Chapter 2

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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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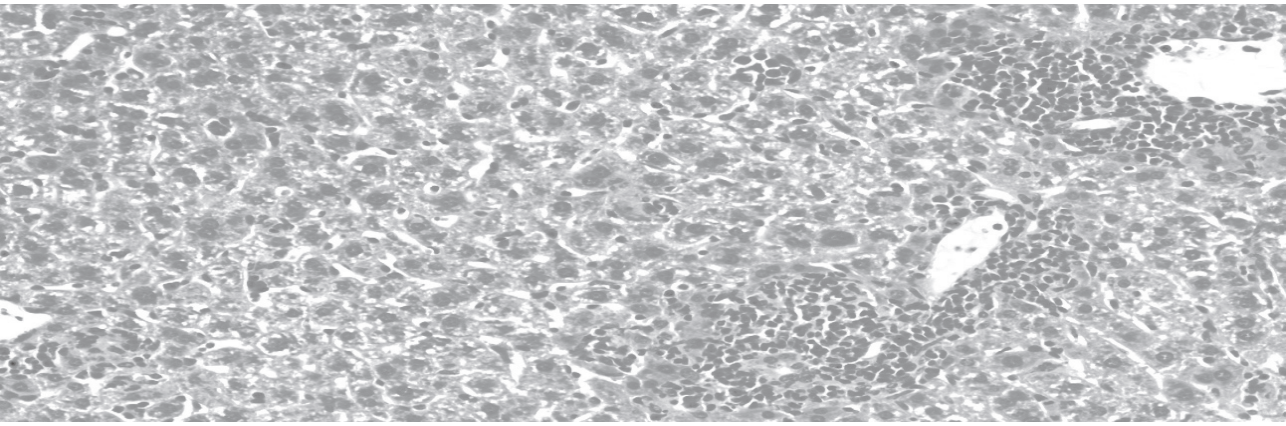

Abstract:
Immune-therapy against tumors using anti-CD40 agonistic antibodies has been extensively studied in pre-clinical animal models and recently also in clinical trials. Although promising results have been obtained, antibody-related toxicity has been a limiting factor. We reasoned that strict local 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.

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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.

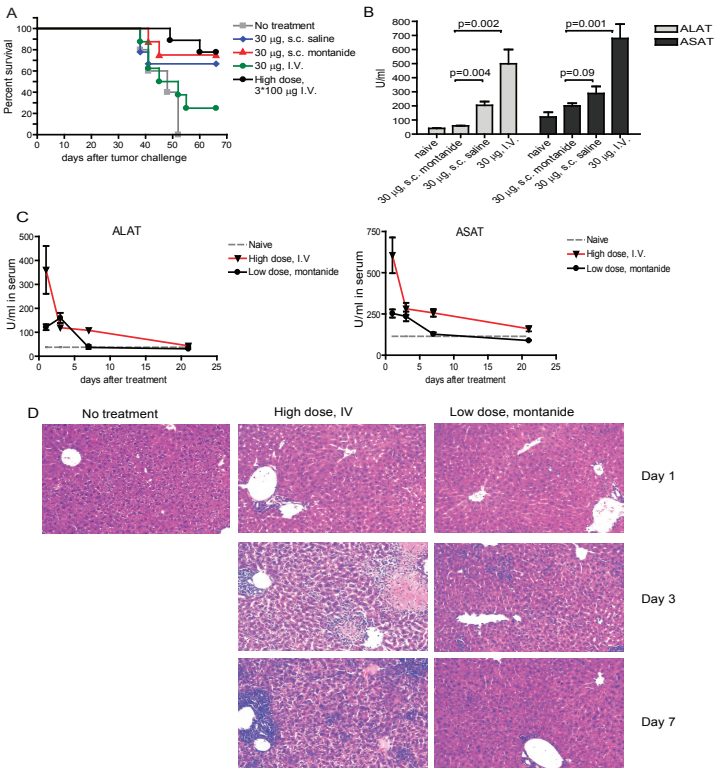

Figure 1: **Low dose local treatment with anti-CD40 gives lower toxicity then high dose systemic treatment.**

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.

