Targeting the tumor-draining area : local immunotherapy and its effect on the systemic T cell response

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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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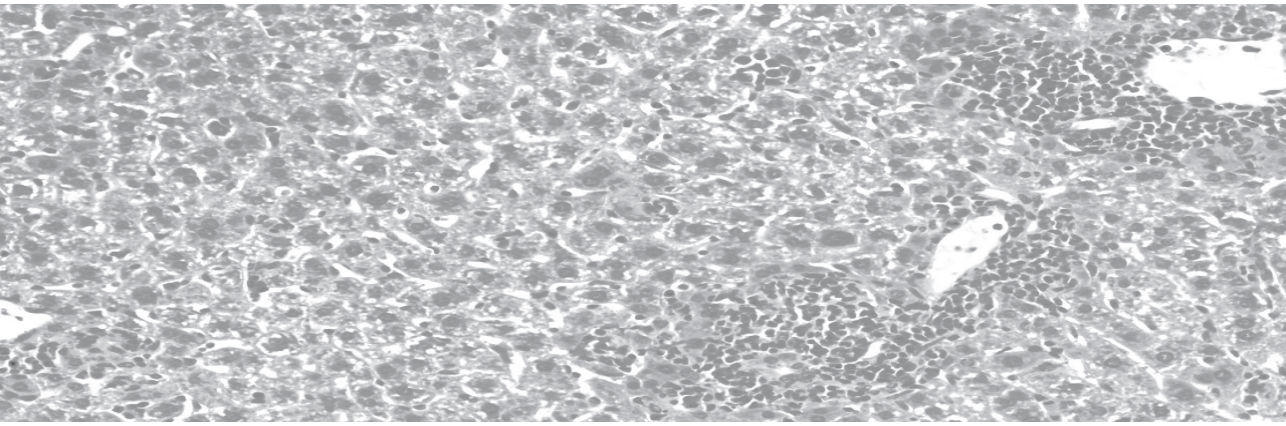

Abstract

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.

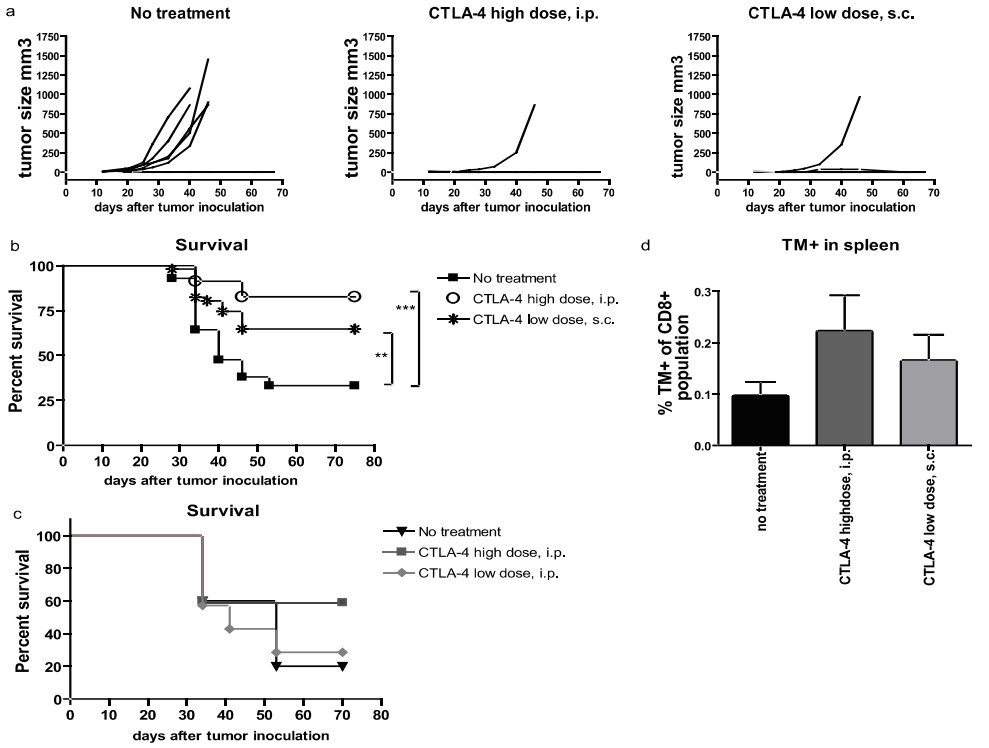

Fig. 1 Local treatment with a low dose of CTLA-4 blocking antibody induces effective tumor eradication. Mice bearing palpable MC-38-ova tumors were treated with two intraperitoneal injections with high dose (2 x 200 µg) of CTLA-4 blocking antibody (standard treatment), or one subcutaneous, local, injection 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.

