatment (A, B) (mean ± SE, n=4 mice per group) data representative of two independent experiments. Activation of dendritic cells in lymph nodes after treatment with low dose (30 µg) anti-CD40 antibody, either in Montanide, or encapsulated in dextran-based microparticles. B7.2 (CD86) expression on CD11c high cells in draining versus non-draining lymph nodes of tumor-bearing mice (C). Students T-test revealed significant difference in B7.2 expression on DC between draining and non-draining lymph nodes of Montanide treated mice (P=0.03) (mean ± SE, n=4 mice per group), data representative of two independent experiments.

Eradication of tumors after dextran-particle treatment

Next we analyzed the capacity of dextran-based CD40 treatment to control tumors in a therapeutic setting. Mice were inoculated with tumor cells and treated with anti-CD40 when tumors were palpable. In figure 4A and B the outgrowth of the tumors and the survival of mice following the different treatments are depicted. The majority of mice treated with high dose systemic anti-CD40 antibody and low dose Montanide anti-CD40 antibody eradicated their tumor, whereas the

majority of non-treated mice and mice treated with low-dose dextran-particle anti-CD40 antibody succumb to tumor burden.

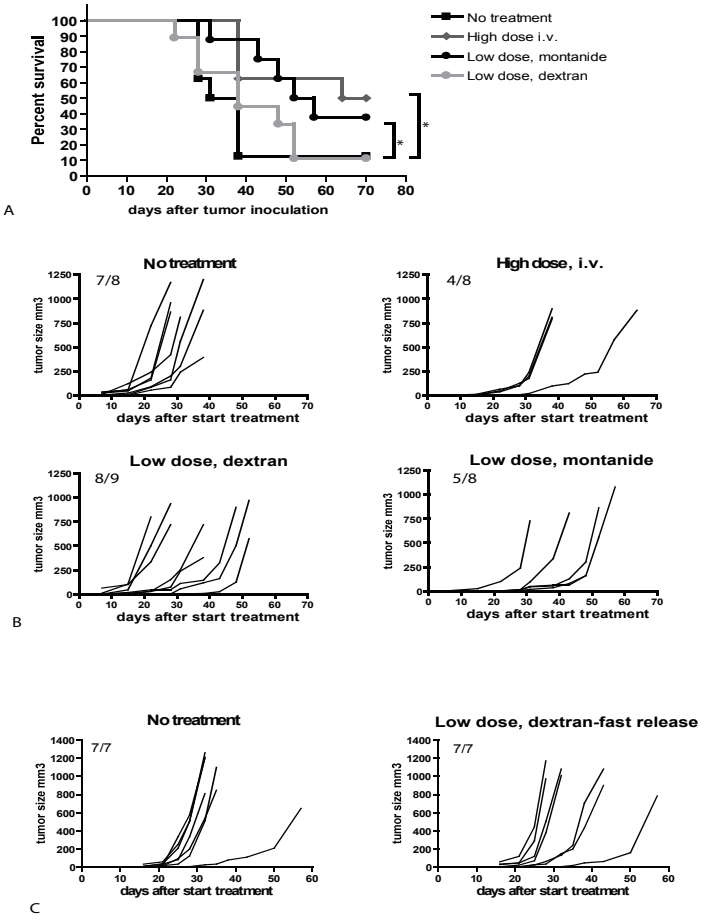

Figure 4: Tumor eradication by low dose, local anti-CD40 antibody treatments. Survival and tumor growth of tumor-bearing mice after treatment with 30 µg of anti-CD40 agonist Ab in Montanide or 30 µg of anti-CD40 agonist Ab in dextran-based microparticles , subcutaneously, compared with high dose (3*100 µg) intravenously, 8 days after tumor inoculation. Data presented as survival with 8 or 9 mice per group. (A); the Kaplan–Meier test revealed a significant difference between local Montanide treatment and no treatment, and between intravenous treatment and no treatment (P = 0.04 and P= 0.01, respectively). (B); data presented as tumor growth in each mouse, 8 or 9 mice per group, number of mice that died of tumor burden indicated in top left-hand corner. (A,B); data representative of 3 independent experiments.

There is a clear delay in tumor outgrowth in most of the mice in the dextran-particle treated group compared to the non-treated group (Fig 4B), but treatment is not effective enough to cure the mice. Most likely due to the slower release kinetics of the dextran-particles, and the slower kinetics of the anti-tumor T cell response, the treatment was suboptimal for tumor-eradication. To determine if the slow kinetics were responsible for the poor tumor eradication we treated

mice with a similar dose of anti-CD40 antibody in dextran-particles with faster release kinetics, namely the 70% water containing particles. As is shown in figure 4C, the faster release particles did not lead to better tumor control.

Treatment with a higher dose of dextran particles.

