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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 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. Tumor size was measured twice weekly in three dimensions and mice were killed when tumor size exceeded 1 cm^3 .

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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 $E1A_{234-243}$-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.

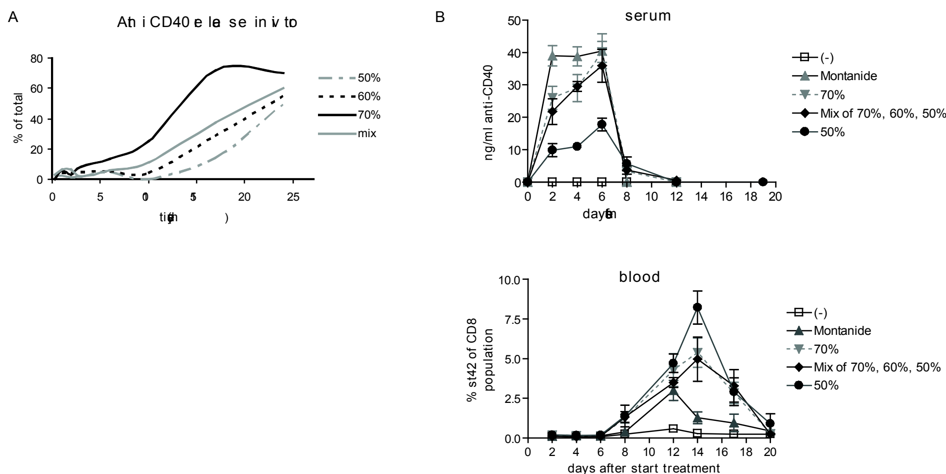

Figure 1: In vitro and in vivo release of dextran-based encapsulated antibody. Dextran-based microparticles were dispersed in PBS, and incubated at 37 ˚C. Samples were taken at regular intervals, and antibody levels were determined by Bradford analysis (A). In vivo release of antibody, correlated with 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.

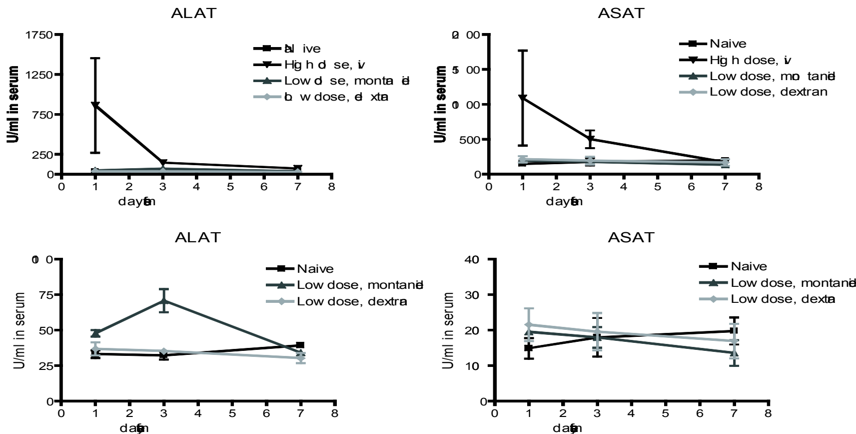

Figure 2: Systemic toxicity caused by antibody treatment. Mice were treated with different dosages and administration methods of anti-CD40 agonist Ab. Serum samples were taken at regular intervals. (A); ALAT and ASAT levels in serum, mice treated with high dose anti-CD40 antibody (2 x 100 µg) intravenously 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.