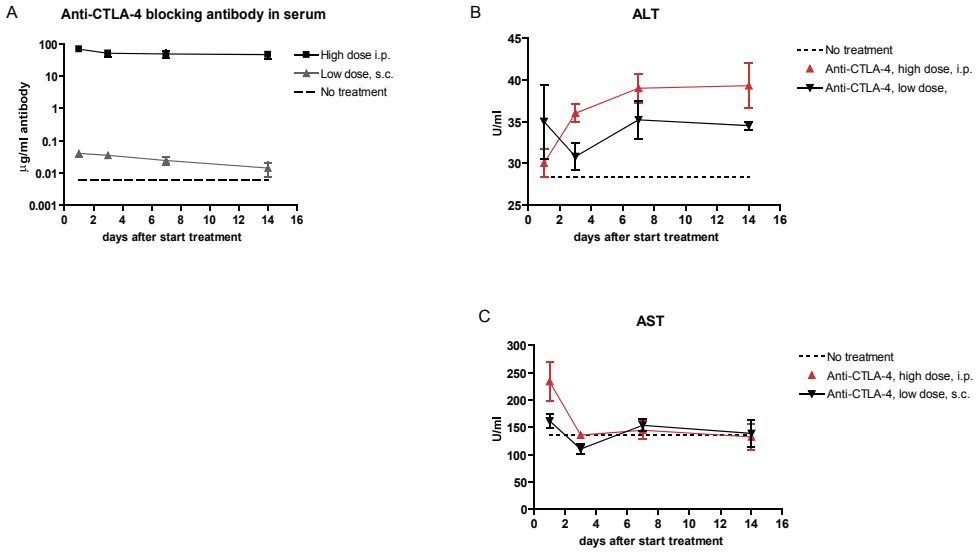

Fig. 2 Local treatment with a low dose of CTLA-4 blocking antibody results in decreased treatment induced toxicity as compared to high dose, systemic treatment. Shown are CTLA-4 antibody concentrations and liver enzyme levels in serum in time after treatment with low dose (50 µg) local treatment or high 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).

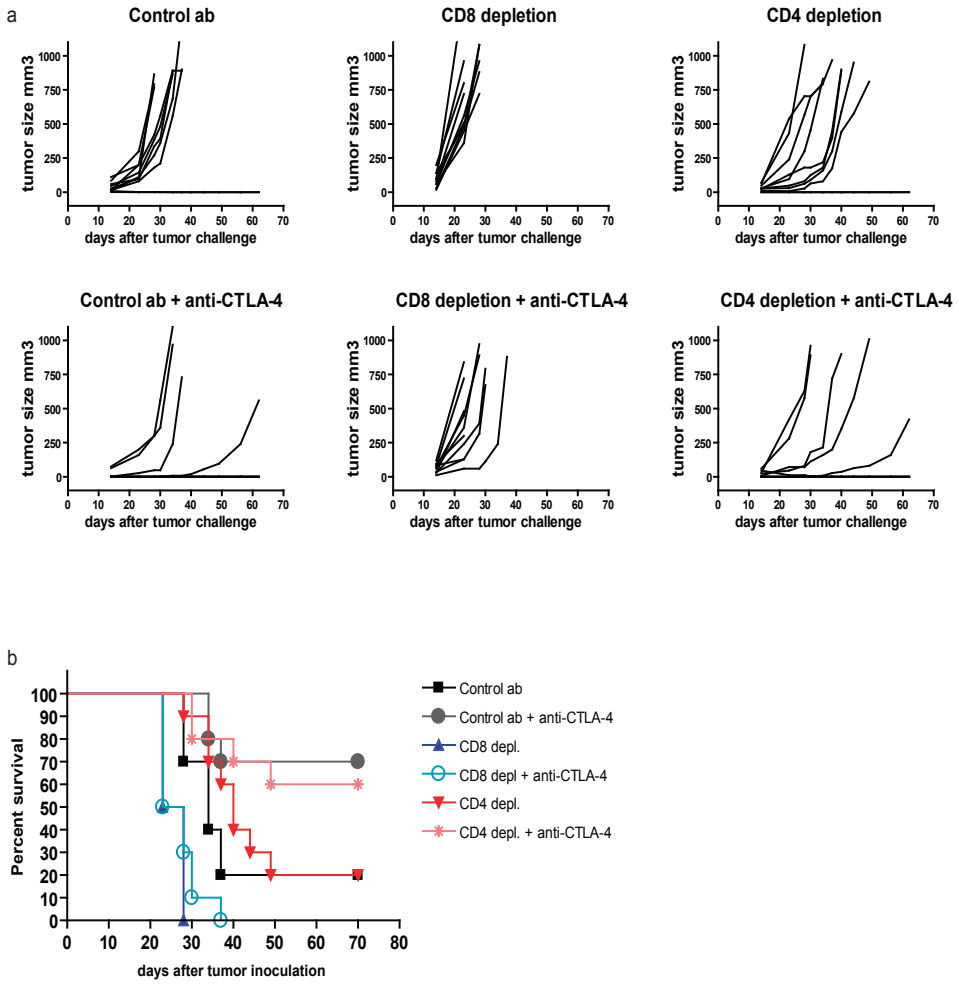

Fig. 3 CD8⁺ T cells are the main effector cells involved in tumor eradication and the main target of local treatment with CTLA-4 blocking antibodies. Mice were depleted of CD8⁺ or CD4⁺ T cell populations, starting one day before tumor inoculation, for three weeks. 8 days after tumor inoculations, when 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].

Acknowledgements

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