Since previous experiments showed that serum levels of antibody and liver enzymes were decreased after treatment with 50% water containing dextran-particles compared to Montanide, we conjectured that there was a window in which we could use a higher dose of anti-CD40 antibody encapsulating dextran-particles in order to achieve improved anti-tumor responses without increasing adverse systemic side-effects from the antibody levels in the serum. We analyzed the tumor eradication after treatment with a 3 times higher dose of antibody in dextran-particles. Since the antibody to dextran-ratio was fixed, we injected tumor-bearing mice with 3 times more dextran particles. Unexpectedly, the tumor in the mice treated with the higher dose of dextran and antibody grew out faster than the untreated group, as is shown in figure 5. Simultaneously, the mice in this group developed large ulcerating subcutaneous lumps at the site of injection. This phenomenon was also observed in mice treated with control dextran-particles, and mice treated with lower doses of dextran particles, but to a much lesser extent. The overall outcome of these experiments led us to conclude that dextran-based microparticles induce undesirable local side-effects and are therefore not recommended as slow-release vehicles for tumor immune therapy.

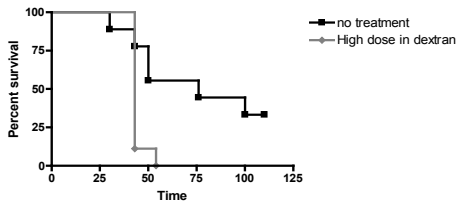

Figure 5: High dose of dextran-based microparticles enhances tumor-outgrowth.Survival of tumor-bearing mice after treatment of 100 µg of anti-CD40 antibody in dextran-based microparticles, compared to non-treated mice. The Kaplan-Meier test revealed a significant difference between microparticles treated mice and non-treated mice (P=0.01) 9 mice per group.

Discussion

We here report the comparison of dextran-based microparticles and Montanide ISA-51 for delivering immune-modulating antibodies in the tumor-draining area in order to activate the anti-tumor T cell response. By comparing dextran-particles with the previously described slow-release agent Montanide-ISA-51 for release kinetics, adverse side-effects, and ability to activate immune cells and mediate tumor eradication, we determined whether dextran-particles could be a suitable slow-release method for the use in tumor immune therapy. We show that dextran-particles are a very effective slow-release agent, with valuable

options to tailor release kinetics and thereby decrease the adverse systemic side-effects of the immune-modulating antibody. However, the dextran-microparticles unfortunately cause serious local inflammatory side-effects, associated with enhanced tumor outgrowth and ulcerating subcutaneous swellings.

We contemplated that the enhanced outgrowth of the tumor cells could be caused by two possible, not mutually exclusive, processes. The ulcerating lumps formed at the injection site can cause attraction of innate immune cells. In a previous study by de Groot et al. histological analysis of dextran-associated subcutaneous masses determined the infiltrate to be macrophages and neutrophils [22]. Innate immune cells, like macrophages and neutrophils have been implicated in creating tumor-promoting micro-environments [23, 24]. The second process possibly responsible for the enhanced outgrowth of tumors is the presence of dextran near the tumor, serving as an additional source of nutrient for the tumor cells. Dextran is composed of large sugar molecules that can be broken down to glucose. Tumor cells reportedly have an elevated glucose metabolism, growing faster in the presence of higher level of glucose [25, 26].

The results obtained in this study are contradictory to an earlier study in which tumor bearing mice were treated with dextran-based microparticles containing the cytokine IL-2 [27]. In this study efficient anti-tumor responses were found, and no adverse events of dextran were observed. The model used in this study was a lymphosarcoma, which was injected intraperitoneally, like the IL-2 containing microparticles. The fact that both tumor and microparticles are in the peritoneal cavity in this model, compared to the subcutaneous location in our model, could explain the differences found. In the peritoneal cavity, the proximity of the microparticles to the tumor was reduced, and the microparticles were dispersed through the cavity, and did not form a depot as they did subcutaneously. Additionally, the glucose metabolism of the two tumor models could be different. Dextran-microparticles could be a potentially attractive formulation for other purposes, such as vaccination, considering the efficient activation of CD8+ T cells by anti-CD40 antibodies, combined with lower serum levels of antibody, and lower systemic toxicity. Since the adverse events were shown to be dextran-specific and deteriorated with increased amounts of injected dextran, it is possible, that by altering the protein to dextran-ratio, dextran-microparticles can be prepared with less adverse events as the particles described in this study.

In the present study, we show that dextran-based microparticles are an effective slow-release delivery system for antibodies. The delivery can be tailored, and the amount of antibodies in the serum remains low, thereby reducing antibody-mediated toxicity. However, adverse events caused by the dextran, make this delivery system less suitable for the use in tumor-immunotherapy. Since Montanide ISA-51 does not display these disadvantages, we conclude

that Montanide is preferred over dextran-based microparticles as slow-release delivery system for the use in tumor-treatment. This study shows that local CD40 agonistic antibody delivery is a very potent approach for cancer treatment, but great care should be taken in selecting a proper slow-release delivery system for use in tumor-immunotherapy.

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Chapter 6

Discussion:

Local targets for Immune Therapy to Cancer: Tumor Draining Lymph Nodes and Tumor Microenvironment.