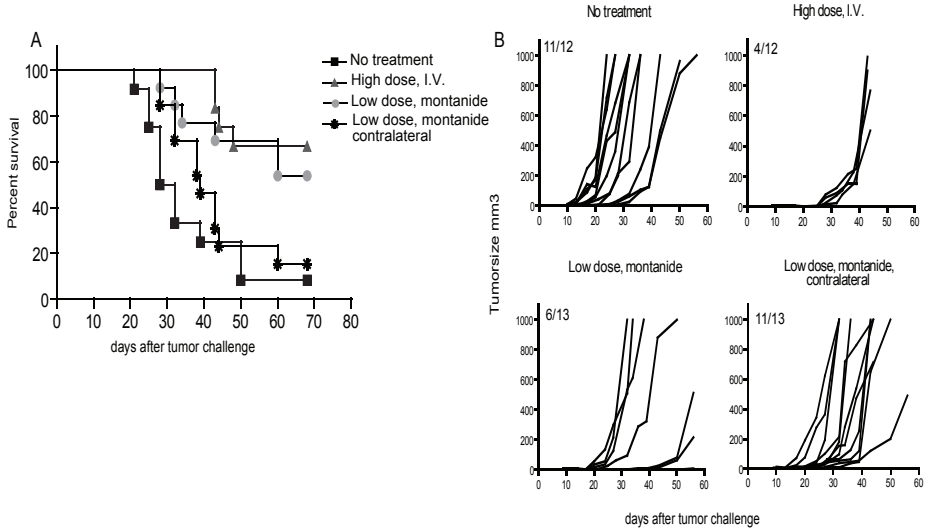

Figure 2 **Low dose local treatment gives similar anti-tumor response as standard treatment, and is a strictly local treatment.**
Survival and tumor-growth of tumor-bearing mice after treatment with 30 µg of anti-CD40 agonist antibody in montanide, subcutaneously compared to standard protocol, and compared 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

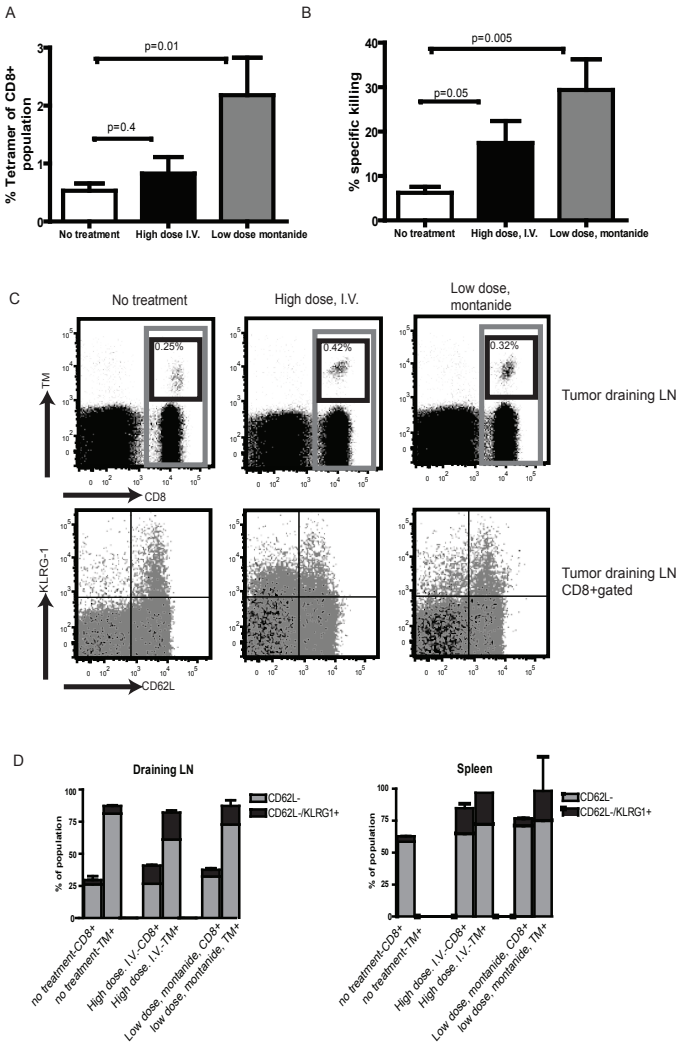

Figure 3 **Systemic CTL response after local, low dose, montanide treatment with anti-CD40 antibody.** Quantity and functionality of the anti-tumor CTL response in blood after local, low dose, montanide treatment with anti-CD40 agonist antibody, compared to standard treatment. 8 days after start of treatment, 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).

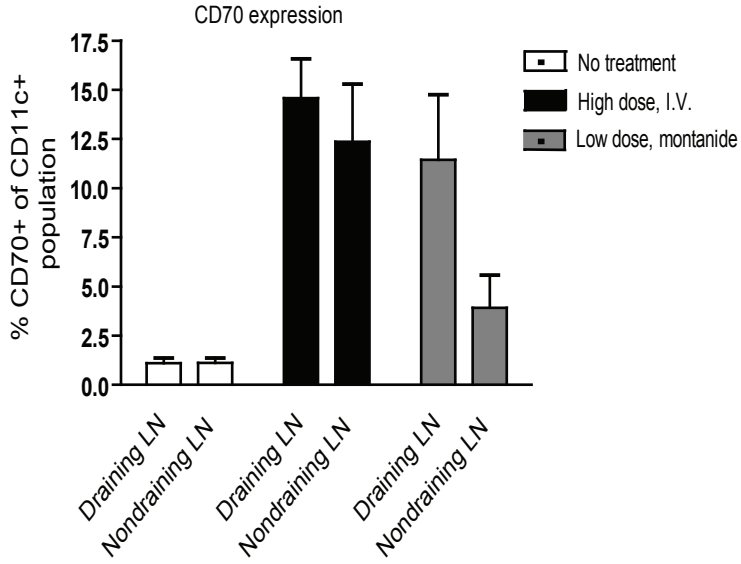

Figure 4 **DC activation in tumor-draining lymph node after local treatment**
CD70 expression on CD11c high cells in tumor-draining and non-draining lymph nodes of tumor-bearing mice, non-treated, high systemic dose treated of low dose, montanide treated. 4 mice per group, one representative experiment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