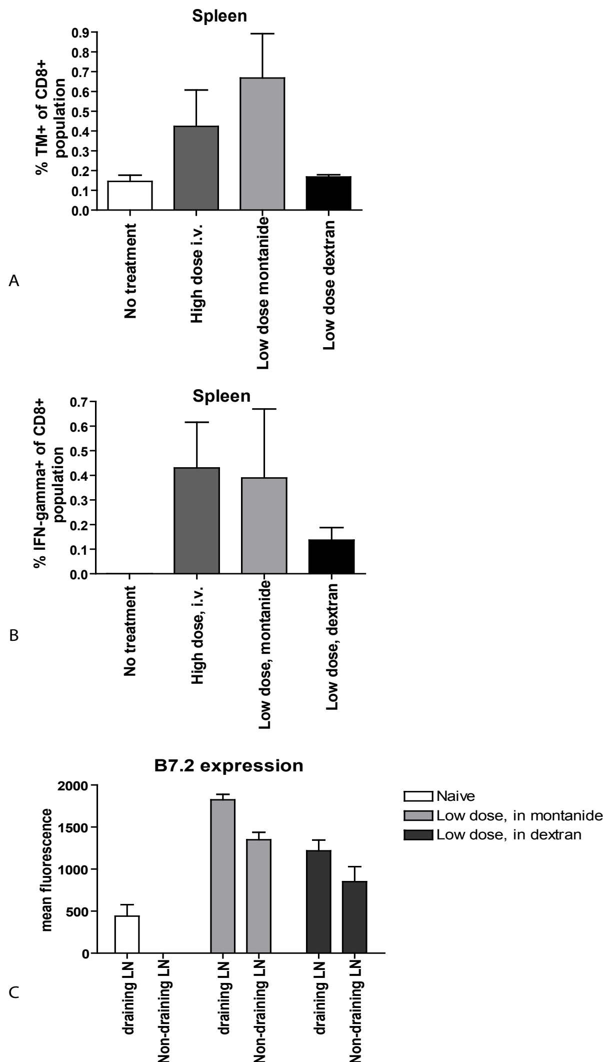

Figure 3: Endogenous immune response after dextran-based treatment. Tumor bearing mice were treated with high dose (3*100 μg) intravenously compared to mice treated with low dose (30 μg) anti-CD40 antibody, either in Montanide, or encapsulated in dextran-based microparticles. Spleen were analyzed for 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.

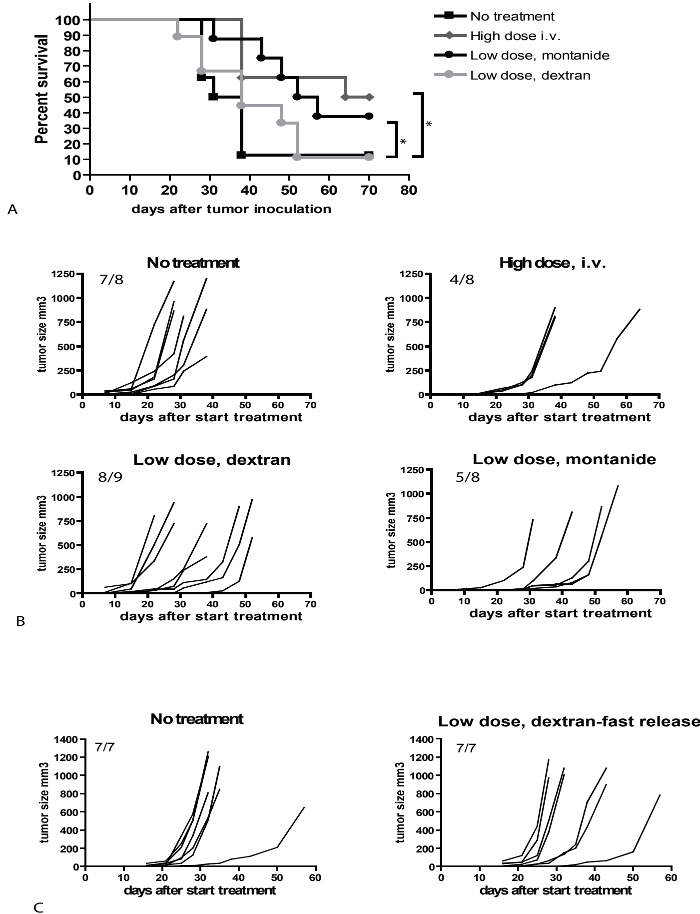

Figure 4: Tumor eradication by low dose, local anti-CD40 antibody treatments. Survival and tumor growth of tumor-bearing mice after treatment with 30 µg of anti-CD40 agonist Ab in Montanide or 30 µg of anti-CD40 agonist Ab in dextran-based microparticles , subcutaneously, compared with high dose (3*100 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.

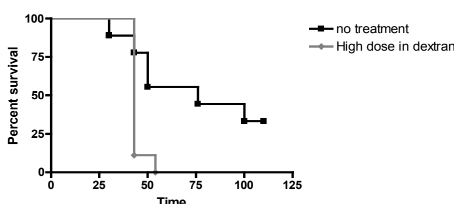

Figure 5: High dose of dextran-based microparticles enhances tumor-outgrowth.Survival of tumor-bearing mice after treatment of 100 µg of anti-CD40 antibody in dextran-based microparticles, compared to non-treated mice. The Kaplan-Meier test revealed a significant difference between microparticles treated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