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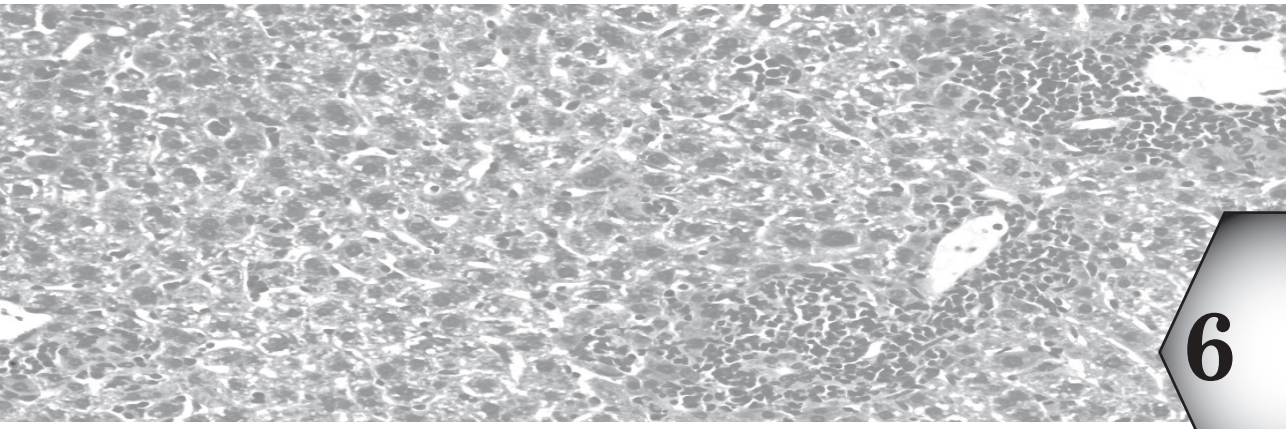

Abstract

In recent years, it has become apparent that in subjects with growing tumors, there is a balance between tumor-eradicating and tumor-promoting immunity. The key players in maintaining this balance are mainly present in the tumor microenvironment and the tumor-draining lymph node. Interventions aimed at shifting the balance towards tumor-eradicating immunity, are therefore most efficient when targeted directly to this area as shown in this thesis. As immune-modulating therapy has been shown to cause many adverse side-effects when administered systemically, we strongly advocate the further development of local treatment for tumor- immune therapy.

6

Introduction:

Lymph nodes (LNs) are organs comprised of lymphoid cells that occupy strategic positions throughout the body and play a pivotal role in the immune system. LNs act as sentinels within the system, filtering the afferent lymph and bringing together cells of the innate and adaptive immune system interact, which in case of acute infection, leads generally to robust priming of naïve T cells. Tumor-draining LNs (TDLNs) have a dubious position as they can induce anti-tumor T cell responses but are at the same time under the direct influence of the tumor microenvironment and can act as route for malignant cells towards distant organ metastasis[1-3] Because of this, the controversial role of surgical removal of sentinel LNs has been a matter of debate for decades[4].

The concept of tumor immune surveillance, the potential of the immune system to keep the formation and outgrowth of malignant cells in check, has been described as early as 1891, by William Coley, and has waned and recurred in scientific publications several times over the last 120 years. The experimental evidence supporting the concept of immune surveillance is still growing (see for recent reviews Swann and Smyth[5] and Vesely MD et al[6]) and was recently extended by Schreiber and colleagues who described the association of tumor cells and lymphocytes actively inhibiting the formation and progression of transformed cells and ultimately causing selective evolution of tumor cells that can evade the immune response, a phenomenon called cancer immunoediting[7,8]. Based on this knowledge many tumor intervention treatments have been designed and studied involving immunotherapy in both pre-clinical models and clinical trials with varying successes and pitfalls. The potential for targeted immunotherapy to the tumor area and more specifically to the TDLN has been brought forward in recent years. In this review we would like to discuss the latest insights into tumor immune therapy and the strategies and advantages of local targeting.

Balancing induction and suppression of tumor immunity.

The growth of a tumor often coincides with both the stimulation of anti-tumor T cell responses and the parallel (and often unwanted) induction of immune suppression. Both processes take place mainly in the tumor area and TDLN. This balance between T cell priming and suppression is one of the key aspects in disease prognosis[9].

In mice the critical importance of the adaptive immune system, especially of T cells, to prevent tumor development was proven by sophisticated experimental tumor models involving the ablation of the specific regulators of the adaptive immune system[10-12]. In cancer patients, significant numbers of T cells specific to tumor associated antigens have been identified and found to be correlated with improved prognosis[9,13-17]. Tumor antigens are presented by APCs within

the tumor or are first carried from the tumor by tumor cells or APCs traveling through lymphatic channels to become cross-presented in LNs to T cells[18,19,20]. The APCs in the tumor mass and TDLN generally display a certain level of maturation due to the presence of endogenous danger signals from the growing tumor, such as heat shock proteins and uric acid from decaying tumor cells. Compared to pathogenic infections however, the APC maturation signals are much lower, which can lead to inadequate T cell priming[20-23]. Especially, the lack of costimulatory signals (e.g. CD80/86) has been linked to dysfunctional T cells. Likely, also the lower production of the pro-inflammatory cytokines IL-12 and IFN-gamma[20,21,23] contributes to the low state of T cell activation to tumor antigens. This phenomenon of inadequate T cell activation has led to different nomenclatures for these T cells including anergic T cells, division arrest T cells, incomplete differentiated T cells, dysfunctional T cells and tolerised T cells. In tumor settings anergic T cells are characterized by inadequate effector function such as the lack of cytolytic molecules (e.g. perforin or granzyme B), expression of low levels of IFN-γ and a division arrest phenotype, which all contribute to reduced capacity to kill tumor cells[19,24-28]. Besides lower "quality" of T cell priming by APCs, both animal models and human studies show that TDLN also harbor lower numbers of DC. Nevertheless, tumor specific T cells with full cytotoxic capacity have been described with respect to phenotype and function[9,25,29], suggesting that transformed cells can lead to proper T cell activation providing hope for immunotherapeutic strategies.