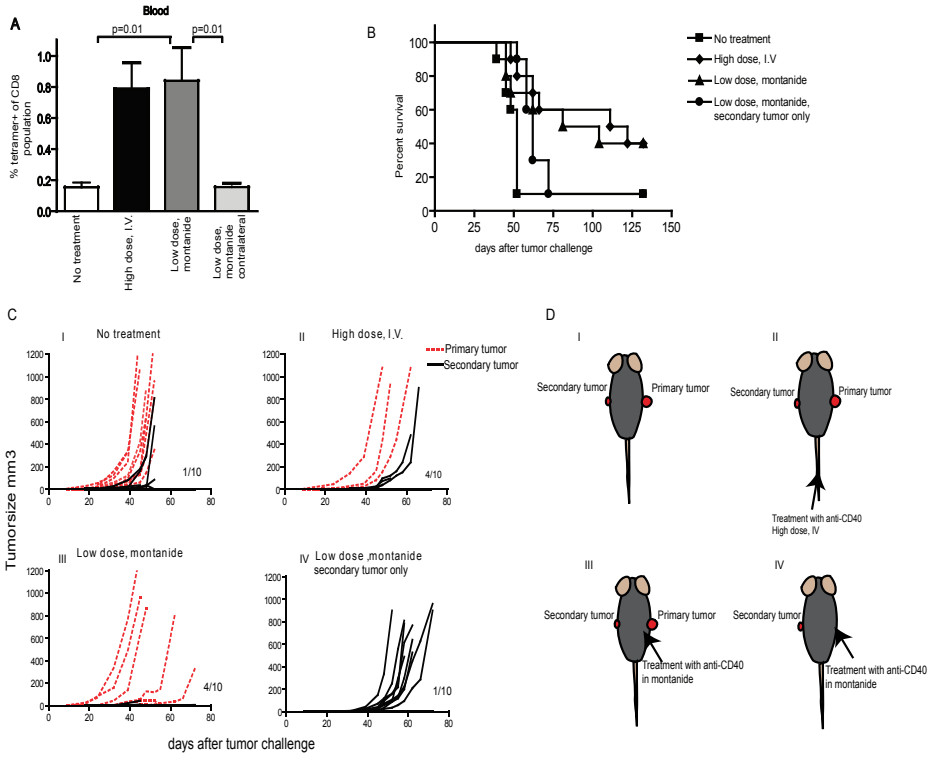

Figure 5 **Local treatment can eradicate a distant tumor.**
Eradication of secondary tumor by tumor-specific CTLs activated by local treatment with low dose anti-CD40 in montanide, compared to standard treatment. Data are presented as cumulative survival (10 mice per group), representative experiment 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).

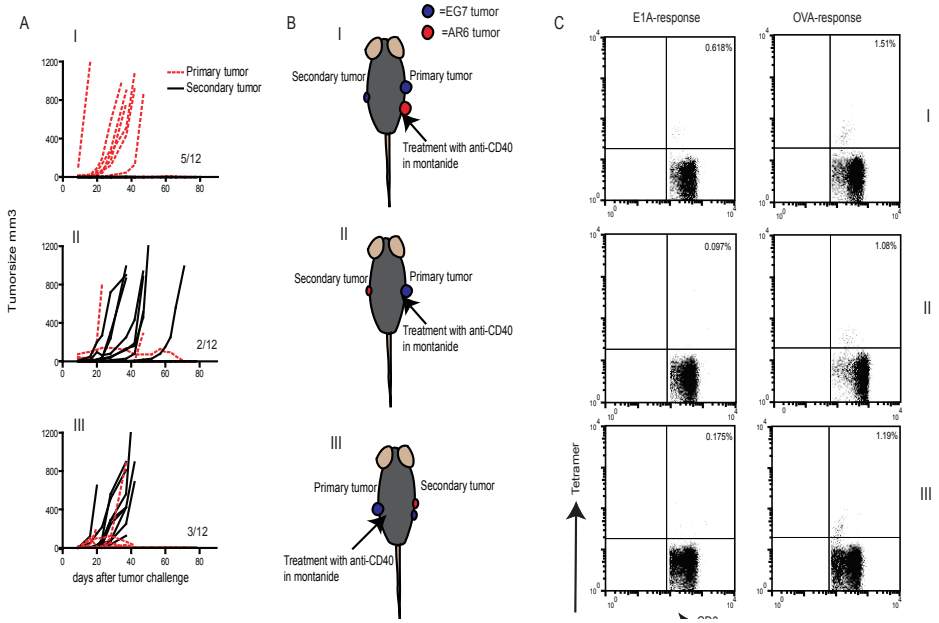

Figure 6 **Tumor-eradicating response is activated by locally available tumor-antigen.**
Eradication of secondary tumor by tumor-specific CTL activated by local treatment with low dose anti-CD40 in montanide. Dependence of tumor-eradicating capacity of CTL on locally available tumor-antigen. Tumor growth 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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