Immune suppression within the tumor microenvironment and TDLN is characterized by an unfavorable concoction of immunosuppressive cytokines, growth factors, and various suppressing cell populations. Well studied suppressive cytokines, produced by tumors or tumor associated macrophages, are IL-10, TGF-ß, VEGF and IL-6. Chemokines, such as CCL2 and CXCL8, secreted by monocytes and tumor associated macrophages, cause tumor progression, myeloid derived suppressor cell (MDSC) and macrophage infiltration and tumor angiogenesis. Effector T cell suppression is mediated by regulatory T cells, MDSCs and tolerogenic DC[21,22,30]. A special type of factor that inhibits the induction of pro-inflammatory immune responses is IDO (indoleamine 2,3 dioxygenase). IDO, expressed on plasmacytoid DC and some types of tumor cells, causing inhibition of T cell proliferation by enzymatically degrading tryptophan leading to tryptophan starvation. This can also lead to conversion of CD4+ T cells to Tregs in TDLN[31].

The increased presence of regulatory T cells (Tregs) in TDLN compared to non-draining LN has been well established in both animal models and cancer patients, in which accumulation of Tregs in DLN of colorectal cancer patients and not in tumor or peripheral blood is correlated with disease progression[22,30,32,33]. Treg

accumulation in tumor bearing animals can result from either proliferation of natural, thymic differentiated Tregs or conversion of naïve $CD4^+$ T cells into Tregs. The mechanisms of Treg suppression are not fully understood yet, but can include IL-2 deprivation, expression of CTLA-4, and secretion of IL-10 and/or TGF-ß. Recent publications showed that Tregs can also limit DC, NK and $CD8^+$ T cell numbers by direct granzymeB and perforin dependent killing in TDLN[31,34]. Further limiting the immune response is the fact that in elderly patients (the majority of cancer patients), the immune system has undergone aging[35]. This phenomenon, also called immunosenescence, is characterized by loss of immunocompetence which limits immune resistance not only to tumors but also to pathogens such as influenza virus, respiratory synticium virus, pneumococci and tubercle bacilli as well as to, chronic persistent viruses such as CMV. Therapies designed in animal models to boost the immune systems against tumors may be imperfect in elderly patients, because of this phenomenon, and more vigorous therapies or different strategies may be necessary. In this aspect, it is interesting to note that Belloni et al recently reported age-dependent differences in side-effects to systemic anti-IL10 receptor antibodies. IL-10 inhibition caused high mortality in older animals, whereas no mortality was observed in young animals. Since cancer patients are often older individuals, these results imply that systemically blocking the IL-10 receptor should be evaluated carefully[36].

Local Immune therapy: targeting the tumor micro-environment and draining lymph nodes.

Decrease of adverse side-effects by local treatment versus systemic treatment.

Recent reports describe the dangers of toxic side-effects of systemic immune activating treatments, emphasizing the need for more targeted therapies. Together with the growing evidence defining the local suppressive effects of the tumor microenvironment and the unique position of the tumor draining lymph node, this calls for exploring the potential of immune intervention strategies that act mainly locally.

Many different strategies have been proposed to re-activate the TDLN resident anergic T cells, and overcome tumor induced immune suppression, some of which specifically target the tumor, tumor draining area and/or tumor draining LN. Many of these strategies were first described in systemic applications of immunostimulatory strategies in experimental models and later in clinical trials. Numerous pre-clinical studies have described that such systemic therapies can overcome T cell anergy, either by activating DCs (using TLR-ligands or agonistic CD40 antibody), blocking inhibitory signals (blockade of CTLA-4, PD-1 or TGF-ß), or addition of pro-inflammatory cytokines (IL-12, IFN-α or IL-2) [37,38] resulting in tumor eradication. Clinical trials, however, did not show a similar

success rate in clearing tumors as observed in some animal models. Frequently, the relative dose of immune stimulating reagents used in rodents is higher then the maximum tolerated dose used in humans (correlated for body weight). Immunologists using animal models are often less focused on side effects than on efficacy. However, more researchers are starting to become aware that in order for pre-clinical animal models to be more representative to the human situation, lower doses of immune stimulating agents should be used, and toxic side-effects in animal models should be meticulously analyzed,[36,39,40] as described in chapter 2.

Moreover, systemic activation of the immune system can cause serious toxicity as shown in a number of clinical trials and animal studies. An example is the catastrophic clinical trial with CD28 superagonist TGN1412. Indeed, potent systemic activation of the entire immune system is unadvisable, and should be applied with utmost caution[41]. In many other studies, adverse events caused by systemic immune activation were dose-limiting and hampered the efficiency. Agonistic antibodies against CD40 and cytokines IL-12 and IL-2 have all been described to have potent effects in enhancing the anti-tumor T cell response, and all have been causing severe toxicity in patients after systemic administration[42-44]. Even GM-CSF administration, which is not directly immune activating and therefore contains a lower risk of causing toxicity, has been shown to have adverse effects when injected systemically. Serafini et al published a paper in which data was presented showing the increase in MDSC in subjects treated with high dose systemic GM-CSF, causing an impaired immune response[45].

Specific targeting of the tumor microenvironment.

One way of reducing systemic side effects is to target exclusively the tumor lymphoid drainage area. For instance, CpG, a toll-like receptor 9 ligand, injected locally enhances DC maturation and migration to TDLN[40,46-48]. When compared to other administration routes, local injection was superior in DC maturation, T cell priming and tumor eradication, in a preclinical model[48]. In a clinical trial, CpG was administered intradermally directly adjacent to the scar of melanoma resection, before the sentinel lymph node (SLN) resection, and the immune response was analyzed in the SLN and PBMC. Patients displayed higher numbers of DC in the SNL associated with upregulation of costimulatory molecules, increased release of pro-inflammatory cytokines and reduction in immunosuppressive Treg frequencies. Fifty percent of these patients had a measurable pro-inflammatory T cell response against melanoma specific tumor antigens in the SLN and in 40% of the patients, a T cell response was also found in blood. This therapy was well tolerated by patients. In another clinical trial, intratumoral injection of CpG was combined with low dose, local irradiation.

An increase in tumor specific T cells was detected in PBMC of patients, and objective responses were found[49 50].

Induction of inflammation in the tumor lymph node draining area leads to upregulation of several factors, like CCR7 on DC and CCL21 on lymphatic endothelial cells which in turn lead to enhanced migration of DC to the lymph node[51,52]. The influx of mature DC into the LN causes the lymph node to increase in size and cellularity, called reactive lymph node. The inflammatory state of the reactive lymph node influences the activation of T cells, as described recently. Especially important for memory recall responses, T cells that had developed in the presence of a reactive lymph node had a significant quantitative advantage over T cells in mice without a reactive lymph node[53](chapter 4). In animal models and clinical trials, genetically engineered tumor cells secreting GM-CSF, CTLA-4 blocking antibody or CCL20 (a DC attracting chemokine), have been studied as local treatment. By injecting the irradiated tumor cells close to the tumor, they serve as antigen and antibody secreting depot to the TDLN, and cause activation of effective anti-tumor T cell responses and tumor eradication, with lower treatment associated toxicity than upon systemic administration[54,55].

Previously we reported that targeting the tumor-draining area with a low dose of agonistic CD40 antibody in a slow-release formulation overcomes tumor-induced immune suppression and induces excellent systemic tumor-specific T cell responses capable of killing metastatic cells located elsewhere in the body. Local therapy therefore can thus lead to systemic responses, with only a fraction of the toxic side-effects[56](chapter 2). Local, slow-release administration of CTLA-4 blocking antibody is also capable of activating tumor-eradicating CD8+ T cells as a monotherapy, as described in chapter 3. This treatment severely reduced the serum-levels of CTLA-4 blocking antibody compared to systemic administration, reducing the risk of auto-immune related side-effects.

It is likely to assume that slow-release formulations are functional in targeting immune stimulating agents to the TDLN, because they keep the tumor-draining area, or regional basin, in a pro-inflammatory status for a prolonged period of time, allowing the T-cell response to fully develop and the immune suppression to remain blocked. In addition, the concentration of immune stimulatory agent remains high only locally and not systemically, thereby preventing undesirable side-effects and unspecific overstimulation. Slow-release formulations such as montanide ISA 51, have been studied for their efficiency in delivering immune modulating antibodies (such as anti-CD40) to the TDLN with strong systemic anti-tumor responses as a result, but no systemic toxicity[56](chapter 2 and 3). The discovery of several new sustained release systems, such as PLGA-based microparticles, opens up possibilities for targeted treatments which can be explored for tumor immunotherapy. [57,58] However, as described in chapter

5, dextran-based microparticles have unexpected local side-effects, causing enhanced tumor-outgrowth, making them inferior as slow-release delivery system to Montanide. Slow-release formulations should therefore be carefully analyzed for their suitability in tumor-area targeted therapy.
Another aspect that strengthens the use of local immunotherapy lies in the fact that many immunosuppressive mechanisms that inhibit tumor-specific effector T cell responses, as described before, are not uniquely operable in the tumor microenvironment, but are mechanisms that have evolved to keep the immune system from attacking self tissue. Interfering with these interactions on a systemic scale, therefore, is risky. Not surprisingly, examples of systemic immunostimulatory tumor immunotherapy causing severe autoimmunity are abundant [38,59,60].

Potential hurdles for local immunotherapy.
Recent studies have shown that elevated levels of MDSCs are present in cancer patients and tumor bearing mice. Since these cells are described to incite systemic suppression, rather then local suppression, targeting of the TDLN is not likely to overcome suppression by these cells[61,62]. Several studies mentioned in this review describing local targeting of the TDLN have been able to overcome local suppression by activating robust anti-tumor T cell responses, which are able to withstand systemic suppression and eradicate distant tumors. However, systemic suppression by MDSC was not analyzed in these studies, and might have been weak, where in other models, it could be stronger, and therefore harder to overcome.
Targeting TDLN might cause the practical problem of inaccessibility of a draining node, since in several types of cancer TDLN's are not within easy reach. In order to solve this problem, new approaches are being studied such as delivery of nanoparticles coupled to tumor-antigen-specific antibodies, which can be injected systemically but, deliver their immuno-modulating content selectively into the tumor from where it will eventually drain to the TDLN[40,46].

Concluding remarks.
The tumor microenvironment and especially the TDLN are the key locations for important anti-tumor immunological processes, and therefore the quintessential targets for immune-modulating therapies in tumor bearing subjects. Since both priming of tumor-specific T cell responses and immune suppression occur in this area, local therapies designed to balance this equilibrium toward more effective anti-tumor T cell response will be most efficient. Whether tumor eradication is most efficiently achieved by promoting the stimulation of DCs presenting tumor

antigens, enhancing tumor antigen presentation, abolishing immune suppressive pathways, or a combination of these, remains to be defined experimentally and clinically. Notably, since most of the tumor-immunotherapy strategies harbor the risk of causing serious toxicity and/or auto-immunity, targeting the TDLN and/or the tumor microenvironment instead of systemic administration should be a focus of future immuno-therapeutic strategies.

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Nederlandse samenvatting voor niet-ingewijden

Ons afweersysteem is ontstaan om ons te beschermen tegen ziekteverwekkers zoals virussen en bacteriën. Het bestaat uit een groot aantal verschillende soorten cellen met elk een specifieke functie, die moeten samenwerken om ziekteverwekkers te kunnen herkennen en opruimen. Het moeilijkste van dit proces voor de afweercellen is onderscheid te maken tussen gezond weefsel en onschuldige indringers (zoals bacteriën in je darmen of deeltjes uit je voeding) en ziekteverwekkers. Als het afweersysteem gaat reageren tegen gezond weefsel kan auto-immuniteit ontstaan, en als het afweersysteem reageert tegen onschuldige indringers, dan kunnen allergieën ontstaan. Om het onderscheid te kunnen maken tussen gezond/onschuldig en ziek moeten afweercellen goed communiceren. Met een strak gereguleerde balans tussen activeren en afremmen moeten ze tot de juiste actie overgaan.
Sinds een tijd weten we dat het afweersysteem ook belangrijk is bij kanker. Bij patiënten die een verminderde weerstand hebben door medicatie (bijvoorbeeld na transplantatie) of ziekte (bijvoorbeeld bij AIDS patiënten) ontstaan sneller tumoren. In veel kankerpatiënten en proefdiermodellen van kanker zijn bewijzen gevonden van een afweerrespons tegen de kankercellen. De afweerrespons is niet sterk genoeg om tumoren volledig op te kunnen ruimen. Dit komt voor een groot deel door de strakke regulering van de balans van het afweersysteem. Doordat kankercellen lichaamseigen cellen zijn en er geen sprake is van duidelijke schade zoals dat bij virale of bacteriële infectie wel het geval is, blijft de balans van het afweersysteem op afremmen in plaats van activeren. In dit proefschrift wordt onderzoek beschreven naar therapieën om de afweerrespons tegen tumoren te activeren, zonder daarbij een te groot risico te lopen op het opwekken van auto-immuniteit.

Een bepaald soort cel van het afweersysteem, de dendritische cel (DC), fungeert als verkenner in het lichaam. Deze cellen bevinden zich op alle plaatsen waar indringers het lichaam zouden kunnen binnendringen. Een DC speurt continue de omgeving af en verzamelt materiaal door bijvoorbeeld dode cellen op te nemen. Als er veel dode cellen en schade zijn, bijvoorbeeld bij een infectie, dan worden DCs geactiveerd waarna ze migreren naar de dichtstbijzijnde lymfeklier. Daar bevinden zich vele andere soorten afweercellen die met de DC kunnen communiceren. De DC laat kleine stukjes van het materiaal dat hij heeft opgenomen aan zijn celoppervlakte zien. Daarmee laat de DC aan de afweercellen in de lymfeklier zien wat er in het lichaam aan de hand is. Meerdere cellen scannen de DCs; wanneer ze iets herkennen, gaan ze tot actie over. Een bepaald soort cel van het afweersysteem, de CD8 T cel, kan, als hij het juiste signaal van

de DC heeft gekregen, overgaan tot het doden van geïnfecteerde cellen. Elke CD8 T cel herkent iets anders; pas als de CD8 T cel in contact is gekomen met een geactiveerde DC die dat specifieke stukje (antigeen) heeft gepresenteerd, en daarbij nog enkele activerende signalen heeft gegeven, gaat deze cel heel hard delen. Als er voldoende cellen zijn die allemaal dat specifieke stukje antigeen herkennen, gaan de CD8 T cellen door het lichaam op zoek naar cellen die ook dat antigeen hebben en maken deze cellen dood. Die stukjes antigeen kunnen afkomstig zijn van virussen of bacteriën, maar dat kunnen ook stukjes materiaal van kankercellen zijn.
Als alle cellen die de stukjes antigeen hebben zijn opgeruimd, gaat het grootste deel van de CD8 T cellen dood. Een klein deel blijft leven en wordt geheugencellen. Deze geheugencellen reageren veel sneller wanneer eenzelfde virus/bacterie nogmaals het lichaam binnendringt. Dit is het principe van immuniteit tegen bepaalde ziekte en ook het principe van vaccinatie. Door bewust afweercellen te activeren tegen stukjes van bepaalde virussen of bacteriën kunnen we zorgen dat er geheugencellen ontstaan, zodat ons afweersysteem adequaat en snel kan reageren als we later met dezelfde virussen of bacteriën in aanraking komen.

<u>Hoofdstuk 2:</u> Lokale toediening van anti-CD40 activerend antilichaam in een langzame afgifte depot geeft goede tumor opruiming en sterk verlaagde toxiciteit

In hoofdstuk 2 wordt een manier beschreven om de CD8 T cellen die kankercellen kunnen herkennen te activeren, zonder daarbij het hele afweersysteem te activeren. In een proefdiermodel laten wij zien dat er in de poortwachter lymfeklier, de lymfeklier die het dichtst bij de tumor ligt, tumor-specifieke CD8 T cellen zijn. Deze tumor-specifieke CD8 T cellen zijn niet voldoende geactiveerd en hebben niet genoeg gedeeld om de tumor op te kunnen ruimen. In eerdere onderzoeken is al aangetoond dat als je deze muizen injecteert met anti-CD40 activerend antilichaam, de CD8 T cellen wel voldoende worden geactiveerd en de tumor gaan opruimen. Dit werkt als volgt; de anti-CD40 activerende antilichamen binden aan het molecuul CD40 op de DCs, en geven een activerend signaal. In de poortwachter lymfeklier zitten DCs die stukjes van de tumorcellen presenteren, en als deze door de anti-CD40 activerende antilichaam worden geactiveerd, kunnen ze op hun beurt de tumorspecifieke CD8 T cellen activeren. Het probleem met deze therapie is dat het anti-CD40 activerende antilichaam in het hele lichaam alle DCs activeert, en nog een heleboel andere afweer cellen, die ook het molecuul CD40 hebben. Dit geeft onnodige schade en een verhoogd risico op auto-immuun ziektes. Ons onderzoek toont aan dat als je het anti-CD40 antilichaam inspuit dicht bij de tumor en de poortwachter lymfeklier, in een

emulsie waaruit het antilichaam langzaam vrijkomt, de tumorspecifieke CD8 T cellen door de DCs in de poortwachter lymfeklier worden geactiveerd, maar dat er niet zoveel van het antilichaam in de rest van het lichaam terecht komt dat alle immuuncellen geactiveerd raken. De bijwerkingen van deze manier van toediening zijn veel lager dan wanneer het anti-CD40 antilichaam in het bloed wordt ingespoten. Daarnaast is veel minder van het antilichaam nodig bij lokale toediening voor een goed effect. Bovendien, doordat de tumor-specifieke CD8 T cellen in de lymfeklier goed worden geactiveerd en gaan delen, kunnen ze daarna in het hele lichaam op zoek gaan naar tumorcellen. Eventuele uitzaaiingen kunnen dan wel worden opgeruimd door de CD8 T cellen, ook als het anti-CD40 antilichaam er niet bij in de buurt komt.

<u>Hoofdstuk 3:</u> Lokale toediening van CTLA-4 blokkerend antilichaam in een langzame afgifte depot geeft goede tumor opruiming via activering van CD8 T cellen

In hoofdstuk 3 wordt dezelfde lokale toediening in een emulsie toegepast met CTLA-4 blokkerend antilichaam. CTLA-4 is een molecuul op de oppervlakte van T cellen dat betrokken is bij de regulering van activatie van T cellen. CTLA-4 geeft een remmend signaal aan T cellen om responsen tegen lichaamseigen cellen te voorkomen. Het blokkeren van CTLA-4 wordt gebruikt om bij tumor-specifieke T cellen die remmende werking op te heffen. Dit antilichaam is onlangs goedgekeurd in de Verenigde Staten voor gebruik in patiënten met vergevorderde huidkanker. Bij deze patiënten wordt het blokkerende antilichaam toegediend in het bloed. Er zijn goede resultaten geboekt met remming van de groei van tumoren en activering van de T cellen tegen tumoren. Een groot deel van de patiënten die wordt behandeld met dit antilichaam krijgt ook last van auto-immuun gerelateerde bijwerkingen, zoals ernstige diarree en huidontstekingen. Dit is logisch omdat het blokkeren van CTLA-4 niet alleen bij tumor-specifieke T cellen gebeurt, maar bij alle T cellen. Wij laten zien dat voor het CTLA-4 blokkerende antilichaam hetzelfde geldt als voor het anti-CD40 activerende antilichaam. Als je het dicht bij de tumor en de poortwachter lymfeklier spuit in een emulsie die voor langzame afgifte zorgt, krijg je met een veel lagere dosis van het antilichaam een even goed effect op tumoren als bij een toediening van een hoge dosis in het bloed. Dit geeft minder bijwerkingen en verlaagt het risico op auto-immuniteit.

<u>Hoofstuk 4:</u> De invloed van een lokaale geactiveerde lymfeklier op de ontwikkeling van de CD8 T cellen

In hoofdstuk 4 wordt beschreven wat de bijdrage is van het geactiveerde milieu van een lymfeklier in een afweerrespons. Tijdens een infectie wordt de lokale lymfeklier geactiveerd. Door een sterke influx van cellen zwelt de lymfeklier op en worden allerlei activerende stoffen uitgescheiden. In ons onderzoek hebben wij een methode opgezet waarmee we precies de invloed van deze geactiveerde omgeving kunnen bepalen. CD8 T cellen worden kort in kweekschalen geactiveerd onder precies dezelfde omstandigheden, waarna ze in muizen met of muizen zonder een geactiveerde lymfeklier worden gespoten. De cellen worden daarna op verschillende momenten geanalyseerd op aantallen, capaciteit tot het doden van hun doelwit cellen en ontwikkeling tot geheugencellen. De resultaten laten zien dat de CD8 T cellen in muizen met een geactiveerde lymfeklier voordeel hebben ten opzichte van de T cellen in muizen zonder geactiveerde lymfeklier. Dit uit zich vooral in een grotere hoeveelheid geheugencellen en een snellere en sterkere reactie van deze geheugencellen op een tweede infectie.

Hoofdstuk 5: Een emulsie van olie in water is geschikter om antilichamen voor tumor therapie langzaam af te geven dan microscopisch kleine bolletjes opgebouwd uit dextran-suikers

In hoofdstuk 5 wordt onderzoek beschreven waarin twee manieren om antilichamen langzaam af te geven in het lichaam met elkaar worden vergeleken. In hoofdstuk 2 en 3 wordt beschreven dat lokale toediening van anti-CD40 activerend antilichaam en CTLA-4 blokkerend antilichaam in een langzame afgifte formulering een goede tumor-specifieke T cel activatie geeft met veel minder bijwerkingen. De langzame afgifte formulering die wij in de studies beschreven in hoofdstuk 2 en 3 gebruikten is Montanide, een olie in water emulsie. Hier vergeleken wij Montanide met een nieuwe methode, namelijk microscopisch kleine bolletjes gemaakt van dextran-suikers (dextranbolletjes), waarin het antilichaam is verpakt. Deze bolletjes vallen in het lichaam vanzelf langzaam uit elkaar waardoor het antilichaam vrij komt. De snelheid van uit elkaar vallen kan worden bepaald door de manier waarop de bolletjes worden gemaakt, met meer of minder water in het mengsel. Wij beschrijven dat de langzame afgifte van anti-CD40 antilichaam uit de dextran-bolletjes goed werkt; zelfs bij lage snelheid van uit elkaar vallen worden nog steeds tumor-specifieke T cellen geactiveerd. De bijwerkingen van de therapie met deze dextranbolletjes zijn zelfs nog minder dan bij de montanide-emulsie. Helaas blijken de dextranbolletjes toch minder geschikt voor gebruik als tumor-therapie aangezien de tumoren sneller gingen groeien van de dextran-bolletjes die er dichtbij werden gespoten. Het feit dat de langzame afgifte efficiënt is, is dus nog geen maat voor de goede werking bij tumor-therapie. Dit onderzoek geeft aan dat Montanide emulsie meer geschikt

is voor tumor-therapie dan dextran-bolletjes.

Hoofstuk 6: Discussie

In het laatste hoofdstuk worden alle onderdelen van het proefschrift samengevat en bediscussieerd aan de hand van vergelijkbare studies uit de literatuur. In een opinie artikel bespreek ik onderzoek waarover de afgelopen jaren is gepubliceerd. Ik gebruik die publicaties, samen met onze eigen bevindingen, om de stelling te onderbouwen dat immuun-activerende therapie van tumoren het beste gericht kan worden op de poortwachter lymfeklier in plaats van in het bloed gespoten te worden.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 13 juni 1975 in Grave. In 1993 haalde ze haar VWO diploma aan het Dukenburg College in Nijmegen, waarna ze Farmacie ging studeren aan de Universiteit Utrecht. In 1995 maakte ze de overstap naar de Hogeschool voor Natuur en Techniek in Utrecht, waar ze in 1998 haar diploma behaalde met als afstudeerrichting Biotechnologie.
Na een jaar als diagnostisch analist te hebben gewerkt in het VU ziekenhuis te Amsterdam, werd ze eind 1999 aangenomen als research analist bij de Tumorimmunologie groep van de afdeling IHB in het LUMC in Leiden. Zes-en-een-half jaar werkte ze als analist, assisteerde meerdere AIO's bij het afronden van hun promotietraject, behaalde haar proefdierkunde certificaat, en werkte uiteindelijk op een eigen project onder begeleiding van een postdoc. Op 1 mei 2006 begon ze aan het promotie onderzoek beschreven in dit proefschrift.
Sinds 1 januari 2011 is ze werkzaam als postdoc op een project van Professor Ossendorp, binnen de groep Tumorimmunologie in het LUMC.

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Nawoord:

Wie had gedacht, toen ik 12.5 jaar geleden bij de tumorimmunologie-groep als analist kwam werken, dat ik nu een proefschrift uit zou delen? Ikzelf in ieder geval niet. Maar na al die jaren ben ik nog steeds heel blij en dankbaar voor de keuzes die ik heb gemaakt, de mensen waarmee ik heb samengewerkt, en de kansen die ik heb gekregen.

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